ACCELERATED ARTICLE

Simultaneous Inhibition of SRC and STAT3 Induces an Apoptotic Response in Prostate Cancer Cells

Sherman Leung[1,3], Elizabeth Duval[2], and Olga Timofeeva[4]
Student[1], Teacher[2]: *Science, Mathematics, and Computer Science Magnet Program*, Montgomery Blair High School
51 East Boulevard, Silver Spring, MD, 20901
Intern[3], Mentor/Assistant Professor[4]: *Lombardi Comprehensive Cancer Center,* Georgetown University
3800 Reservoir Road, N.W. Washington, DC 20007

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Abstract

Prostate cancer is the most frequently diagnosed non-cutaneous cancer and the second leading cause of cancer-related deaths in American men. Recent studies have suggested that sarcoma inducing kinase (SRC) and its downstream signaling targets are implicated in prostate cancer, but the mechanisms behind how SRC inhibition induces apoptosis are still poorly understood. This study focused specifically on the interactions between SRC and its one of its downstream targets, Signal transducer and activator of transcription 3 (STAT3), in prostate cancer cells. Contrary to studies on the SRC/STAT3 pathway, western blotting showed that SRC inhibition had minimal effects on phosphorlyated-STAT3 levels in DU145 prostate cancer cells. Simultaneous inhibition of both SRC and STAT3 through PP2 (inhibits SRC expression) and STAT3 siRNA, respectively, led to more distinct poly (ADP-ribose) polymerase 1 (PARP-1) cleavage, a hallmark indicator of apoptosis. qRT-PCR analysis showed a two-fold and three-fold decrease between simultaneous versus exclusive treatments in levels of induced myeloid cell leukemia (MCL-1), a pro-survival gene. Together, these findings suggest that the inhibition of STAT3 through SRC is ineffective and that the independent inhibition of STAT3 induces a stronger apoptotic response. Additionally, the study suggests that the simultaneous inhibition of SRC and STAT3 may be a novel and promising treatment for prostate cancer.

Introduction

Prostate cancer affects 35% of all American men[1,2], and is the most frequently diagnosed non-cutaneous cancer in males. Men who develop androgen-independent prostate cancer (AIPC) are at a loss for any established effective treatments; however, recent studies have suggested that sarcoma inducing kinase (SRC) is a possible target in treating AIPC cells[3,4]. It has been shown that a subset of patients with AIPC, who exhibit an increase in SRC activity during the transition of prostate cancer to an androgen-independent state, have a poorer prognosis and a reduced overall survival[3]. When deprived of androgen simulation, AIPC cells develop the ability to survive and thrive by up-regulating oncogenic pathways where tyrosine kinases like SRC play a crucial role[3,4]. SRC inhibitors, currently in phase III of clinical trials, have been suggested as a viable and promising treatment option for patients with AIPC[4]; yet the mechanism by which SRC inhibitors trigger cancer cell death and prevent metastasis is still not completely understood.

Signal transducer and activator of transcription 3 (STAT3), a downstream phosphorylation target of SRC[4], has been identified as a substrate of v-src (viral src) necessary for enabling v-src induced adhesion-independence and malignant transformation[5]. As a transcription factor, STAT3 activates the expression of pro-survival genes (e.g. MCL-1, BCL2, BCL-xL) and genes that drive the cell cycle (e.g. c-MYC, cyclin D1) by binding to their promoters[6]. This results in increased survival and proliferation of cancer cells[6,7]. Significantly, SRC has been shown to induce cell transformation through STAT3-mediated gene regulation[7]. Recent research has suggested that the knockdown of STAT3 expression via RNA interference may be effective in inhibiting growth of prostate cancer cells[8]; yet, the mechanism by which STAT3 interacts with SRC in prostate cancer has not been investigated.

The initial aim of this study was to investigate the results of SRC inhibition. It was expected that SRC inhibitors would slow cancer growth and induce apoptosis through the inhibition of STAT3. However, western blotting and a MTT assay showed that the inhibition of SRC kinase with PP2, a commonly used Src family kinase inhibitor[9], induces growth inhibition in DU145 prostate cancer cells without inhibiting STAT3 phosphorylation. Stemming from this observation, the study focused on the functional interactions between SRC and STAT3 for prostate cancer cell survival, and whether the simultaneous inhibition of both STAT3 and SRC could induce a stronger apoptotic response in prostate cancer cells.

