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REVIEW ARTICLE

Mechanism and effect of stress granule formation in cancer and its potential roles in breast cancer therapy

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Abstract Stress granules are non-membranous cytoplasmic foci induced by various stress conditions. It is a protective strategy used by cells to suppress overall translation during stress. In cancer cells, it was thought that the formation of stress granules could protect them from apoptosis and induces resistance towards anti-cancer drugs or radiation treatment which makes the stress granules a potential target for cancer treatment. However, most of our understanding of stress granules are still in the stage of molecular and cell biology, and a transitional gap for its actual effect on clinical settings remains. In this review, we summarize the mechanism and effect of stress granules formation in cancer and try to illuminate its potential applications in cancer therapy, using breast cancer as an example.

Introduction

In animals, stress granules (SGs) were first named and morphologically characterized by Collier *et al* in 1988 as dense cytoplasmic bodies formed in restressed chicken embryonic fibroblast cells.[1] Subsequent studies have revealed that SGs can be formed in many other eukaryotic cells ranging from yeast to human cells and induced by various stress conditions including heat, oxidative stress, and hypoxia, indicating that the formation of SGs is an evolutionally conservative strategy to protect cells from stress conditions.[2,3] The detailed structure, composition, and function of SGs have been extensively studied during the past three decades, mainly using laboratory cancer cell lines. Noteworthily, SGs have also been recently detected *in vivo*, both in human pancreatic adenocarcinoma tissue

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and mouse xenograft of human osteosarcoma cell line.[4,5] Specific oncogenic mutations including KRAS mutations in pancreatic adenocarcinoma and DDX3X mutations in medulloblastoma have been found to drive the spontaneous formation of SGs.[4,6]

Messenger RNA (mRNA), RNA-binding proteins TIA-1 and TIAR was the first identified components of SGs.[7] Later, an inactivated 48S initiation complex which is composed of translation initiation factors eIFE, eIF4G, eIF3, and 40S ribosome subunit was detected in SGs.[8,9] The compositions indicate SGs is a type of messenger ribonucleoprotein particle (mRNP) similar to other mRNPs like Cajal bodies and P bodies. After exposure to stress, two subsequent processes were necessary for SGs formation, the inhibition of translation initiation[10,7] and RNA-protein aggregation nucleated by RNA-binding proteins.[11] The interactions contributed to SGs formation include RNA—protein, RNA—RNA interactions and protein—protein interactions.[12] Proteomic analysis of SGs proteins found that 15% of SGs proteins have Prion-like domain (PrLD) which is a type of intrinsically disordered region (IDR) and more than 50% of stress granules proteins have RNA-recognition motif.[13,14] For many stress granule proteins, these two domains are found to be essential for their recruitment of SGs.[15—17] Both domains and their combination are also important for the inducing of liquid—liquid phase separation (LLPS).[13,18,19] Given the dynamic property of SGs, they are considered to be liquid droplets separated from cytoplasm through complex RNA and protein interactions.[20] Meanwhile, super-resolution fluorescent microscopy and FRAP examination of SGs revealed that SGs harbored a biphasic structure, composed of multiple high-density solid cores and surrounding shell structure which is believed to be formed by LLPS. The cores harboring mRNA and proteins are relatively more condense and stable than the out shell structure which is more dynamically flexible with proteins transporting in and out rapidly.[13,21] The composition of SGs formed in different conditions can be diverse. Different from the arsenite-induced SGs which are called canonical SGs, selenite induced SGs lacked many important classical proteins including RACK1 while chronic nutrition starvation-induced SGs lack 40S ribosome which is present in all of the other reported SGs.[22,23]

Functionally, the formation of SGs is closely related to cancer, neurodegeneration diseases, and viral infection.[24—26] In this review, we focus on the role of stress granules in cancer development and progress, aiming to summarize and provide more details about the association between SGs and cancer cell behavior.

The effect of chemotherapy on the formation and disassembly of SGs

It is known that cancer cells are continuously confronted with environmental stresses including hypoxia, the toxicity of chemotherapy and radiation therapy and their responses to stresses make a critical aspect of their biological behavior.[27—29] The formation of SGs is one type of protective strategy for cancer cells to survive from stresses. Numerous studies have reported the formation and function of SGs in cancers mostly using cancer cell lines, only one paper has reported the existence of SGs in human cancer tissues,[4] probably due to the invisibility of SGs under normal optical microscopy and its tendency to disassemble when stress is relieved. Many anti-cancer chemicals can affect the formation and the disassembly of SGs.

