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Functional analysis of PDCD4 on the assembly of stress granule

조 선 대 학 교 대 학 원

생물신소재학과

박 라 영

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Stress granule 형성에 있어

PDCD4 의 기능적 연구

2014년 8월 25일

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지도교수 온탁범

이 논문을 이학박사학위 신청 논문으로 제출함

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Abstract

Functional analysis of PDCD4 on the assembly Of stress granule

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Programmed cell death 4 (PDCD4) is a novel tumor suppressor that function in the nucleus and the cytoplasm and appears to be involved in the regulation of transcription and translation. Stress granules (SGs) are cytoplasmic foci at which untranslated mRNAs accumulate when cells exposed to environmental stresses. Since PDCD4 has implicated in translation repression through direct inhibition of eukaryotic translation initiation factor 4A (eIF4A), we here investigated if PDCD4 has a functional role in the process of SG assembly under oxidative stresses. Using immunofluorescence microscopy, we found that PDCD4 is relocalized to SGs under oxidative stresses. Next, we tested if knockdown of PDCD4 has an effect on the assembly of SG using PDCD4-specific siRNA.

Interestingly, SG assembly was accelerated and this effect was caused by

sensitization of phosphorylation of eIF2a and dephosphorylation of eIF4E binding protein (4E-BP). We demonstrate that knockdown of PDCD4 accelerated polysome disassembly and causes eIF4F complex disruption and ehances eIF4E:4EBP1 interaction under H_2O_2 stress. Moreover, Overexpression of PDCD4 decrease stress granule assembly under stress conditions.

These results suggest that PDCD4 has an effect on SG dynamics and possibly involved in cap-dependent translation repression under stress conditions.

국문초록

Stress granule 형성에 있어 PDCD4 의 기능적 연구

Programmed cell death 4 (PDCD4)는 새로운 종양 억제자이고 핵과 세포질에서 전사와 번역을 조절하는 기능을 한다. Stress granules (SGs)는 세포가 환경적 스트레스에 노출이 되면 비번역 mRNA가 축적된 세포질 군집을 일컫는다. 한때 PDCD4는 진핵세포의 번역개시인자 4A (eIF4A) 억제를 통하여 번역 억제와 연관이 있는 것으로 알려졌다. 여기서 우리는 PDCD4가 산화적 스트레스에서 SG 형성의 과정에 기능적인 역할을 하는지 연구했다. 우리는 면역형광염색법을 이용하여 산화적 스트레스에서 PDCD4가 SGs에 국한되는 것을 발견했다. 다음에 우리는 PDCD4 특이적 siRNA를 사용하여 감소된 PDCD4가 SG 형성에 영향을 주는지 평가했다. 흥미롭게도 SG 형성이 eIF2α의 인산화와 eIF4E 결합 단백질 (4E-BP) 의 탈인산화의 결과로 촉진되어지는 것을 확인했다. 우리는 PDCD4의 감소는 polysome disassembly을 촉진 시키고 과산화수소 스트레스에서 eIF4F 복합체를 파괴시키고 eIF4E:4EBP 상호작용을 강요한다. 게다가, PDCD4의 과발현은 스트레스 상태에서 SG 형성을 감소시킨다.

이러한 결과는 PDCD4가 SG에 역학적인 역할 형성에 관여하고 스트레스 상태에서 cap 의존성 번역 억제에 관여 한다는 것을 밝혔다.

I. Introduction

In eukaryotic cells exposed to environmental stress, inhibition of translation initiation conserves energy for the repair of cellular damage. Untranslated mRNAs that accumulate in these cells move to discrete cytoplasmic RNA-protein complexes known as stress granules (SGs) [1]. Stress granules contain nontranslating mRNAs, translation initiation component, and many additional protein affecting mRNA function [10]. The assembly of SGs helps cell survival under adverse environmental conditions by modulating various aspects of cell metabolism [2]. Stress granules typically contain poly(A)+ mRNA, 40S ribosomal subunit, eIF4E, eIF4G, eIF4A, eIF4B, poly(A) binding protein (PABP), eIF3, and eIF2, although the composition can vary. Many stress responses inhibit translation upstream of 48S complex formation by impairing eIF4E function or via phosphorylation of eIF2, which then limits the formation of a 43S complex containing eIF2, the initiator tRNA, eIF3, and the 40S subunit [3,4,5]. This results from reduced assembly of the pre-initiation complexes eIF4F (composed of eIF4E, eIF4G, and eIF4A) [6, 7, 8] and 43S (composed of the small ribosomal subunit in association with several initiation factors). Assembly of the eIF4F complex is inhibited by eIF4E-binding proteins (4EBPs) that interfere with interactions between eIF4E and

eIF4G [18-21]. Stress-induced inactivation of the PI3K-mTOR pathway reduces the constitutive phosphorylation of 4EBPs to promote the assembly of inhibitory eIF4E:4EBP complexes [16, 17]. Assembly of the 43S complex is inhibited by stress-induced activation of PKR, PERK, GCN2 and HRI, kinases that phosphorylate eIF2α, a component of the eIF2-GTP-tRNAMet ternary complex essential for 43S assembly [9, 12]. These complementary mechanisms are primarily responsible for the global repression of protein synthesis observed in cells subject to adverse environmental conditions [13, 14, 15]. Thus, SGs are thought to promote cell survival under stress conditions by modulating various aspects of cell metabolism.

Programmed cell death 4 (PDCD4) is a novel tumor suppressor encodes a multi-function in the nucleus and the cytoplasm that is involved in the regulation of transcription and translation [22, 23]. Furthermore, PDCD4 plays an important role in suppressing tumorigenesis by regulating several other genes involved in related processes including apoptosis, cell cycle, and cell proliferation [24]. Studies in cultured ovarian cancer cells, for example, suggest that PDCD4 suppresses proliferation and cell cycle progression and induces apoptosis. Acting via the MA-3 domain, PDCD4 acts as a translation inhibitor, thereby influencing protein patterns in the cells.

Additionally, PDCD4 has been shown to be regulated by a diverse set of molecules, including topoisomerase-inhibitors, COX-2 inhibitors, Akt, and various mitogens. Recently, down decreased expression of Pdcd4 has been identified in various human solid neoplasias (human colon cancer, ovarian cancer, lung cancer, primary pancreatic cancer, and glioma) [25-28]. As a translation regulator, Pdcd4 interacts with the eukaryotic translation initiation factor eIF4A, a RNA helicase that catalyzes the unwinding of mRNA secondary structures in 5'-untranslated regions (UTRs) [29]. Binding of Pdcd4 to eIF4A is mediated by the MA-3 domains, whose structure and complex formation with eIF4A have been analyzed [30-34].

Because binding of Pdcd4 to eIF4A inhibits the helicase activity of eIF4A [35, 36] it is believed that Pdcd4 functions as a suppressor of cap-dependent translation of mRNAs with structured 5'-UTRs. This assumption was supported by studies, which assessed the effects of Pdcd4 on the translation of artificial RNAs containing 5'-hairpin structures [36]; however, because physiological translational target mRNAs for Pdcd4 have not yet been unambiguously identified, it is presently unclear whether translation of all RNAs containing structured 5'-UTRs are suppressed by Pdcd4 or whether there are mechanisms that allow Pdcd4 to target specific mRNAs.

Reactive oxygen (ROS) are an important trigger for SG assembly

[37]. Oxidative stress elicits elevated levels of ROS, such as hydrogen peroxide (H2O2), superoxide (O2−) and hydroxyl radical (HO−), which cause increased permeability of the blood-brain barrier, tubulin alterations, and perturbation in synaptic transmission [38], and are known to play an important role in neuronal cell death [39]. Therefore, ROS induction becomes a prominent feature of many neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Increasing evidence has further shown that under pathological conditions, excessive amounts of ROS can readily react with thiol groups of proteins, thereby disrupting the structure of cellular proteins and altering their functions, and also activate related signaling pathways, leading to neuronal apoptosis or neurodegeneration [40-46].

In the present study, we found that knockdown of PDCD4 of accelerates stress granule assembly through sensitization of phosphorylation of eIF2α and dephosphorylation of eIF4E binding protein(4E-BP) under oxidative stress. Furthermore we show knockdown of PDCD4 triggers SG assembly by disrupting the eIF4F complex under H_2O_2 stress. PDCD4 is a key component of the translation initiation machinery. Our results reveal that depletion of PDCD4 triggers SG assembly that may have unique properties in the regulation of the stress response program.