In order to assess the effectiveness of simultaneous inhibition, DU145 cells were transfected with STAT3 siRNA to knockdown STAT3 expression in addition to PP2 treatment. DU145 cells with the simultaneous inhibition of SRC and STAT3 exhibited more distinct Poly (ADP-Ribose) Polymerase-1 (PARP-1) cleavage, an indicator of apoptosis[10]. To investigate the mechanisms behind this cooperation between STAT3 and SRC inhibition, levels of induced myeloid leukemia cell differentiation Mcl-1 (MCL-1), a downstream target of STAT3[6], and a pro-survival gene[11,12], were measured through qRT-PCR analysis. It has been shown that a decrease in MCL-1 levels induces apoptosis in cancer cells[11]. DU145 cells with both treatments led to 3-fold and 2 fold decreases in MCL-1 expression when compared to the exclusive treatments of STAT3 and SRC, respectively. This data suggests that an increase in apoptosis from the combination of both treatments might be due to a decrease in MCL-1 levels.

Results of the study show that the simultaneous inhibition of SRC and STAT3 in DU145 prostate cancer cells is more effective in inducing the apoptotic responses of PARP-1 cleavage and lowered MCL-1 expression when compared to the exclusive inhibition of either SRC or STAT3. This study suggests that the simultaneous inhibition of SRC and STAT3 may prove to be a novel, effective therapeutic strategy for the treatment of prostate cancer patients.

Materials and Methods

Transfection and qRT-PCR: DU145 cells (ATCC) were plated in two sets of 6-well plates (2 * 105 per well) and then transfected with TransIT-TKO (Mirus Bio), using GAPDH siRNA, STAT3 siRNA-1, and STAT3 siRNA-2 (Invitrogen). After 24 hours of incubation, PP2 (Calbiochem) was added to one plate, at a final concentration of 5μM, in each set and all plates were incubated for a total of 48 hours. RNA was then extracted and purified using the RNeasyMiniKit (Qiagen). RNA concentration was measured using Thermo Scientific's NanoDrop 2000. cDNA was made using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Bio-Rad's iCycler Thermal Cycler with the following reaction conditions: 25°C for 10 mins, 37°C for 2 hours, 85°C for 5 mins, and 4°C for incubation. qRT-PCR was then performed using MCL-1 primers in MicroAmp fast Optical 96-well reaction plates using the 7900HT Fast Real-Time PCR System (Applied Biosystems). ***Protein Extraction and Western Blotting:*** After aspirating medium and washing DU145 cells with 1x phosphate buffered saline, a solution containing Tween-20 (TPBS), 100 μL of 1x Lysis Buffer (Millipore), Phosphatase Inhibitor Cocktail 2 (Sigma), and 1 protease inhibitor cocktail tablet (Roche) was added to each well. The cells were transferred to 1.5 mL tubes and kept on ice for 15-30 mins (vortexing every 5 mins). They were then centrifuged at max speed for 10-15 mins at 4°C to remove cell debris. Protein concentration was measured via the Bio-Rad protein assay. Protein samples were then denatured with 2x sample buffer (Invitrogen) at 95°C for 5 mins and loaded into Tricine gels (Novex) that were placed into the XCellSureLock Mini-Cell electrophoresis system (Invitrogen). Immobilion-P Membranes (Millipore) were soaked in methanol for 1 min. The gels, support pads, gel blotting paper (Whatman), and the transfer membrane were all washed in 1x transfer buffer(ThermoFisher Scientific) before being stacked inside the Owl VEP-2 Mini Tank Electroblotting System (ThermoFisher Scientific). Membranes were retrieved and washed with TPBS and blocked with 5% non-fat milk for 1 hour at RT*. Membranes were incubated with anti-phosphorylated STAT3 and anti-PARP-1 primary antibodies (Cell Signaling Technology) in 1x TPBS (1:1000) overnight at 4°C. Membranes were then washed three times with TPBS for 5 mins each. Secondary donkey anti-rabbit antibodies (ThermoFisher Scientific) (1:10,000) were incubated with the membranes for 1 hour at RT. Membranes were washed again and prepared with Western Lightning Plus-ECL. Antibody–epitope binding was detected using AFP Imaging Mini-Medical Automatic Film Processors. After films were processed, membranes were blocked again with milk and allowed to incubate with primary antibodies for total STAT3 (1:1000) and b-actin (1:5000) and a secondary donkey anti-rabbit antibody for STAT3 and donkey anti-mouse antibody for b-actin (1:5000). ***MTT assay:*** 10 μL of MTT (Roche) was added to each well of the 96-well plate and put into a Water Jacketed Co2 Incubator (ThermoFisher Scientific) for 4 hours. After 50 μL of SDS (Roche) was added to each well to dissolve the crystals, the absorbance was measured using a Beckman DU-64 plate reader.