Formation of SGs

Many anti-cancer chemicals can induce SGs. They cover a wide range of anti-cancer working mechanisms from anti-metabolites like 5-fluorouracil (5-FU) to tyrosine kinase inhibitors like sorafenib. They also induce SGs through different mechanisms and signaling pathways which are summarized and presented in Table 1 and Fig. 1. Here, we focused on the current and potential therapeutics in breast cancer.

5-Fluorouracil (5-FU), one of the oldest and effective therapy for breast cancer, was reported to be SGs inducing.[30] Its pro-drug capecitabine is widely used for metastatic breast cancer. Experiments have shown that the therapeutic dose of 5-FU can induce the formation of SGs in cultured HeLa cells. Among all the activities, its incorporation into RNA is necessary for promoting SGs formation while others are not.[31] Noteworthily, other two FDA-approved RNA-incorporating anti-cancer drugs, 5-azacitidine and 6-thioguanine can also induce the formation of SGs although in much higher concentration than their clinical dosage.[31] SGs formation induced by the RNA-

Table 1 Reported chemicals and drugs that can induce SGs formation.

Drug generic name	Anti-cancer mechanism	SGs inducing target	Ref.
5-FU#	Anti-metabolic; DNA and RNA incorporation	eIF2α (PKR)	30, 31
Sorafenib#	Tyrosine kinase inhibitor	eIF2α (PERK)	32, 36
Lapatinib#	Tyrosine kinase inhibitor	eIF2α (PERK)	37
Selenite#	ROS inducing; Antophagy inhibiting	eIF4E	22, 67
MG132#	Proteasome inhibitor	eIF2α (GCN2)	51
Bortezomib	26S proteasome inhibitor	eIF2α (HRI)	51, 90
Arsenite	ROS Inducing; Cell cycle arrest	eIF2α (HRI, PERK, PKR)	74, 87
Thapsigargin	ER stress Inducing	eIF2α (PERK)	40
PateamineA	Translation inhibition	eIF4A	42
Hippuristanol	Translation inhibition	eIF4A	41—44
Silvestrol	Translation inhibition	eIF4A	41—44
Oxaliplatin	DNA crosslinking Immunogenic cell death	eIF2α	46—48

Ps. The # marked entries are physiologically relevant drugs that can possibly induce SGs in their therapeutic concentrations.