II. Materials and Methods

II-1. Cell culture and transfection.

The human osteosarcoma (U2OS) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 500 U/ml penicillin-streptomycin at 37 ℃ in a humidified atmosphere of 5 % CO_2 in air. U2OS cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or Fugene 6 (Roche, Indianapolis, IN, USA). After 36 h, the cells were lysed by using RIPA buffer (150 mM sodium cloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, and 50 mM Tris-HCl pH 8.0).

II-2. Reagents and antibodies.

The Sodium arsenite, hydrogen peroxide (H_2O_2) and cyclohexamide were purchased from Sigma. The 4EBP1, eIF4E, p-4EBP1 antibodies were purchased from Cell Signaling (Danvers, MA, USA). The PDCD4, β-actin (AC15), eIF4A1, RPL13A antibodies were purchased from Abcam (Cambridge, MA, USA). p-eIF2α (Ser52) was purchased from Biomol (Farmingdale, NY, USA). The eIF3b, G3BP, eIF4G, PABP and GFP was purchased from Santa Cruz Biotechnology. The FLAG-M2 antibody was

purchased from Sigma. Secondary antibodies conjugated with horseradish peroxidase (HRP) were from GE Healthcare. Cy2-, Cy3-, and Cy5-conjugated secondary antibodies were purchased from Jackson Immunoresearch Labs.

II-3. Construction of plasmids.

The PDCD4 coding region was amplified from pCI-neo-flag-PDCD4 for 35 cycles (94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min) using EX polymerase (TAKARA) and primers with *XhoI* and *BamHI* cloning sites GATCCTCGAGTCATGGATGTAGAAAATGAG and GATCGGATCCTCAGTAGC TCTCTGGTTTAAG respectively. The inserts were cut with *XhoI* and *BamHI* and cloned in-frame with the C-terminal in pmCherry that was similarly cut. The G3BP1 coding region was amplified from pEF1α-flag-biotag for using primers with *XhoI* and *BamHI* cloning sites GATCCTCGAGATGGTG ATGGAGAAGCCTAG and GATCGGATCCTCACTGCCGTGGCGCAAGCC respectively. The inserts were cut with *XhoI* and *BamHI* and cloned in-frame with the C-terminal in pEGFP that was similarly cut.

II-4. Preparation of cell lysate and western blot analysis.

The U2OS cells were washed with PBS twice and lysated in RIPA

buffer supplemented with protease and phosphatase inhibitor cocktails. After incubation on ice for 15 min, the supernatants were collected by centrifugation at 13.000 r.p.m. for 15 min. Protein concentration was determined using a Bradford protein assay (BioRad, Hercules, CA, USA). Aliquots containing 20~35 μg protein were separated on a 12 % SDS-polyacrylamide gel and transferred to a PVDF membrane. The protein-bound membrane was incubated with an antibody, followed by HRP-conjugated secondary antibodies, and visualized by chemiluminescence (ECL).

II-5. Immunofluorescence microscopic analysis.

Cells were grown on glass coverslips, transfected, and incubated as indicated above, then washed twice in PBS-A (1X PBS with 0.02 % sodium azide), immediately fixed with 4 % (v/v) formaldehyde in PBS for 15 min at 4 ℃, and permeabilized with Methanol for 5 min at RT. Protein was detected using a primary antibody for overnight at 4 ℃, then washed twice in PBS-A, briefly stained with Cy2- or Cy3-conjugated immunoglobulin (Jackson ImmunoResearch, West Grove, PA, USA) and Hoechst 33258 (Sigma) for 1 h at RT in dark condition, washed twice in PBS-A, and the coverslips were mounted on slide glasses. Fluorescence microscopic images were taken using a Nikon Eclipse 80i fluorescence

microscope (40 X) and compiled using Adobe Photoshop software (San Jose, CA, USA).

II-6. Polysome profiling analysis.

U2OS cells ($1*10^6$) were plated and treated with siCONT or siPDCD4 for 72 hrs. Before harvest, cells were incubated with or without 0.15 Mm sodium arsenite for 40 min prior to addition of 10 mg/ml cyclohexamide, washed with cold PBS, then scrape harvested into 1 ml polysome lysis buffer (20 mM HEPES (pH 7.6), 125 mM KCl, 5 mM MgCl2, 2 mM DTT, and DEPC water) supplemented with protease inhibitor cocktail (EDTA free, PIERCE), and RNAsin (Ambion). After 15 min tumbling in the cold room, and insoluble material was pelleted by centrifugation at 13 000 r.p.m. for 10 min. The resulting supernatant extracts were then loaded onto a 17.5~50 % sucrose gradient prepared with polysome profiling buffer and ultracentrifuged for 2.5 h at 35,000 r.p.m. in a SW41-Ti rotor (Beckman, Brea, CA, USA). Following centrifugation, the gradients were fractionated using a fraction collector (Brandel, Gaithersburg, MD, USA).

Fractions were acetone-precipitated, and processed for further analysis.

II-7. RNA interference.

U2OS cells were transfected with siRNAs (40 nM) using Lipofectamine 2000 (Invitrogen). After 36 h, cells were trypsinized, reseeded, and transfected again for another 40 h. For single transfection, cells were treated for 48~72 h and processed for the next step. Knockdown efficiencies were verified by western blot analysis.

GFP siRNA (GGCUACGUCCAGGAGCGCACC) and PDCD4 siRNA (GCAUGGAGAUACUAAUGAA) was purchased from Bioneer.

II-8. Methyl Cap Pull-Down Assay

Association of PDCD4 with the cap mRNA-eIF4F complex was assessed using 7-methyl-GTP sepharose beads, which simulate cap mRNA pool *in vivo*. U2OS cell were treated with SA and H_2O_2 after knockdown contol or PDCD4 and lysed in buffer A (50 mM Tris-HCl at pH 7.4, 50 mM KCl, 1 mM EDTA, 0.5 % NP-40 and protease inhibitors). Equal amounts of protein were used in pull-down assays using 7-methyl-GTP sepharose beads for overnight at 4 °C. Sepharose-associated PDCD4-depleted supernatant was collected and assessed for PDCD4-eIF4A and PDCD4-eIF4G associations following PDCD4 immunoprecipitation. The resin was washed twice with buffer A and immunoblotted for eIF4A and eIF4G along with PDCD4 immunoprecipitates.

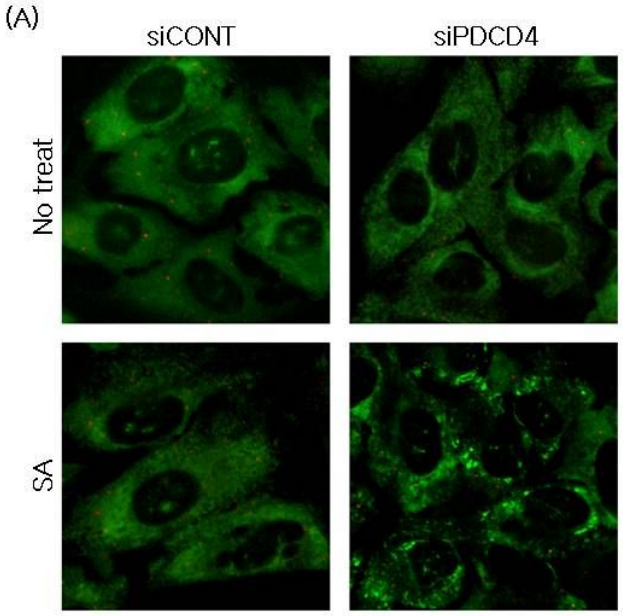
II-9. Cytosolic and Nuclear Protein Fractionation

U2OS seeded at a density of $2*10^6$ cells/ 60-mm petridish were treated with SA (0.5 mM). At the end of the experiment, nuclear and cytosolic protein fractions were isolated according to manufacturer's instructions (BioVision). Following protein estimation, equal amounts of protein were used for immunoblotting experiment as described above. Purity of the extracts was confirmed by immunoblotting for lamin B1 (nuclear).