*(RT) = Room Temperature

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Results

Inhibition of SRC delays growth of prostate cancer cells

Treatment of PP2 (5μM), a widely used inhibitor of SRC family kinases, showed a clear decrease in the survival of DU145 cells with an average decrease of 26.2% from untreated cells (Figure 1).

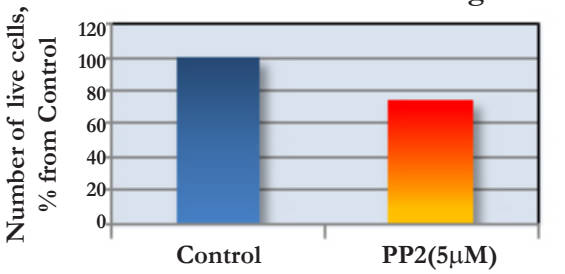

Figure 1: MTT Assay demonstrates that 5μM PP2 induces growth inhibition in DU145 cells.

Inhibition of SRC does not affect STAT3 levels

Phosphorylated STAT3 (pSTAT3) levels of cells treated with PP2 were analyzed to investigate how inhibition of SRC affects STAT3 phosphorylation. Most notably, the levels of pSTAT3 were not decreased by PP2 (Figure 2). Re-blotting the membranes for total STAT3 levels as a control for protein loading did not reveal any major inconsistencies(Figure 2).

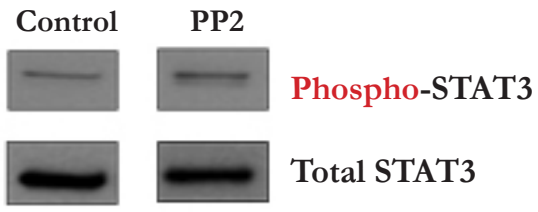

Figure 2: PP2 does not inhibit phosphorylation of STAT3. Western blot analysis performed on control DU145 cells and cells treated with 5 μM PP2 for 48 hours.

Knockdown of STAT3 by RNA interference

siRNAs were used to knockdown STAT3 expression. The transfection reagent, TransIT-TKO, was used as a control for toxicity from the transfection procedure. GAPDH siRNA was used as a control in order to ensure that GAPDH knockdown does not show the same effects on apoptosis as STAT3 knockdown. Two siRNAs targeting different regions of STAT3 mRNA were used to knockdown STAT3 in order to ensure that the observed effects were due to STAT3 knockdown, and not due to off-target effects. Western blotting confirmed that both phosphorylated and total levels of STAT3 were visibly decreased by STAT3 siRNAs (Figure 3). In agreement with data presented in Figure 2, treatment of cells with 5μM PP2 (Figure 3B) did not show any visible difference in phosphorylated STAT3 levels from the cells left untreated (Figure 3A).