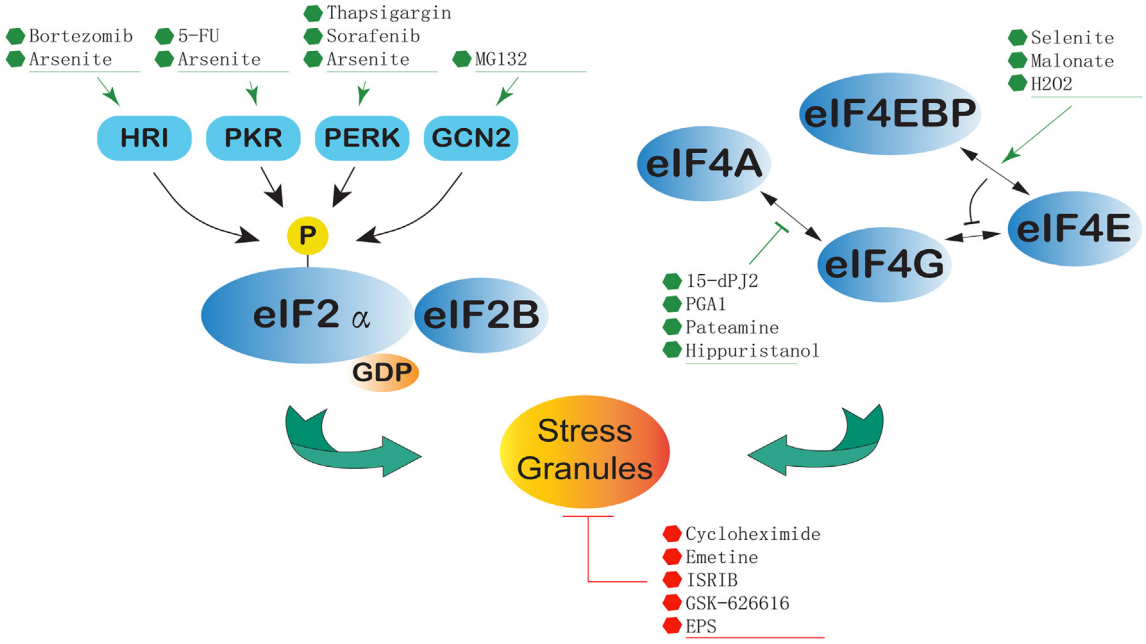

Figure 1 Chemicals and drugs that can affect the assembly and disassembly of SGs through various pathways. Green hexagons represent molecules that can promote the assembly of SGs and red hexagons represent the molecules that can promote the disassembly of SGs. The formation of SGs can achieved by blocking either the eIF2α or the eIF4F complex represented by large green arrow. The upstream pathways of SGs formation were indicated by small arrows.

incorporating pathway was mediated by the phosphorylation of eIF2α. The detailed underlying mechanism, along with the question of whether all RNA-incorporating drugs are able to induce SGs formation remain to be revealed.

Sorafenib is a multikinase inhibitor targeting BRAF, CRAF, and VEGFR.[32] Although it is currently used to treat advanced renal cell carcinoma and hepatocellular carcinoma, lots of trials have been launched to evaluate its effect as monotherapy or combined with others for advanced breast cancer.[33–35] Sorafenib can induce SGs formation in various cancer cell lines via phosphorylation of eIF2α by PERK. Disruption of the SGs formation would make cells more sensitive to the sorafenib.[36] Another tyrosine kinase inhibitor lapatinib was shown to be able to induce SGs formation in T-47D breast cancer cell line at therapeutic level.[37] Lapatinib targeting HER2 and EGFR pathway and is used in combination therapy for HER2-overexpressed breast cancer. Same with sorafenib, it induces SGs formation via phosphorylation of eIF2α by PERK. Moreover, both sorafenib and lapatinib were found to activate PERK via the altered expression of GRP48/BiP, an ER chaperone binding to PERK to inhibit its dimerization.[38,39] Thus a potential mechanism about the controlling role of GRP48/BiP in SGs formation was worth further exploration.

Other SGs inducing breast cancer therapeutics include selenite which induces SGs through generating ROS,[23] thapsigargin is an extract from the plant and can induce SGs by generating ER stresses,[40] both of them are currently in the clinical trial phase. Three eIF4A inhibitors, pateamine, hippuristanol, and silvestrol are also proved to promote SG formation and they all showed a certain degree of anti-cancer activity for both cell lines and xenograft tumors.[41–44]

Cisplatin, as common salvage chemotherapy and a potential first-line therapy in BRCA mutated patients, failed to induce SGs formation. While its analog oxaliplatin which was applied in ovarian and colorectal cancer was found to have the activity of SGs inducing via phosphorylation of eIF2α.[45–48] It indicates the SGs inducing is not related to the shared DNA damage effect between them but is caused by the extra effect of oxaliplatin like the inducing of immunogenic cell death.[49,50]

It's worth reminding readers that different cancer cell lines have different SG forming sensitivity towards specific stress conditions. For example, SGs can be successfully formed in many cell lines including Hela and Calu-1 by bortezomib, while its formation cannot be found in breast cancer cell line Hs578T,[51] indicating that SGs formation is under sophisticated regulation. Thus, for the study of SGs, the experiments and conclusions should be processed in a drug- and cancer-specific manner.

SGs assembly pathways targeting by different anti-cancer chemicals

As mentioned above, translation initiation inhibition and macromolecular aggregation are two core steps for SGs forming. Although the above chemicals induce SGs through different initiating mechanisms, all of them targeted specific translation initiation factors in the process of SGs forming (see Fig. 1).

