The Effects of Cell Stress (Serum Deprivation and Leptomycin B) on JunD Nuclear Export Inhibition

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June 10, 2016

Abstract

Cell stress can cause multiple factors within a cell, including, but not limited to, the location of RNA within a cell. Messenger RNAs follow specific pathways, playing key roles in retroviruses and their proliferation. The mRNA junD in HEK293 cells were used to see where they finally rested during normal conditions and serum deprived states. This allows us to see if we could suppress retroviruses via targeting and suppressing junD, so long as the pathway junD uses to leave nucleus and enter the cytoplasm was identified. It was originally hypothesized that junD used CRM1 pathways to move through the nucleus and into the cytoplasm. This hypothesis was disproved by serum deprivation of HEK293 cells, their fractionation, and the use of the CRM1 pathway-suppressor Leptomycin B to test it. The results showed that Leptomycin B did nothing to inhibit the path of junD out of or into (via reverse transcription) the nucleus.

Introduction

Team Tuuli's research was focused on the mRNA junD. Its main function is to defend the cell from apoptosis and regulate many of its cellular processes (1) and cell types (3). TNF-α induces apoptosis which triggers junD's defense mechanism (1). JunD is not limited to its cell in its defense strategy; it can use different signaling pathways to protect surrounding cells from apoptosis (2). AP-1, the transcription factor complex that junD is part of, is modulated by a

specific cofactor, FHL2. This cofactor is only expressed by the addition of serum, which could indicate that serum deprivation inhibits FHL2/junD expression. FHL2 is also shown to promote junD activity (7) The targeting of junD via miRNAs (9) and salinomycin (10) in human non-small cell lung cancer also reduces the proliferation of the cancer cells.

Team Tuuli's objective was to study the relationship between cell stress by serum deprivation, and junD reverse transcription and nuclear export pathway mechanisms.

Serum deprivation removes important nutrients that a cell needs to continuing functioning effectively. Serum deprivation is a cell stressor because it causes the environment surrounding the cell to change, thus forcing the cell to adapt in order to survive. In most cases when a cell undergoes serum deprivation, its mitochondria becomes a target for apoptosis. Cell cycle arrest in the G2/M phase is the next step, and is the cell's way of attempting to sustain life. However, if deprivation continues it will ultimately result in photoreceptor cell death, which mimics neurotrophin withdrawal. This results in uncontrolled cell death (3). Serum deprivation can also cause DNA fragmentation (4), as we observed in lab two. The transcription process of junD is not induced by addition or deprivation of serum. However, when used for stimulation, the protein will degrade, then re-emerge and increase as the cell progresses to the G1 phase. This indicates that junD is regulated by unique post-transcriptional and post-translational control mechanisms. The translation of junD, however, is dependent upon ribosome scanning. JunD is also regulated--positively--by mitogen-activated protein kinase (2).

Various kinds of stress have different effects on how mRNA behave or act within the cell, but it is known that the nuclear pore complex becomes altered under duress (6). JunD is becoming known for its ability to control healthy transcription during oxidative stress, though this ability changes as a mammal ages. It acts like NADPH inhibition, when NADPH impairs nitric oxide

levels. Of course, the disruption of junD results in increasing DNA fragmentation in this process. (5). Heat stress affects and changes the chaperone proteins that associate with their corresponding mRNA, and keeps the mRNA within and on in the rim of the nucleus, while osmotic stress causes the binding proteins to exit the nucleus into the cytoplasm (6).
Team Tuuli hypothesizes that cell stress will have an effect on the mRNA junD. This was observed by serum deprivation and inoculation of leptomycin B.

Cells were cultured by growing in Dulbeccos Modified Eagle Medium in preparation for serum deprivation. Results were examined by running RT- PCR reactions of cDNA of junD. The expectation was that we would see definite junD appearance within the total and cytoplasmic fractions with added serum mixes. We expected to see little to nothing in the negative serum fractions.

Methods

Cell culture:

HEK293 cells were grown in growth medium (Dulbeccos Modified Eagle Medium, 10% FBS, 1% antibiotic). Twenty-four hours in advance, treatment cells were seeded in two six-well plates at a density of 3×10^5 cells/well. To serum-starve the HEK293 cells, the well plates were washed three times with 1x PBS. This was followed by culturing in either 10% or 0.5% FBS media for twenty-four hours.

Cell harvest and subcellular fractionation:

HEK293 cells were harvested from the serum-deprivation experiment by washing each well with 1x DPBS and adding 1 mL of 1x DPBS. Cell suspensions underwent centrifugation at 4℃ for 5 minutes at 300 x g and supernatant was separated and discarded from cell pellet. Subcellular fractionation was conducted by resuspending cell pellets in 175 mL of chilled Buffer RLN-lysis (18 µL Tris-HCl pH 7.4, 10.8 µL NaCl, 0.54 µL $MgCl_2$, 18 µL Nonidet P-40, 13.5 µL RNase OUT Ribonuclease Inhibitor, 299.16 µL RNase-free water). Cell suspensions were then incubated on ice for five minutes and centrifuged at 300 x g and 4℃ for 2 minutes, separating nuclear and cytoplasmic extracts. 100 µL of Buffer RLN-wash (12.5 µL Tris-HCl, 7.5 µL NaCl, 0.38 µL $MgCl_2$, 12.5 µL Nonidet P-40, 217.12 µL RNase-free water) was added to nuclear pellet and centrifuged under the same conditions. Supernatant was removed and discarded and nuclear fractionate was incubated on ice.