III. Results

III-1. Knockdown of PDCD4 accelerates stress granule assembly.

To conclusively determine whether knockdown of PDCD4 affects SG assembly, U2OS cell were transfected with control or PDCD4-directed siRNA and then left untreated or treated with SA (Fig. 1A). Western blot analysis verified that siRNA targeting PDCD4 downregulated PDCD4 protein levels (Fig. 1B). We found that depletion of PDCD4 resulted in accelerates in SG assembly induced by SA.



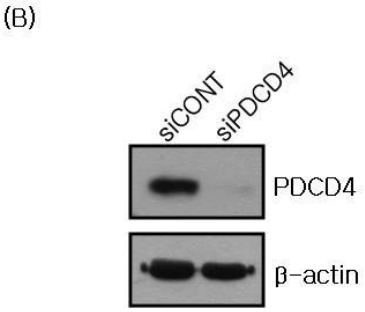


Figure 1. Knockdown of PDCD4 accelerates stress granule assembly.

(A) U2OS cells were transfected with non-target control (siCONT) or PDCD4-specific (siPDCD4) siRNAs, then treated with or without oxidative stress induced by 150 μM sodium arsenite for 40 min before processing for immunofluorescence microscopy. Stress granules were visualized as green foci (GFP-eIF3b) and p-bodies were visualized with red foci (RFP-RCK). (B) Knockdown of PDCD4 was confirmed by Western blot analysis.

III-2. PDCD4 is component of SGs under oxidative stresses.

eIF3b has been known SG marker under various stress conditions. Therefore, we next examined whether PDCD4 is component of SGs. PDCD4 was found to be localized in the nucleus of U2OS cell. Upon treatment with 0.5 mM arsenite, PDCD4 was localized in the cytoplasm and predominantly in the SGs containing eIF3b in U2OS cells. Colocalization study of endogenous PDCD4 with a SG component eIF3b confirmed that PDCD4 is also a SG component.

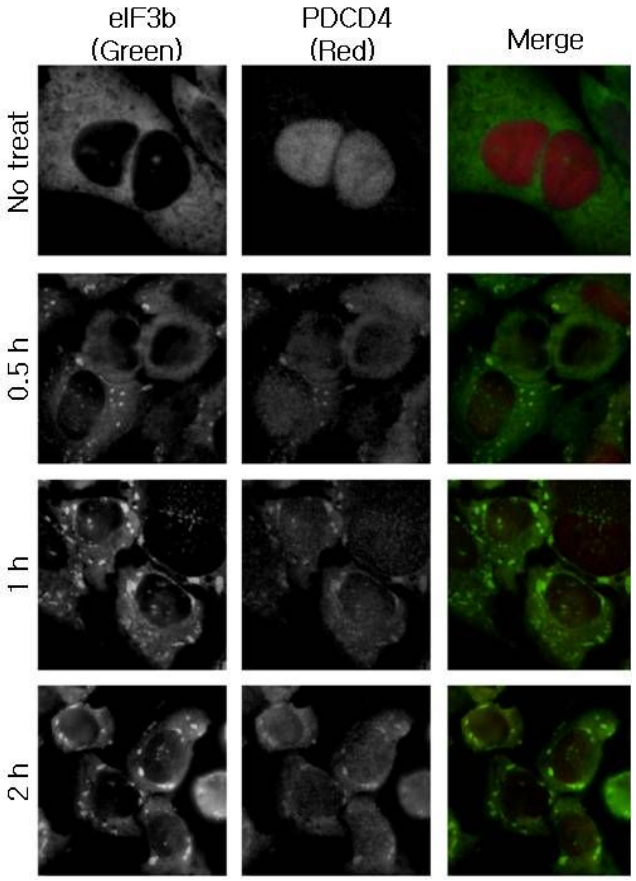


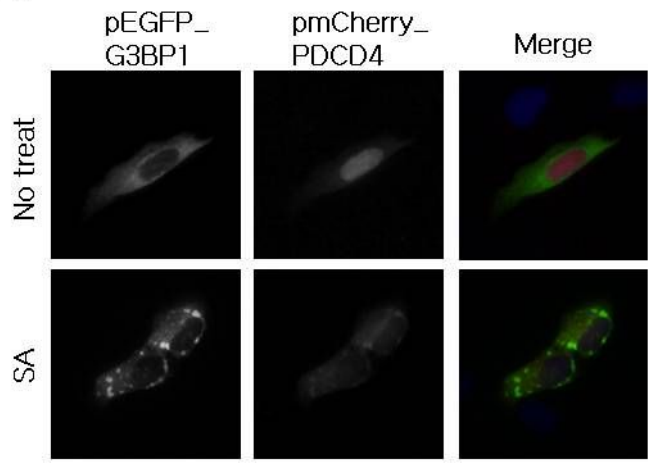
Figure 2. PDCD4 is component of SGs under oxidative stresses.

A) U2OS cells were treated with sodium arsenite (0.5 mM) for the indicated times prior to processing for immunofluorescence microscopy. Anti-eIF3b antibodies were used to visualize SGs.

III-3. PDCD4 is colocalized to SGs under oxidative stresses.

G3BP1 has been shown to be essential for the assembly of SGs under various stress conditions. Therefore, we next examined whether PDCD4 is localized in SGs. To confirm the result, pmCherry-PDCD4 and pEGFP-G3BP constructs were co-transfected and expressed for 24 hrs in U2OS cells. Upon treatment with 0.5 mM arsenite, PDCD4 was localized in the cytoplasm and predominantly in the SGs containing G3BP1 in U2OS cells (Fig. 3A). Western blot analysis show that PDCD4 antibody recognize distinct N-terminal pmCherry-PDCD4 detected PDCD4 protein in U2OS cell (Fig. 3B). These results show that PDCD4 is recruited into SG in cells exposed to stress.

(A)



(B)

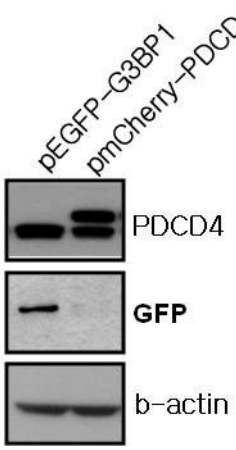


Figure 3. PDCD4 is colocalized to SGs under oxidative stresses

(A) To confirm the result, pmCherry-PDCD4 and pEGFP-G3BP constructs were co-transfected and expressed for 24 hrs in U2OS cells, SGs were then induced by treating sodium arsenite (0.5 mM, 1 hr) prior to immunofluorescence microscopy. (B) Overexpression of PDCD4 was confirmed by Western blot analysis.

III-4. Relocalization of PDCD4 from nucleus to cytoplasm under oxidative stress condition.

Programmed cell death 4 (PDCD4) is a multi-function in the nucleus and the cytoplasm that is involved in the regulation of transcription and translation. Therefore, we assessed cytoplasmic and nucleus PDCD4 expressions following SA treatment. Immunoblot analysis showed that SA transfer from nucleus PDCD4 to cytoplasmic PDCD4 (Fig. 4). Purity of the cytosolic fraction was further confirmed by α-tubulin. PDCD4 has been shown to localize in the nucleus in U2OS cell, while the SA induced cytoplasmic PDCD4.

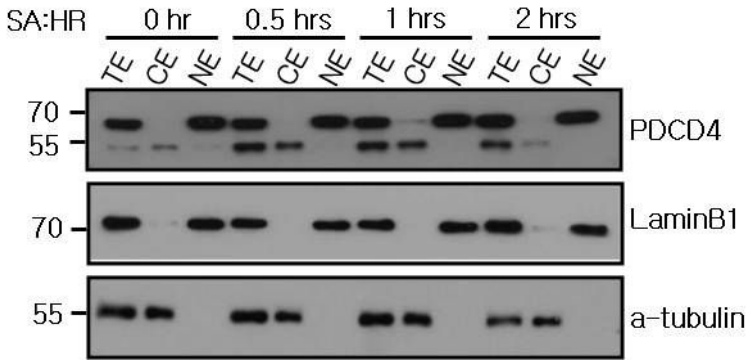


Figure 4. Relocalization of PDCD4 from nucleus to the cytoplasm by sodium arsenite treatment. Western blot analysis using α-tubulin and lamin B1 antibodies was performed on cytoplasmic extracts and nucleus extracts from control and SA-treated U2OS cell.