The eIF2α is a translation initiation factor whose phosphorylation at Ser51 serves as a classic SGs inducing step. Once eIF2 is phosphorylated at Ser51, it would bind strongly with eIF2B, a guanidine exchange factor (GEF) to form a

stable p-eIF2α-eIF2B complex. The GEF function of eIF2B promoting the conversion between eIF2α-GDP and eIF2α-GTP is disabled due to the tight and rigid interaction with p-eIF2α. Subsequently, the inactive eIF2α-GDP complex accumulates in cells with inadequate eIF2-GTP for translation initiation.[52] eIF2α ser51 can be phosphorylated by four kinases PRK, PERK, GCN2 and HRI. For anti-cancer chemicals, MG132 promotes SGs formation through phosphorylation of eIF2α Ser51 by GCN2 while bortezomib enhances the eIF2α phosphorylation by HRI.[51] PERK activation is targeted by sorafenib.[19] Arsenite induced SG formation also involves eIF2α phosphorylation by HRI, PKR and PEKR. 5-FU can also induce the phosphorylation of eIF2α through PKR. In some SG forming conditions, eIF2α phosphorylation can be observed but are not necessary for SG formation, as in the case of selenite and H_2O_2.[53,23] In a screening experiment trying to find SGs-inhibiting compounds, EPS (a mixture of beta-estradiol, progesterone, and stanolone) was found to inhibit SGs formation in HeLa through PKR-mediated eIF2α phosphorylation.[54] Moreover, inhibition of SGs promoted HeLa cells' sensitivity to anticancer drug cisplatin.

The eIF4F complex is another essential translation initiation complex that can capture the 5' m7 cap of mRNA and activate mRNA for translation. eIF4F complex is comprised of three individual proteins eIF4A, eIF4E and eIF4G. Inactivation of any of them would cause translation initiation arrest and SGs formation.[23]

Small molecule compounds, pateamine, hippuristanol and silvestrol are proved to promote SG formation through inhibiting eIF4A.[55–58] Two prostaglandins 15-d-PGJ2 and PGA1 can bind directly with eIF4A to inhibit the binding between eIF4A and eIF4G, thus suppressing the formation of eIF4F complex.[59]

The binding between eIF4E and eIF4G is also necessary for the assembly of eIF4F complex. eIF4E binding protein (eIF4EBP) binds to the dorsal surface of eIF4E which is the same binding site shared by eIF4G. Thus, eIF4EBP competes with eIF4G for the binding of eIF4E. As a regulation strategy, the binding affinity of eIF4EBP with eIF4E is affected by its phosphorylation status. In hypophosphorylation status, it binds strongly with eIF4E and thus inhibits the formation of eIF4F complex. SGs are subsequently formed due to translation initiation arrest. When it is phosphorylated by mTORC1, its affinity with eIF4E decreases and releases eIF4E for translation activation. Selenite induces SGs formation mainly by promoting eIF4EBP hypophosphorylation. eIF2α phosphorylation can also enhance selenite induced SGs but is not indispensable, unlike the case for arsenite-induced SGs. Meanwhile, malonate and H_2O_2 also induce SG forming via eIF4E-BP hypophosphorylation.[53,60]

Disassembly and persistence of SGs

SGs are transient structures whose formation is reversible. The dynamics of SGs disassembly is poorly understood, although polysomes and autophagy are known to be involved. Consistently, anti-cancer chemicals that regulate polysomes and autophagy are reported to affect the clearance and persistence of SGs.

Polysomes are complex consist of multiple ribosomes attaching to one mRNA chain for translating. Studies have shown that under stress conditions the majority of mRNA would disassociate from polysomes as a result of overall translation suppression.[61] It is hypothesized that those depleted mRNA depleted would get sequestered into SGs. Indeed, research showed that arsenite induced SGs formation got strongly inhibited and its disassembly was accelerated by stabilizing polysomes with well-established polysome stabilizing agents, cycloheximide or emetine, which indicated the existence of exchanging between SGs and polysomes. Moreover, as heat-induced SGs forming is inhibited by cycloheximide, cell apoptosis rate caused by heat shock would greatly increase. They both stabilize polysome by binding to ribosomal subunits and suppress translation elongation.[62] The SGs inhibition effect makes them potential therapy for cancer or cancer chemoresistance. However, cycloheximide cannot be applied in the clinic due to its side effects including DNA damage and teratogenesis.