RNA extraction:

To isolate RNA from the six samples, 600 µL of Buffer RLT was added to cytoplasmic lysate and nuclear pellet. After vortexing for one minute, 430 µL of 96-100% ethanol was added to each homogenized lysate. 700 µL of each sample was transferred to RNeasy spin column and centrifuged for fifteen seconds at 8000 x g and 25℃. Note that flow-through should be discarded after every centrifuge. Addition of 700 µL Buffer RW1 to each spin column, centrifuged for fifteen seconds at 8000 x g and 25℃. Addition of 500 µL of Buffer RPE to each spin column, centrifuged for two minutes at 8000 x g and 25℃. Addition of 500 µL of Buffer RPE to each spin column, centrifuge for fifteen seconds at 8000 x g and 25℃. Added 500 µL of 80% ethanol to each spin column, centrifuged for two minutes at 8000 x g and 25℃. Moved RNeasy spin columns to new collection tube and centrifuged for one minute at 16,000 x g and 25℃. Moved

RNeasy spin columns to new collection tube (1.5 mL) and added 50 µL 65℃ RNase-free water to each sample. Centrifuged for one minute at 8,000 x g and 25℃ to elute RNA, and place on ice. Stored at -20℃ long-term. In order to validate efficient RNA isolation, samples were run on 1% gel electrophoresis.

RT-PCR:

To amplify a region of junD 16 µL of RT master mix (112 µL 1x buffer RT, 14 µL dNTP mix, 28 µL of random hexamers, 7 µL RNase OUT Ribonuclease Inhibitor, 7 µL Omniscript Reverse Transcriptase, 42 µL Rnase-free water) was aliquoted into each reaction tube. Then 4 µL of our RNA template was added to each sample and incubated at 37℃ for one hour. PCR was conducted by aliquoting 45 µL of PCR master mix (360 µL 1x Taq Polymerase Buffer, 8 µL dNTPs, 16 µL $MgCl_2$, 8 µL forward primer+ (CGCGCCTGGAAGAGAAAGTG) , 8 µL reverse primer+ (GGCGCAGCTCAAGCAGAAAG) , 8 µL Taq polymerase, 272 µL sterile water) into each reaction tube, and then adding 5 µL of our cDNA template. All tubes were placed in the thermal cycler at set conditions. Water was used as a negative control and GAPDH as a positive one by acting as a nontargeting control.

Agarose gel electrophoresis:

To prepare RT-PCR reactions for agarose gel electrophoresis 25 µL of each tube was aliquoted into PCR tubes. 5 µL of 6X loading dye was added and mixed. A total of 30 µL was loaded into appropriately-labeled wells of prepared 1.5% agarose-EtBr gel. Electrophoresis was run at 150V for 20-30 minutes.

Results

To examine the effects of cell stress on the biology of junD RNA, HEK293 cells were first cultured under the conditions of 10% serum media or 0.5% serum media for 24 hours. Under observation, the serum deprived HEK293 cells were 60% confluent (Table 1). The HEK293 cells incubated in 10% serum media were healthy at a 90% confluency. The drug Leptomycin B (LMB) was added to the next round of 24 hour serum deprivation treatment using HEK293 cells. The HEK293 cells drugged with LMB and incubated with 0.5% serum media were observed to have a 30% confluency (Table 1). The other HEK293 cells treated with LMB and incubated in 10% serum media were observed to have 90% confluency (Table 1).

Table 1: Observations of HEK293 cells throughout experimental treatments (plating, serum-starving, serum-starving and Leptomycin B drugging, subcellular fractionation and harvest)

Date	Overall health and confluence (%)	Experiment
4/13/2016	95	Plating HEK293 cells for serum deprivation
4/14/2016	60	Serum-starved HEK293 cells
4/15/2016	90- 10% serum media 30- 0.5% serum media	Harvest and subcellular fractionation
5/11/2016	99	Plating HEK293 cells for serum deprivation
5/12/2016	60	Serum-starved HEK293 cells and inoculated with LMB
5/13/2016	25	Harvest and subcellular fractionation

Gel electrophoresis was run using 10% of extracted RNA in order to confirm the validity of successful RNA extraction of the treated HEK293 cells. The results of Figure 1 show a successful extraction of RNA in the total, cytoplasmic and nuclear fractions (Fig. 1 lanes 1, 2, 3). Noting the present 28S and 18S ribosomal RNA bands, and that the 28S is approximately twice the size of the 18S, it indicated that the mRNA remained intact. Bands appeared more in the fractions that contained the 10% serum media (+serum) and of the 0.5% serum (-serum) bands were only observed in the cytoplasmic fraction (Fig.1 lane 6).