III-5. SG assembly was accelerated by sensitization of phosphorylation of eIF2α under sodium arsenite stress.

Several reports have shown that SA are oxidative stress that induce the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α). And eIF2α phosphorylation was shown to be essential for SG assembly in response to several stresses. We determined whether eIF2 phosphorylation is required for SG assembly in depletion of PDCD4.

We confirmed that knockdown of PDCD4 upregulate the phosphorylation of eIF2α in U2OS cell that induced by SA. As shown in Fig. 5, knockdown of PDCD4 accelerated SG assembly by sensitization of phosphorylation of eIF2α under sodium arsenite stress.

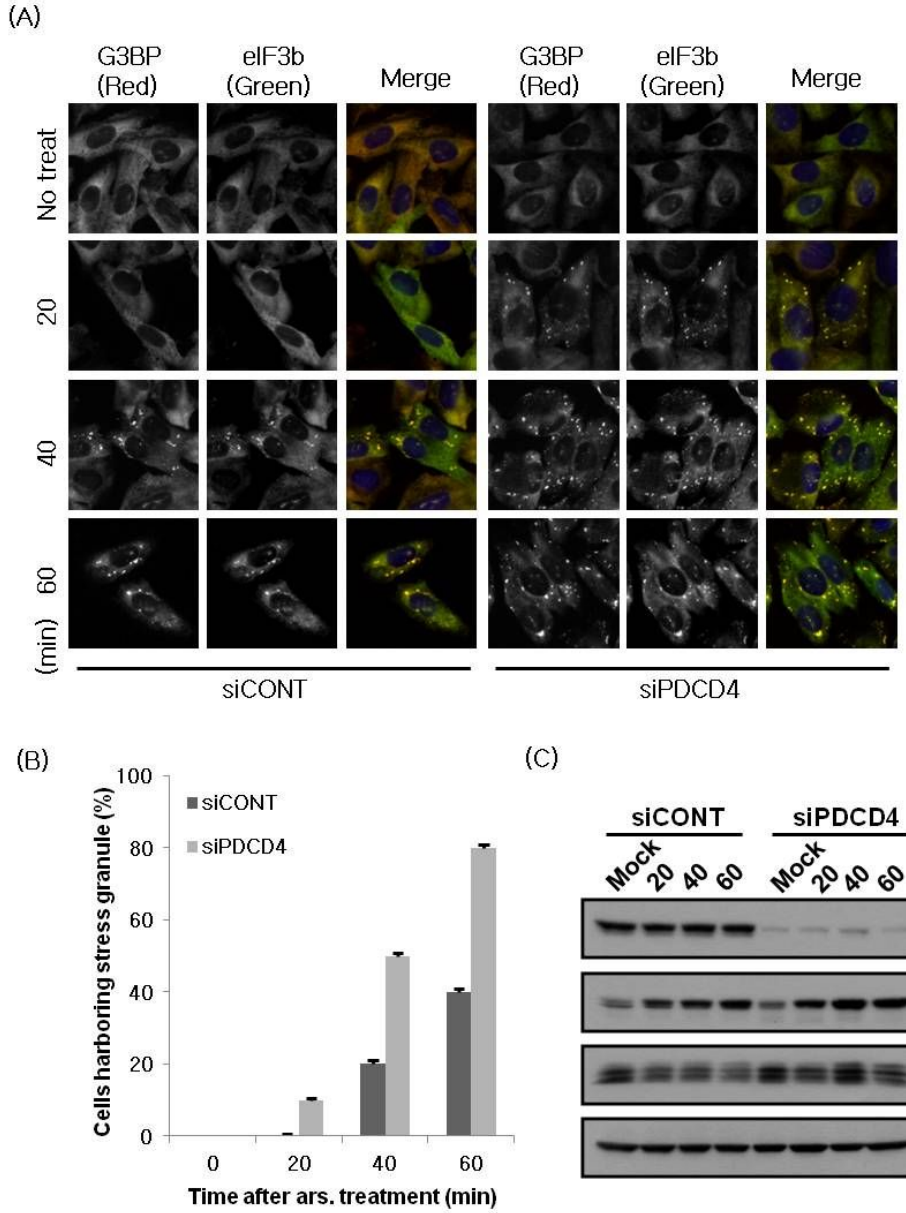


Figure 5. SG assembly was accelerated by sensitization of phosphorylation of eIF2α under sodium arsenite stress. (A) U2OS cells were treated with either control (siCONT) or PDCD4-specific (siPDCD4) siRNAs prior to treatment with sodium arsenite (0.15 mM) for the indicated times before processing for immunofluorescence microscopy. Cells were stained with eIF3b (SG marker; green), G3BP (SG marker: red). (B) Cells containing SGs were quantified from each time course. (Error bars indicate the standard deviations of the mean (n = 3, /p = 0.00036). (C) Sensitization of phosphorylation of eIF2α was confirmed by Western blot analysis.

III-6. SG assembly was accelerated by sensitization of dephosphorylation eIF4E binding protein under H_2O_2 stress.

Reactive oxygen species (ROS) are an important trigger for SG assembly. To determine whether PDCD4 accelerated SG assembly by H_2O_2, U2OS cells were transfected with control or PDCD4 directed siRNAs and then treated H_2O_2. Western blot analysis verified that siRNAs targeting PDCD4 downregulated PDCD4 protein levels (Fig. 6C). Knockdown of PDCD4 accelerates SG assembly and this effect was caused by sensitization of dephosphorylation eIF4E binding protein (4E-BP) under H_2O_2 stress (Fig. 6C).