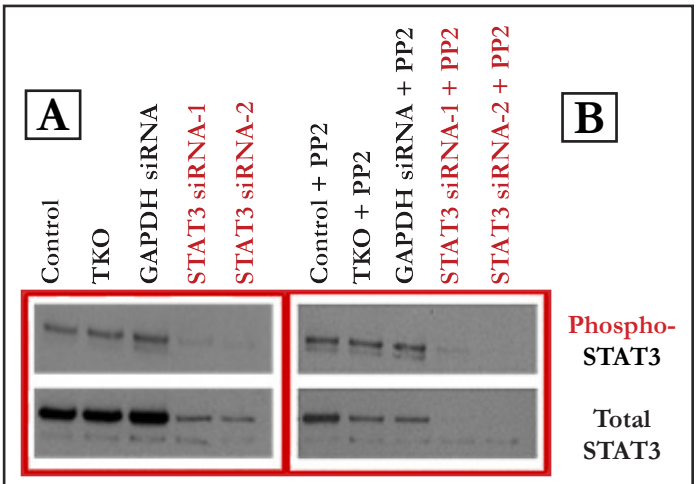

Figure 3: STAT3 siRNA knockdown significantly decreased levels of STAT3 in DU145 cells. Western blot analyses were performed on two sets of DU145 cells: **(A)** a control set and **(B)** a set treated with PP2. Each set was treated with a transfection reagent, TransIT-TKO, GAPDH siRNA, and two STAT3 siRNAs targeting different regions of mRNA. After protein transfer, membranes were probed with anti-pSTAT3 and anti-STAT3.

Simultaneous inhibition of SRC leads to PARP-1 cleavage

Western blotting showed that DU145 cells treated with PP2 exhibit PARP-1 cleavage in all samples (Figure 4B). In contrast, the cells transfected with STAT3 siRNA-1 and siRNA-2 show only faint signs of PARP-1 cleavage (Figure 4A).The highest proportion of the proteolytic cleavage of PARP-1 protein (89 kDa) is found in cells transfected with STAT3 siRNA and treated with PP2 (Figure 4B). It should be noted that cells treated with TKO exhibit a small amount of PARP-1 cleavage because of the cell toxicity caused by this transfection reagent which is amplified with the additional treatment of PP2 (Figure 4B).

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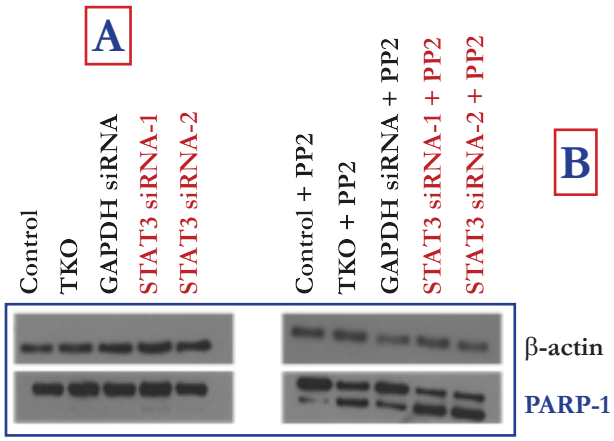

Figure 4: Simultaneous inhibition of SRC and STAT3 induces PARP-1 cleavage. (A) Control DU145 cells and **(B)** DU145 cells treated with PP2 were blotted using an antibody against the C-terminal domain of PARP-1 that recognized full-length PARP-1 protein (116kDA) and its proteolytically cleaved isoform (89kDa) that is generated during apoptosis. The membranes were then re-blotted with anti-β-actin as a loading control.

Simultaneous inhibition decreases MCL-1 levels by 3-fold

RT-PCR analysis was performed with primers for MCL-1 on two sets of DU145 cells, both treated and untreated with PP2. The cycles at threshold (Ct) levels of MCL-1 were taken and subtracting for corresponding samples to find the Ct difference for each corresponding pair. Additionally, the Ct levels for control DU145 cells treated with PP2 and TKO were subtracted from simultaneously treated cells to find the difference between simultaneous inhibition and an exclusive treatment of PP2. These differences were raised exponentially to the power of 2 to find the fold difference and summarized into a bar chart (Figure 5). Close analysis of the fold differences show that cells simultaneously treated with STAT3 siRNA and SRC showed a 3-fold decrease in MCL-1 expression when compared to the knockdown of STAT3 alone and a 2-fold decrease when compared to the exclusive treatment of PP2. (Figure 5).