Autophagy is another cellular response to various stresses responsible for the degradation of various cellular constituents.[63] Moreover, SGs were found to be cleared by autophagy,[64,65] and autophagy promoter agent rapamycin can accelerate the disassembly of SGs while autophagy inhibitor wortmannin attenuated the disassembly of SGs. Rapamycin, as the suppressor of mTOR pathway, has shown therapeutic potential for breast cancer,[66] and its influence on SGs dynamics should be considered in the further study of pharmacodynamics. Selenite as a SGs inducing agent can also induce autophagy,[67] however the mutual relations between autophagy and SGs under selenite were not revealed yet.

Regarding the persistence of SGs, studies have indicated that mTOR pathway might be intimidatedly involved although the underlying mechanisms remain to be addressed. The manipulation of both kinases in the mTOR pathway, DYRK3 and S6K2, has been reported to promote the persistence of SGs. While inhibition of DYRK3 kinase by a small compound GSK-626616 can promote the persistence of arsenite induced SGs,[68] overexpression of S6K2 can prolong the presence of SGs.[69] When there is almost no SGs retained in the control group 2 h after recovery, SGs retained more than 60% in S6K2 overexpressing cells. A recent study has identified the role of raloxifene in preventing the dissolution of hypoxia induced SGs possibly by inhibiting the re-activation of mTOR pathway.[70] Conversely, a small molecule called ISRIB has been shown to induce rapid disassembly of formed SGs and inhibit eIF2α phosphorylation dependent SGs formation, making it a potential drug for the treatment of chemoresistance through SGs.[71]

Radiation therapy and SGs

Radiation therapy was used in almost every stage of breast cancer to reduce the recurring risk. It works by generating DNA damage with X-ray and causing cell apoptosis. Even though radiation itself can not directly induce SGs, research found that SGs can help induce radiation resistance of cancer cells.[72] Cancers are commonly in hypoxia status which is an

SGs inducing stressor. However, shortly after the application of radiation, the cancer tissue would be reoxygenated and gradually recover to its original hypoxia status later. In the reoxygenation process, SGs are destabilized due to the elimination of hypoxia stress and the sequestered mRNAs get released. Among them are many hypoxia-inducible factor (HIF)-regulated transcripts and the release of them makes the HIF1 signaling enhanced. It is known that activated HIF1 pathway would elevate the expression of VEGF and bFGF in vascular endothelial cells, promote their survival and thus cause radiation resistance.[73]

Protective role of SGs in cancer cells

Indeed, numerous researches have provided evidence for the anti-apoptosis and drug resistance inducing effect of SGs. A recent study has directly proven that by adding SGs inhibiting drug compounds EPS, the hypoxia-induced drug resistance of HeLa cells can be relieved.[54] Moreover, as SGs have been detected in human cancer tissues, more attention should be paid to the potential role of SGs as a new cancer biomarker and a treatment target. Here, we aimed to summarize the drugs, proteins and pathways that induce SGs as well as the effect of SGs on cancer cells.

SGs formation triggers overall translation suppression as a protective strategy of cells. In cancer cells, it is known that the formation of SGs can protect cells by inhibiting apoptosis and promoting drug resistance. The related mechanisms have been summarized below.

Anti-apoptosis effect of SGs

The anti-apoptosis effect of SGs has been observed in many studies. Zou *et al* showed that arsenite treated intestinal epithelium cells with SGs forming are more resistant to apoptosis when exposed to TNF-α/CHX than non-arsenite-treated cells.[74] In the treatment of bortezomib, non-SGs forming breast cancer cell line Hs587T showed higher sensitivity and higher apoptotic rate than other SGs forming cell lines.[51] It was believed that its anti-apoptotic effect was mainly achieved by sequestering pro-apoptotic proteins activated in other stress reponse pathways including the hyperactivation of mTOR pathway, the JNK pathway and the accumulation of reactive oxygen species (ROS). These include tumor necrosis factor receptor-associated factor 2 (TRAF2), receptor for activated C kinase 1 (RACK1) and plakophilin3[23] which are summarized and presented in Figure 2. Hyperactivation of mTORC1 complex which was composed of mTOR and its binding proteins raptor and PRAS40 was known to be pro-apoptosis.[75] Astrin, as a raptor interactor, competes with mTOR for the binding of raptor which froms the astrin-raptor complex. Under stress conditions, the astrin-raptor complex was recruited to SGs, leading to reduced mTORC1 formation and reduced apoptosis rate.[76,77] The mTOR effectors, S6K1 and S6K2, are also incorporated in the SGs. Although the two of them share 83% amino acid identity, their roles in SGs are found quite different with S6K1 enriched significantly only in SGs formed by mild arsenite stress but not in SGs formed by acute arsenite stress while S6K2 showed robust localization in SGs formed by both conditions. Their ortholog in *C. elegans*, RSKS-1, was found in SGs and can prevent stress-induced apoptosis. The above findings indicate a sophisticated role of the mTOR pathway in the anti-apoptosis mechanism of SGs.

The stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) pathway can be activated by stress including cisplatin and heat shock, and it has been reported to promote apoptosis.[78–80] The binding RACK1 with MTK1 is essential for stress induced pathway activation. When the cells are treated with arsenite or in hypoxia, the formed SG will effectively chelate RACK1 protein instead of MTK1, resulting in the inability of MTK1 and SAPK/JNK pathways to activate and preventing the subsequent pro-apoptosis effect.[81] Another protein essential for the stress induced JNK pathway activation is ROCK1. It is responsible for the phosphorylation of JIP-3 which is necessary for the activation of JNK pathway. Under heat shock stress, the activated form of ROCK1 is recruited into SGs while the inactive form remains free in cytoplasm, making the JNK pathway inactivated and apoptosis inhibited.[82]

It is well known that ROS is an apoptosis inducer.[83,84] However, the level of ROS is found to be reduced in cells when G3BP1 is overexpressed and SGs are formed. This effect is mediated by a G3BP1 interacting protein USP10 which has antioxidant activity. In a steady-state, G3BP1 inhibits the antioxidant activity of USP10. When SGs are formed, they are both recruited to SGs and this inhibition effect was inactivated, subsequently, the unrecruited free USP10 then decreases the ROS level and apoptosis is prevented. When USP10 is knockout, cells would have increased arsenite-induced apoptosis rate which can be rescued by adding ROS scavenger, revealing the close relationship between SGs-regulated ROS level and apoptosis.[85]

Drug resistance induced by SGs

Besides anti-apoptosis, SGs can also protect cancer cells by inducing drug-resistance. It is known that cancer cells are commonly in a hypoxia status due to fast growth and insufficient blood supply. Meanwhile cells adapt to hypoxia through making metabolic changes which subsequently cause drug resistance.[86] Shikshya *et al* reported that, when the hypoxia-induced SGs gets inhibited by EPS compounds, drug sensitivity of HeLa cells can be recovered to normoxia.[54] Moreover, drug resistance can also be restored through overexpressing G3BP1 and new SGs formation indicating the direct relations between drug resistance and SGs.[54]

Glass *et al* showed that repeated sodium arsenite exposure would cause the development of cells resistant to SGs-inducing chemotherapeutic agents including arsenite itself and diclofenac sodium, although the resistance would disappear when arsenite is removed for 10 cell passage time.[87] This resistance phenomenon is probably mediated by the altered cytokine secretion (the elevation of serpin E1 and decrease of MCP1 by resistant cells) and functions in a paracrine way. More works are needed to elucidate the mechanism behind.[87,88]