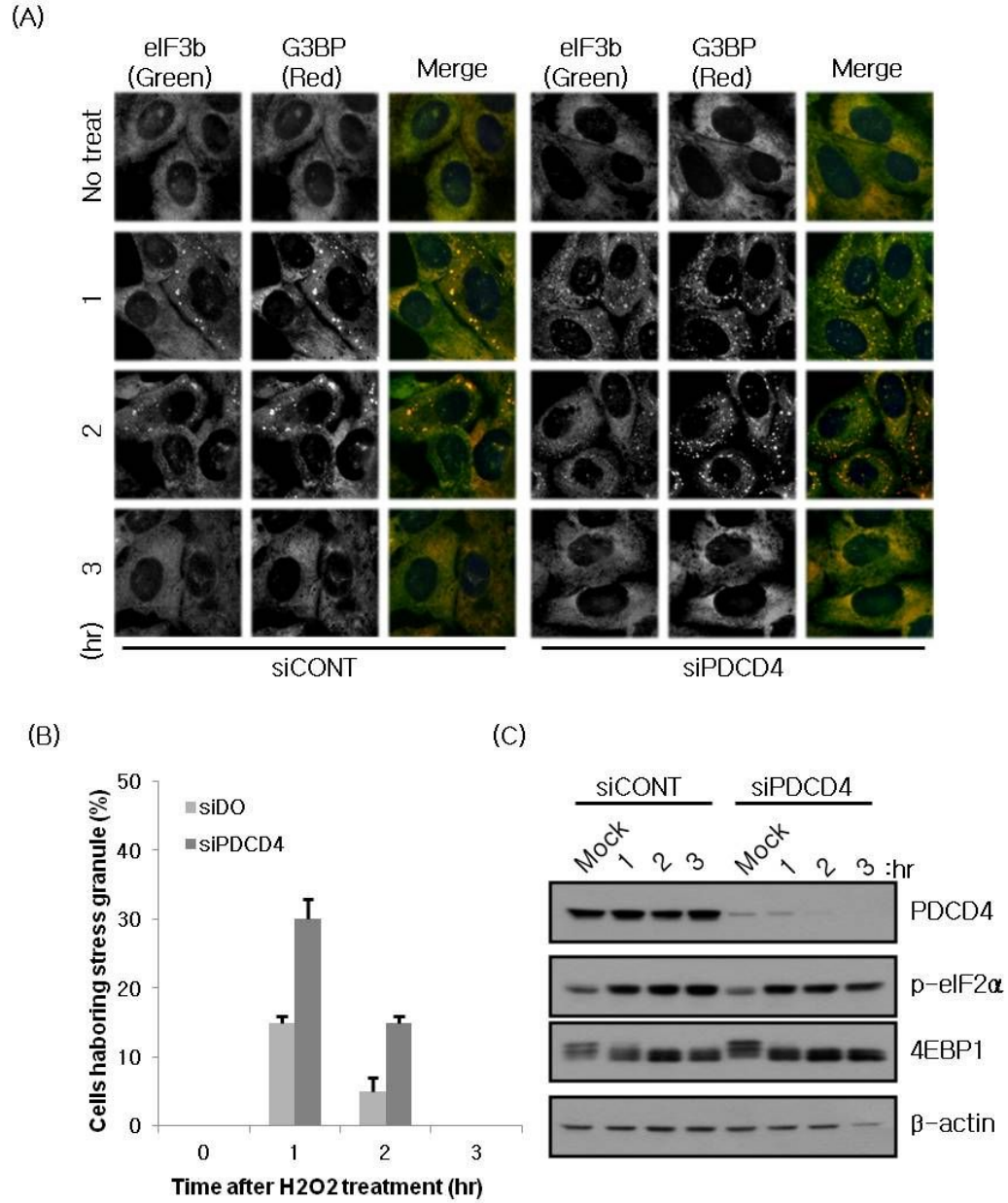


Figure 6. SG assembly was accelerated by sensitization of dephosphorylation eIF4E binding protein under H_2O_2 stress.

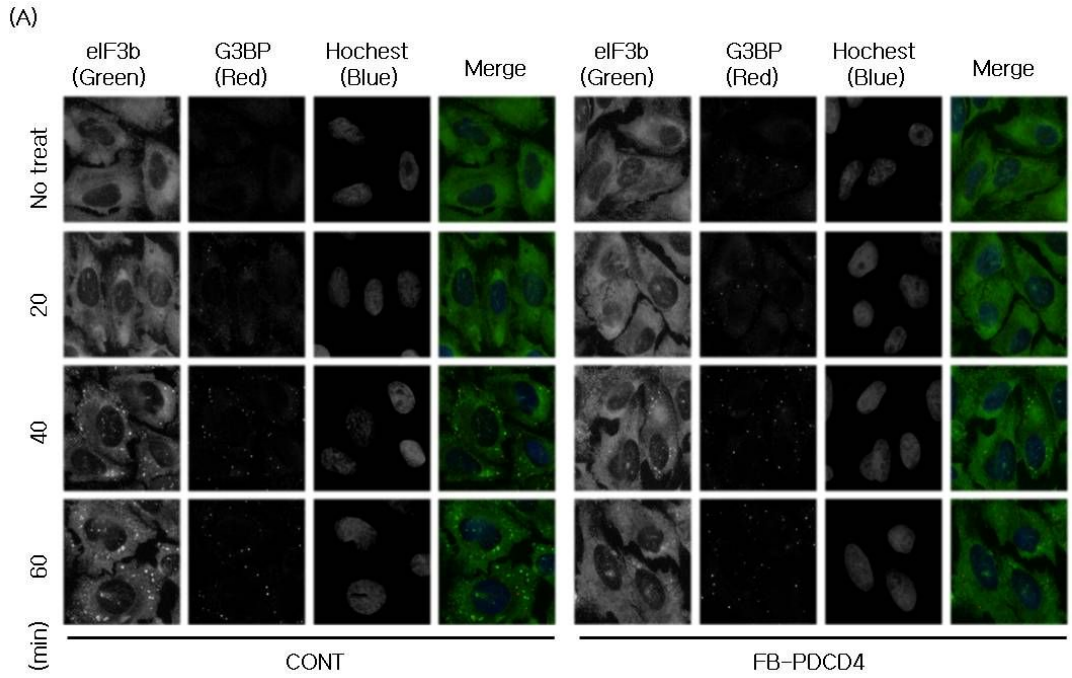
(A) U2OS cells were treated with either control (siCONT) or PDCD4-specific (siPDCD4) siRNAs prior to treatment with H_2O_2 (1 mM) for the indicated times before processing for immunofluorescence microscopy. Cells were stained with eIF3b (SG marker; green), G3BP (SG marker; red). (B) Cells containing SGs were quantified from each time course. Error bars indicate the standard deviations of the mean (n = 3, /p = 0.00036). (C) Sensitization of dephosphorylation of 4E-BP was confirmed by western blot analysis.

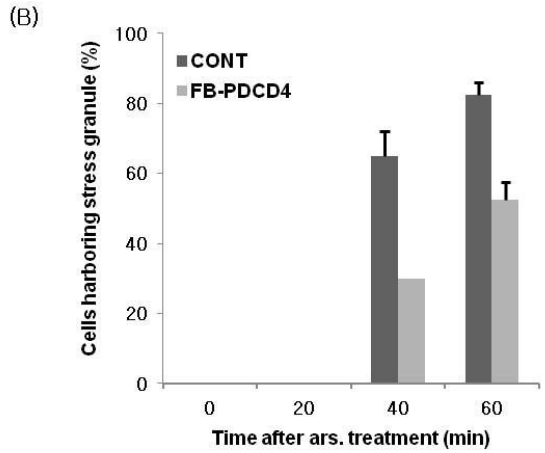
III-7. Overexpression of PDCD4 decrease stress granule assembly under SA stress

Our data suggest a novel role knockdown of PDCD4 in stress granule assembly in U2OS cells under oxidative stress. We therefore wondered if, and how, when overexpression of PDCD4 are stress granule assembly.

To address this we transfected FB-PDCD4 constructs and expressed for 24 hrs in U2OS cells, SGs were then induced by treating sodium arsenite (0.15 mM) for the indicated times before processing for Immunofluorescence microscopy. The result, stress granule assmbly was significantly decreased (Fig. 7A). And we confirmed that overexpression of PDCD4 downregulate the phosphorylation of eIF2α in U2OS cell (Fig. 7C).

These observations suggested that the suppressive effect of PDCD4 is diminished under DNA-damaging conditions. Taken together, our data suggest PDCD4 have an effect on phosphorylation of eIF2α for stress granule assembly under oxidative stress.





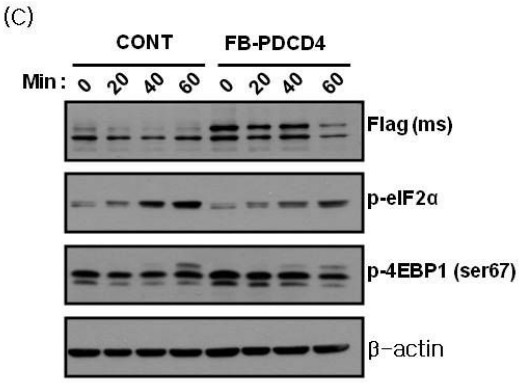


Figure 7. Overexpression of PDCD4 decrease stress granule Assembly.

(A) To confirm the result, FB-PDCD4 constructs were transfected and expressed for 24 hrs in U2OS cells, SGs were then induced by treating sodium arsenite (0.15 mM) for the indicated times before processing for immunofluorescence microscopy. Cells were stained with eIF3b (SG marker; green), G3BP (SG marker; red). (B) Cells containing SGs were quantified from each time course. Error bars indicate the standard deviations of the mean (n = 3, /p = 0.00036). (C) Overexpression of PDCD4 and sentization of phosphorylation of eIF2α was confirmed by western blot analysis.

III-8. Knockdown of PDCD4 accelerated polysome disassembly under SA stress.

We used sucrose gradient analysis to compare polysome profiles in cells expressing reduced levels of PDCD4. U2OS cells were treated with control, PDCD4 targeted siRNAs, then cultured in the absence or presence of arsenite (0.15 mM) for 40 min prior to harvesting for sucrose gradient fractionation. In control cells, arsenite-induced translational arrest results reduces of polysome profiles (Fig. 8, left panel). Knockdown of PDCD4 has no effect on the polysome profile from cells cultured in the absence of stress, but arsenite-induced polysome remarkably reduces the accumulation of polysomal RNPs (Fig. 8, right panel). Thus, PDCD4 are required for efficient polysome assembly.