Gel electrophoresis was run on RT-PCR reactions to observe subcellular localization patterns.The results of Figure 2 showed bands observed in the total and cytoplasmic fractions of junD (Fig. 2A lane 2, 4). 0.5% reactions were run again due to inconclusiveness in previous results. JunD's nuclear fraction is affected by cell stress specifically (Fig. 2C).

To assess the effects of leptomycin B (LMB) on RNA nuclear export gel electrophoresis was run. Figure 3 is the RNA results of nuclear export inhibition using Leptomycin B (LMB). It was hypothesized that this treatment would result in thick bands appearing in the nucleus and total lanes (Fig. 3 lanes 1, 3, 4, and 6), and little-to-no bands in the cytoplasm lanes (Fig. 3 lanes 2 and 5). The results matched expectations, indicating that RNA was sufficiently suppressed by LMB, and stayed within the nucleus.

To determine whether or not junD uses the CRM1 pathway gel electrophoresis. Figure 4 is the RT-PCR result of HEK-293 cells for junD (a) and GAPDH (b). For junD, positive PCR bands were not detectable for lanes 2, 3, 4, and 8, which reflect that there was no RNA in those samples. Lanes 2 or 3 were expected to show positive PCR products, indicating the presence of junD because they are both plus reverse transcriptase and plus serum. However, PCR products were not observed. Lanes 4 and 8 demonstrated non-detectable PCR products, which was given

for lane 4 had no serum and lane 8 did not undergo RT. The bands in lanes 5-7 and 9-12 were weaker than expected, showing that only very little junD is present in the minus serum samples and the minus reverse transcription samples. There was a visible band observed for lane 13, the water control, which should have had no band, indicating that there may have been some contamination in this sample and possibly others. GAPDH was used as a control to insure that the reverse transcriptase was working correctly. The GAPDH lanes turned out as expected meaning that there was a visible band for every lane, excluding lane 13 the water control.

Discussion

Based upon the results and contemporary readings, cell stress alters the production of junD in the cell. When a cell undergoes serum deprivation it becomes stressed it starts producing very little RNA, and what can be observed on an agarose gel is mostly found in the cytoplasm. Under normal conditions, the cell produces large amount of RNA clearly visible on an agarose gel, and the RNA is primarily in the cytoplasm. Cell stress alters the amount of RNA produced by the cell. These conclusions are the same when Omniscript Reverse Transcriptase is used to amplify junD from the rest of the RNA. Leptomycin B alters the localization of RNA dependent on the CRM1 pathway by shutting down that pathway, so that they cannot exit the nucleus. When leptomycin B was applied to the cells, there was no alteration in the localization of junD, so junD must use the TAP/NXF1 nuclear export pathway. This is in line with the hypothesis. It was expected that cell stress would have an affect on junD, and it was observed that cell stress results in decrease in production of junD.

The findings of this research are significant to the understanding of RNA metabolism during cell stress. They are important because the findings show that junD, an mRNA that protects against apoptosis (1), is less abundant during cell stress. This means that when a cell is stressed its mechanism to protect against death no longer works and the cell can die. So a decrease in RNA metabolism is a regulatory effect in and of itself. It shuts down certain pathways, which were preventing other pathways from operating. One significant conclusion that can be drawn from the results is how junD relates to cancer. It has already been shown that may junD pathway contribute to inhibiting hepatocellular carcinoma invasion and metastasis by JunD-regulated MMP9 expression(10). Another possibility is that junD prevents apoptosis by blocking TNF-α, which induces apoptosis (1). A lack of junD results in cell death, according to the results. If junD could be removed from cancerous cell through serum starvation or target miRNAs (9), it may result in the death of cancerous cells. Another very significant conclusion is how the findings relate to HIV. For HIV-1 to be expressed, it is dependent on interactions between host cellular *cis*-regulatory factors and the HIV-1 long terminal repeat (TLR). JunD is a part of AP-1, a transcription factor complex (7), which is important to HIV-1 for virus infectivity (11). Potentially, if junD is removed from the cell, either throughs serum deprivation, target miRNAs, or another method, the spread of HIV-1 could be halted because a lack of junD would cause the AP-1 to function incorrectly. If AP-1 cannot function, it will not bind to the long terminal repeat and thus, prevent the induction of HIV-1 (11).

For future studies, it is proposed that steps are taken to investigate junD and cancer to see if removing junD from cancerous cells to see if it allows TNF-α to induce apoptosis and kill the cancerous cell. The next experiment that should be conducted is serum-starving cancerous cells

to observe confluency, and also to extract the RNA and study the levels of junD to see if there is a correlation between confluency and the amount of junD. The overall future direction of this research should be to finding a possible cancer treatment related to junD, like a specific miRNA to target junD in cancerous cells. Future studies could also conduct experiments to study the effects of removing junD from HIV-1 infected cells to see if it has an affect on the infectivity of the virus. This could be done by serum starving the HIV-1 and healthy cells, and extracting the RNA to see if there is a correlation between the spread of the virus and the levels of junD. The overall objective of this research should be to find a possible treatment for HIV-1 that either stops the spread of the virus or cure the disease altogether.

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