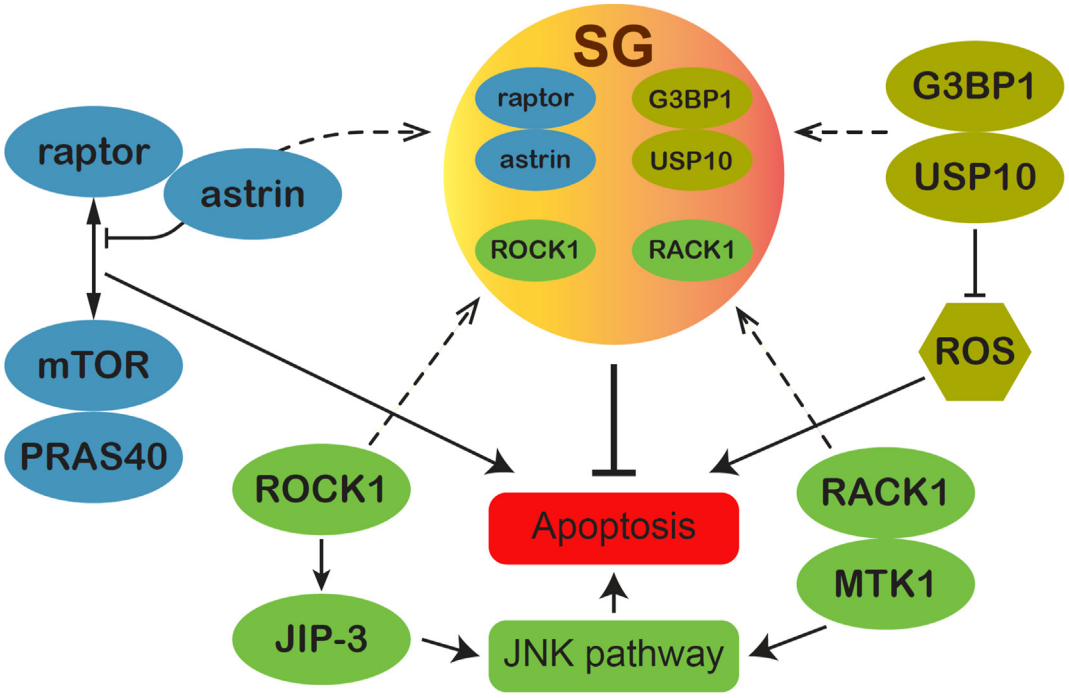

Figure 2 Anti-apoptosis mechanism of SGs. Various apoptosis-promoting proteins can be recruited into SGs (represented by the orange circle) including ROCK1 and RACK1 of the JNK pathway, Raptor and Astrin in the mTORC1 pathway and the G3BP1 and USP10 in ROS production pathway which are represented by the dotted line. The solid lines represent a promoting or contributing effects.

Looking into the future: the potential of SGs in anticancer therapy

Translation and stress response has attracted lots of attention in the research field of anticancer therapy.[41,89] Formation of SGs under stresses suppresses overall translation, protects mRNA from degradation and stimulates stress-adaptive protein synthesis. Its protective role and its potential as a novel anticancer target have been proved both *in vitro* and *in vivo*. In breast cancer research field, the related SGs inducing anticancer drugs including 5-FU, sorafenib, lapatinib, selenite and rapamycin. Further experiments to test whether strategies that combine suppressors of SGs with anticancer drugs may be effectives in preventing resistance are worth investigating.

However, since the formation of SGs is regulated in a sophisticated manner and is involved with various pathways including ER stress, ROS, ubiquitin proteasome and autophagy, how to target SGs for anticancer or anti-chemoresistance therapy is still a great challenge. Taken bortezomib treatment as an example, researches showed that maintaining eIF2α hyperphosphorylation can enhance the efficiency of bortezomib treatment by eradicating the survival quiescent cells.[90] Whereas previous studies revealed that hyperphosphorylation of eIF2α can induce the formation of SGs and contribute to cell survival. Thus, the balance or cross-talk between SGs and ER stress should further be elucidated to have a better understanding of the mechanism.

Meanwhile, the reported components and functions of SGs are still expanding, possibly revealing more potential treatment targets. A recently conducted comprehensive transcriptome analysis of SGs identified 215 SGs enriched ncRNA compared to cytoplasmic level.[84] Also, Anthony *et al* reported that the small RNA binding protein argonaute-2 which plays an essential role in RNA silencing process is recruited into SGs in a miRNA-dependent way,[91,92] indicating the existence of correlation and possible regulation pathway between SGs and ncRNA. A recent study showed dynamics of stress granule are also affected by circadian clock via oscillating eIF2α expression.[93] More studies, especially the *in vivo* studies involving SGs and systematic studies including deep transcriptomic and proteomic data is need for better understanding.

Last but not the least, the identified SGs inducing anticancer drugs are just a small part of all the currently available anti-cancer drugs which may be due to the lack of research on large-scale screening of drugs and molecules that affect SG. Though, a recent drug screening study has identified the effect of raloxifene on the persistence of SGs in a pool of 1120 drugs.[70] More similar efforts should be paid to achieve a more comprehensive understanding of SGs.

Conflict of interests

Authors declare no conflict of interests.

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