(A)

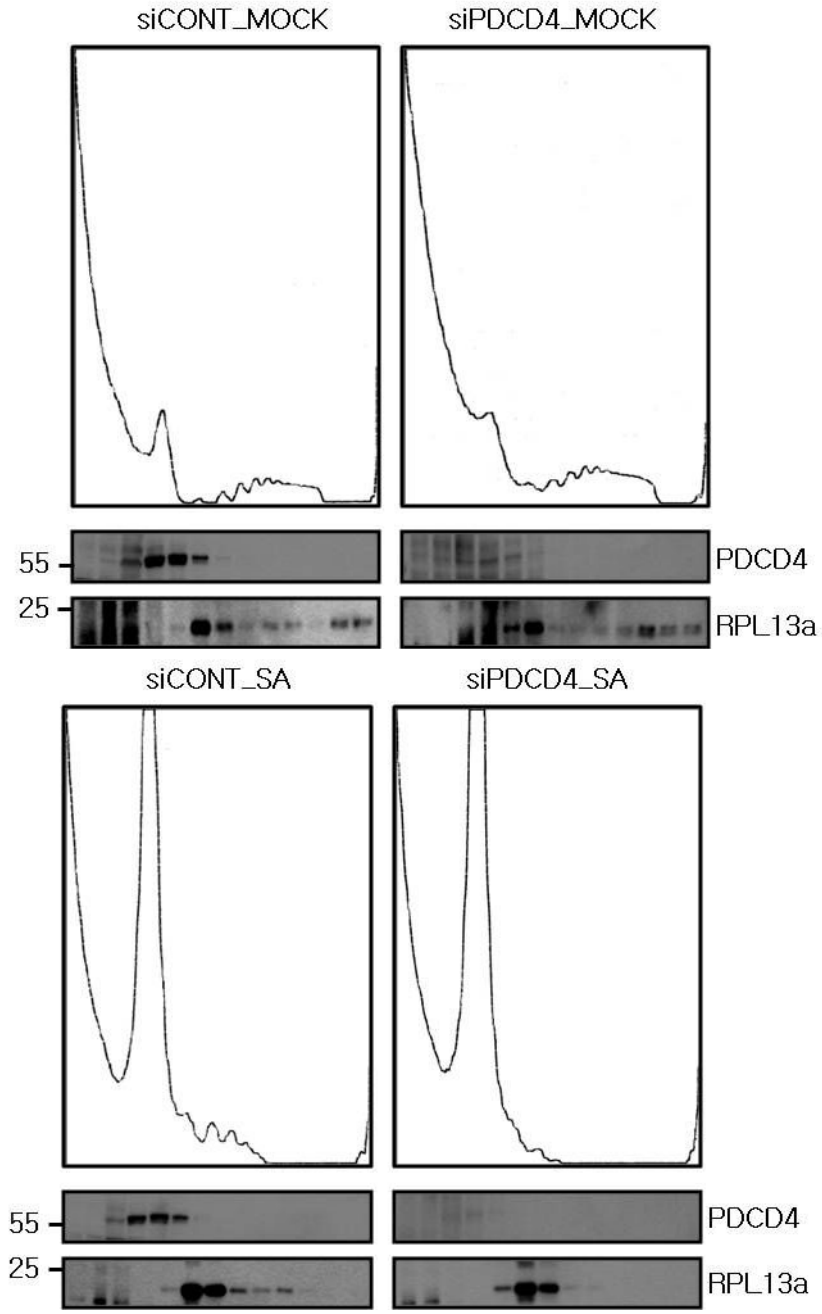


Figure 8. Knockdown of PDCD4 accelerated polysome disassembly.

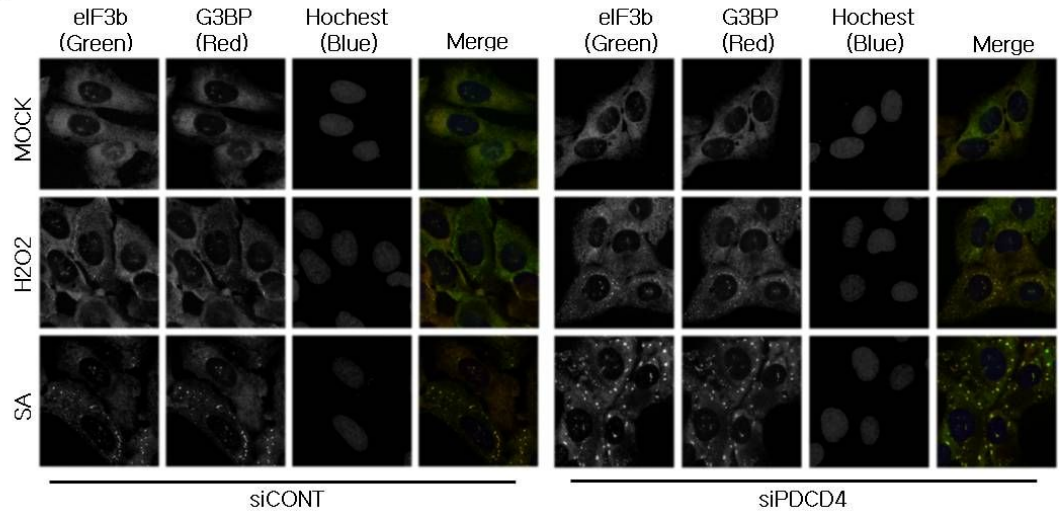
(A) U2OS cells were transfected with control (siCONT)), PDCD4-specific siRNA (si-PDCD4) then cultured in the absence (MOCK) or presence (SA) of 0.15 mM sodium arsenite before processing for sucrose gradient analysis. Fractions from control and PDCD4 knockdown sucrose gradients were analyzed by Western blotting using antibodies reactive with RPL13a.

III-9. Knockdown of PDCD4 causes eIF4F complex disruption and enhance eIF4E:4E-BP1 interaction under H_2O_2 stress.

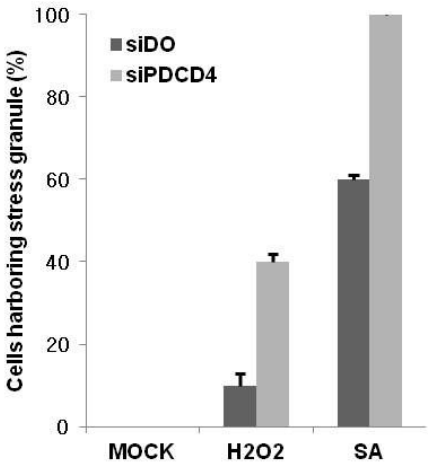
To determine whether eIF4E:4E-BP1 interaction is essential for H_2O_2-induced SG assembly, U2OS cells were transfected with control or PDCD4-directed siRNAs and then untreated or treated with H_2O_2 or SA. Western blot analysis verified that siRNAs targeting downregulated eIF4E protein levels (Fig. 9C). We found that depletion of PDCD4 resulted only in a strongly increase in SG assembly induced by SA and H_2O_2 (Fig. 9A). Inhibition of translation initiation correlates well with SG assembly in cells subjected to different types of stresses. Since H_2O_2 inhibits global protein synthesis in different types of cells primarily at the level of translation initiation it is likely that H_2O_2 targets the translation initiation machinery and thus induces SG formation. To test this hypothesis, we pulled down the eIF4F complex from lysates of SA or H_2O_2- treated U2OS cells using m7GTP-Sepharose (Fig. 9C). Although SA had no effect on the assembly of the eIF4F complex, remarkably, H_2O_2 significantly disrupted the eIF4F complex. This analysis revealed that H_2O_2 displaces eIF4G and eIF4A from eIF4E (Fig. 9C), and at the same time, H_2O_2 promotes interactions between 4E-BP1 and eIF4E. It has been well documented that dephosphorylated 4E-BP1 isoforms binds to eIF4E to displace eIF4G and inhibit translation initiation. Knockdown of PDCD4 affected H_2O_2-induced

SG assembly, resulting in more SGs (Fig. 9A and 9B). These data suggest that eIF4E:4E-BP1 complexes play an essential role in the H_2O_2-induced assembly of SGs.

(A)



(B)



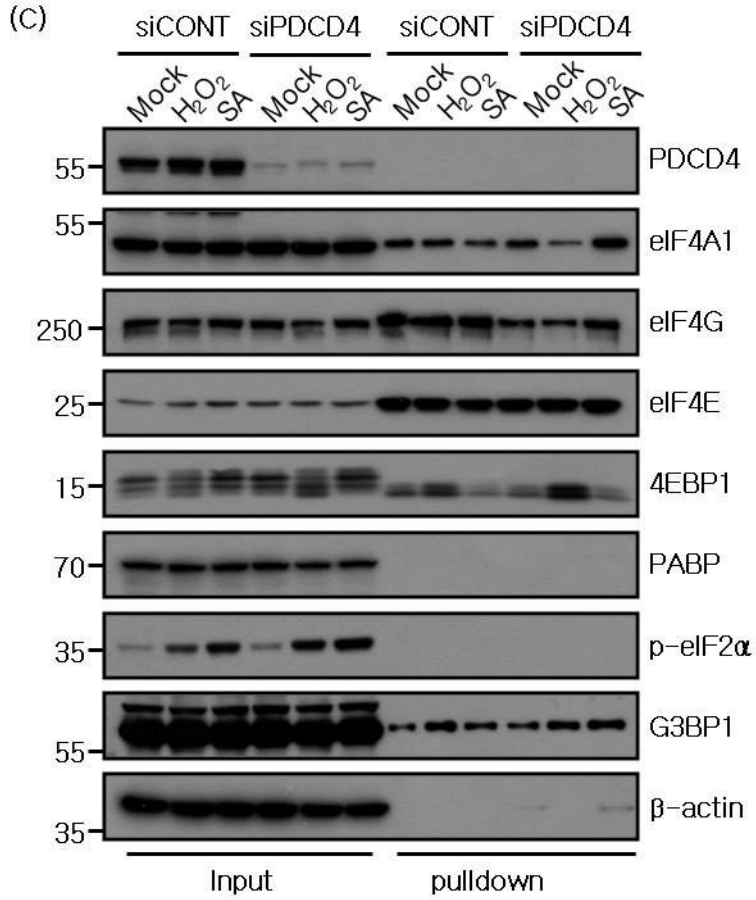


Figure 9. Knockdown of PDCD4 causes eIF4F complex disruption and enhance eIF4E:4E-BP1 interaction under H_2O_2 stress.

(A) Immunofluorescence microscopy showing SG assembly in U2OS cells after PDCD4 depletion. Cell treated with the indicated siRNAs were left untreated (No treat, upper pan or treated with 1 mM H_2O_2 (middle panels), or 100 mM SA (lower panels) and then stained with SG markers eIF3b and G3BP (red). Nuclei are stained with Hoechst. (B) Percentage of cells with SGs after PDCD4 depletion. . Error bars indicate the standard deviations of the mean (n = 3, /p = 0.00036). (C) U2OS cells without (MOCK) or with drug treatment (sodium aresenite (SA) and H_2O_2) were assembled on m7GTP-Sepharose. Western blot analysis showing the knockdown efficiency of PDCD4 in U2OS cells transfected with control siRNA (si-CONT) or PDCD4-specific siRNA (si-PDCD4). G3BP1 was used as a loading control.

IV. Discussion

PDCD4 is a well-known tumor suppressor whose levels are decreased in a variety of cancers, and a link has been identified between decreased levels of PDCD4 and increased invasiveness and tumors exhibiting greater aggression. Recent studies have been aimed at elucidating the mechanism of PDCD4 action. It has been shown that PDCD4 is able to bind to eIF4A and eIF4G, thus inhibiting the helicase activity of eIF4A and preventing cap-dependent translation, which suggests that PDCD4 acts as a general repressor of translation. However, some studies have shown that mRNAs containing a highly structured 5' UTR are preferentially repressed by PDCD4, suggesting an alternative model whereby PDCD4 has specific targets and is not a general inhibitor of translation.

SG assembly results from dynamic re-modeling of global translation in cells subjected to a variety of environmental stresses. Although SG assembly is a conserved phenomenon in a wide range of eukaryotes, the assembly and composition of SGs clearly demonstrate species-specific and stress-specific differences. Mammalian SGs assembled in response to stress-induced phosphorylation of eIF2α are composed of 40S ribosomal subunits in association with the cap-binding complex (eIF4E, eIF4A and

eIF4G), PABP and subunit of eIF3.

Here, we demonstrate that Depletion of PDCD4 accelerates stress granule assembly through sensitization of stress response pathways under oxidative stresses. First, in cultured U2OS cells, knockdown of PDCD4 accelerates SG assembly (Fig. 1). SGs have been shown to harbor untranslated mRNAs that accumulate as a consequence of stress-induced translational arrest. Second, using immunofluorescence microscopy, we found that PDCD4 is a component of SGs formed in response to oxidative stresses (Fig. 2, 3). Third, programmed cell death 4 (PDCD4) is a multi-function in the nucleus and the cytoplasm that is involved in the regulation of transcription and translation. In this regard, immunoblot analysis showed that SA transfer from nucleus PDCD4 to cytoplasmic PDCD4 under SA (Fig 4). Next, we confirmed that knockdown of PDCD4 upregulate the phosphorylation of eIF2α in U2OS cell that induced by SA. As shown in Fig 5, knockdown of PDCD4 accelerated SG assembly by sensitization of phosphorylation of eIF2α under sodium arsenite stress. SA induces eIF2α phosphorylation, the phospho-eIF2α itself is required for SA-induced SGs. On the other hand SG assembly was accelerated by sensitization of dephosphorylation eIF4E binding protein under H_2O_2 stress. H_2O_2 indirectly promotes binding of 4E-BP1 to eIF4E by promoting 4E-BP1 dephosphorylation (Fig 6). This raises the possibility that mTOR which

regulates 4E-BP1 phosphorylation might be involved in H_2O_2-induced SG assembly. Moreover, knockdown of PDCD4 accelerated polysome disassembly under SA stress (Fig. 8). Therefore, knockdown of PDCD4 may directly affect the aggregation of untranslated mRNAs.

In summary, we have identified functional roles of PDCD4 on arsenite-induced stress response and polysome disassembly, and finally, stress granule assembly. PDCD4 has known to be a general translation inhibitor targeting eIF4A, a helicase that function start codon scanning performed by translational preinitiation complex. Recently, PDCD4 has also identified as a target mRNA-specific regulator involved in apoptotic cell death procedure under severe stress conditions. However, this target-specific regulatory role is not clear which is mainly because it doesn't have RNA recognition motifs. Hence, systematic large-scale gene expression profiling analysis searching for genes controlled by PDCD4 would be very valuable and this would allow biological relevance of PDCD4 in the process of apoptosis.

V. References

[1] Keith A. Spriggs, Martin Bushell, and Anne E. Willis, Translational Regulation of Gene Expression during Conditions of Cell Stress, Molecular Cell 40 (2010) 228-237.

[2] Sonenberg, N., and Hinnebusch, A.G. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136 (2009) 731–745.

[3] Hinnebusch, A.G. Translational regulation of GCN4 and the general amino acid control of yeast. Annu. Rev. Microbiol. 59 (2005) 407–450.

[4] Bushell, M., Wood, W., Carpenter, G., Pain, V.M., Morley, S.J., and Clemens, M.J. Disruption of the interaction of mammalian protein synthesis eukaryotic initiation factor 4B with the poly(A)-binding protein by caspaseand viral protease-mediated cleavages. J. Biol. Chem. 276 (2001) 23922–23928.

[5] Jackson, R.J., Hellen, C.U., and Pestova, T.V. The mechanism of eukaryotic translation initiation and principles of its regulation. Nat. Rev. Mol. Cell Biol. 11 (2010) 113–127.

[6] Raught, B., and Gingras, A.C. Signaling to translation initiation. In Translational Control in Biology and Medicine, M. Mathews, N. Sonenberg, and J.W.B. Hershey, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. (2007) 369–400.

[7] Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. Capdependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol. Cell 3 (1999) 707–716.

[8] Tee, A.R., Tee, J.A., and Blenis, J. Characterizing the interaction of the mammalian eIF4E-related protein 4EHP with 4E-BP1. FEBS Lett. 564 (2004) 58–62.

[9] Hinnebusch, A.G., Dever, T.E., and Asano, K. Mechanism of translation initiation in the yeast Saccharomyces cerevisiae. In Translational Control in Biology and Medicine, M. Mathews, N. Sonenberg, and J.W.B. Hershey, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. (2007) 225–268.

[10] Nancy L. Kedersha, Mita Gupta, Wei Li, Ira Miller, and Paul Anderson, RNA-binding Proteins TIA-1 and TIAR Link the Phosphorylation of eIF-2a to the Assembly of Mammalian Stress Granules, JCB 147 (1999) 1431-1441.

[11] Berlanga, J., S. Herrero, and C. De Haro. Characterization of the heminsensitive eukaryotic initiation factor 2a kinase from mouse nonerythroid cells. J. Biol. Chem. 273 (1998) 32340–32346.

[12] Gray, N.K., and M. Wickens. Control of translation initiation in animals.

Annu. Rev. Cell Dev. Biol. 14 (1998) 399–458.

[13] Srivastava, S.P., K.U. Kumar, and R.J. Kaufman. Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. J. Biol. Chem. 273 (1998) 2416–2423.

[14] Harding, H.P., Y. Zhang, and D. Ron. 1999. Protein translation and folding are coupled by an endoplasmic-reticulum resident kinase. Nature. 397 (1999) 271–274.

[15] Murtha-Riel, P., M.V. Davies, B.J. Scherer, S.-Y. Choi, J.W. Hershey, and R.J.Kaufman. 1993. Expression of a phosphorylation-resistant eukaryotic initiation factor 2 alpha-subunit mitigates heat shock inhibition of protein synthesis. J. Biol. Chem. 268 (1999) 12946–12951.

[16] Long Chen, Baoshan Xu, Lei Liu, Yan Luo, Jun Yin, Hongyu Zhou, Wenxing Chen, Tao Shen, Xiuzhen Han, and Shile Huang, Hydrogen peroxide inhibits mTOR signaling by activation of AMPKα leading to apoptosis of neuronal cells, Bipolar Disord 7 (2005) 260-265. Lab Invest 90 (2010):762–773.

[17] Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol. 10 (2009) 307–318.

[18] Sarbassov DD, Guertin DA, Ali SM, et al. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 307 (2005) 1098–1101.

[19] Gao X, Zhang Y, Arrazola P, et al. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol. 4 (2002) 699–704.

[20] Inoki K, Li Y, Zhu T, et al. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol. 4 (2002) 648–657.

[21] Tee AR, Fingar DC, Manning BD, et al. Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc Natl Acad Sci USA. 99 (2002) 13571–13576.

[22] P Singh, L Wedeken, LC Waters, MD Carr and K-H Klempnauer. Pdcd4 directly binds the coding region of c-myb mRNA and suppresses its translation, Oncogene 30 (2011) 4864–4873

[23] Lankat-Buttgereit B, Go¨ ke R. The tumor suppressor Pdcd4: recent advances in the elucidation of function and regulation. BiolCell 101 (2009) 309–317.

[24] Shibahara K, Asano M, Ishida Y, Aoki T, Koike T, Honjo T.. Isolation of a novel mouse gene MA-3 that is induced upon programmed cell death. Gene

166 (1995) 297–301.

[25] Chen Y, Knosel T, Kristiansen G, Pietas A, Garber ME,Matsuhashi S et al. Loss of PDCD4 expression in human lung cancer correlates with tumour progression and prognosis. J Pathol 200 (2003) 640–646.

[26] Afonja O, Juste D, Das S, Matsuhashi S, Samuels HH. Induction of PDCD4 tumor suppressor gene expression by RAR agonists, antiestrogen and HER-2/neu antagonist in breast cancer cells. Evidence for a role in apoptosis. Oncogene 23 (2004) 8135–8145.

[27] Zhang H, Ozaki I, Mizuta T, Hamajima H, Yasutake T, Eguchi Y et al. Involvement of programmed cell death 4 in transforming growth factor-beta1-induced apoptosis in human hepatocellular carcinoma. Oncogene 25 (2006) 6101–6112.

[28] Mudduluru G, Medved F, Grobholz R, Jost C, Gruber A, Leupold JH et al. Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer. Cancer 110 (2007) 1697–1707.

[29] Yang HS, Jansen AP, Komar AA, Zheng X, Merrick WC, Costes S et al. The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Mol Cell Biol 23 (2003a) 26–37.

[30] LaRonde-LeBlanc N, Santhanam AN, Baker AR, Wlodawer A, Colburn NH. Structural basis for inhibition of translation by the tumor suppressor Pdcd4. Mol Cell Biol 27 (2007).147–156.

[31] Waters LC, Veverka V, Bo¨hm M, Schmedt T, Choong PT, Muskett FW et al. Structure of the C-terminal MA-3 domain of the tumour suppressor protein Pdcd4 and characterization of its interaction with eIF4A. Oncogene 26 (2007) 4941–4950..

[32] Suzuki C, Garces RG, Edmonds KA, Hiller S, Hyberts SG,Marintchev A et al. PDCD4 inhibits translation initiation by binding to eIF4A using both its MA3 domains. Proc Natl Acad Sci USA 105 (2008) 3274–3279.

[33] Chang JH, Cho YH, Sohn SY, Choi JM, Kim A, Kim YC et al. Crystal structure of the eIF4A-PDCD4 complex. Proc Natl Acad Sci USA 106 (2009) 3148–3153.

[34] Loh PG, Yang HS, Walsh MA, Wang Q, Wang X, Cheng Z et al. (2009). Structural basis for translational inhibition by the tumour suppressor Pdcd4. EMBO J 28 (2009) 274–285.

[35] Yang HS, Cho MH, Zacowicz H, Hegamyer G, Sonenberg N, Colburn N.

(2004). A novel function of the MA-3 domains in transformation and translation suppressor Pdcd4 is essential for its binding to eukaryotic translation initiation factor 4A. Mol Cell Biol 24 (2004) 3894–3906.

[36] G.W. Cho, M.Y. Noh, H.Y. Kim, S.H. Koh, K.S. Kim, S.H. Kim, Bone marrow-derived stromal cells from amyotrophic lateral sclerosis patients have diminished stem cell capacity, Stem Cells Dev 19 (2010) 1035-1042.

[37] Mohamed M. Emara, Ken Fujimura , Daniele Sciaranghella , Victoria Ivanova , Pavel Ivanov a, Paul Anderson, Hydrogen peroxide induces stress granule formation independent of eIF2a phosphorylation BBRC 423 (2012) 763–769

[38] Baxter LC, Sparks DL, Johnson SC, et al. Relationship of cognitive measures and gray and white matter in Alzheimer's disease. J Alzheimers Dis. 9 (2006) 253–260.

[39] Ruffels J, Griffin M, Dickenson JM. Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: role of ERK1/2 in H2O2-induced cell death. Eur J Pharmacol. 483 (2004) 163–173.

[40] Stadtman E. Protein oxidation and aging. Science.257 (1992) 1220-1224.

[41] Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med. 18 (1995) 321–336.

[42] Figueiredo-Pereira ME, Yakushin S, Cohen G. Disruption of the Intracellular Sulfhydryl Homeostasis by Cadmium-induced Oxidative Stress Leads to Protein Thiolation and Ubiquitination in Neuronal Cells. J Biol Chem. 273 (1998) 12703–12709.

[43] Green KN, Peers C. Divergent pathways account for two distinct effects of amyloid beta peptides on exocytosis and Ca2+ currents: involvement of ROS and NF-κB. J Neurochem. 81 (2002) 1043–1051.

[44] Li Z, Arnaud L, Rockwell P, et al. A single amino acid substitution in a proteasome subunit triggers aggregation of ubiquitinated proteins in stressed neuronal cells. J Neurochem. 90 (2004) 19–28.

[45] Kim S, Moon C, Eun S, et al. Identification of ASK1, MKK4, JNK, c-Jun, and caspase-3 as a signaling cascade involved in cadmium-induced neuronal cell apoptosis. Biochem Biophys Res Commun. 328 (2005) 326–334.

[46] Chen L, Liu L, Yin J, et al. Hydrogen peroxide-induced neuronal apoptosis is associated with inhibition of protein phosphatase 2A and 5, leading to activation of MAPK pathway. Int J Biochem Cell Biol. 41 (2009) 1284–1295.

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