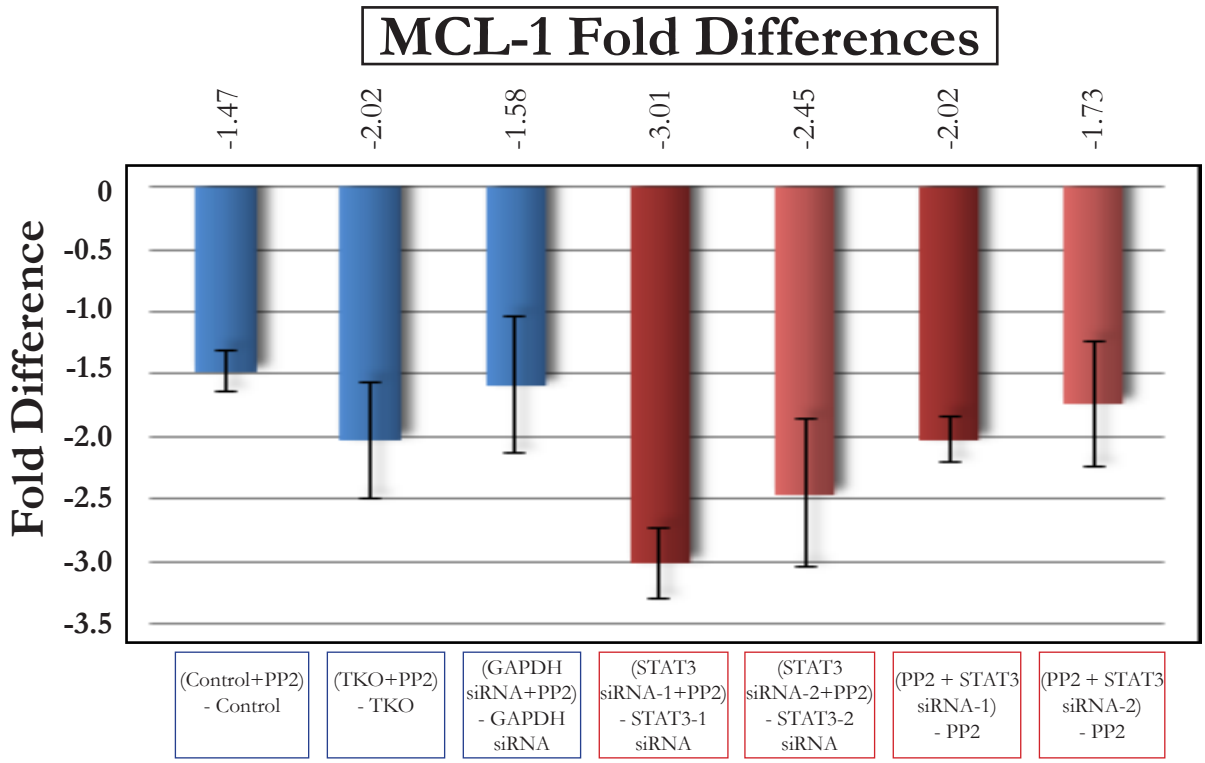

Figure 5: qRT-PCR analysis demonstrates that there is a greater inhibition of MCL-1 mRNA expression levels by STAT3 siRNA knockdown and PP2 compared to single exclusive treatments. The fold differences were found by taking the difference between the Ct levels of corresponding treatments labeled above and raised the values exponentially to the power of 2.

Discussion

Though several lines of evidence suggest that SRC induces the activation of STAT3 pathway[4,5], results of this study show that the inhibition of SRC has no effect on the levels of phosphorylated STAT3 in prostate cancer cells. This suggests the possibility that STAT3 is activated independently of SRC kinase in prostate cancer cells, and may protect cells from apoptosis triggered by SRC inhibitors. The observation also proposes that SRC and STAT3 follow alternate pathways in prostate cancer cells from those suggested in other cancer cell types. Considering that the growth inhibition induced by PP2 in prostate cancer cells was rather modest, it was hypothesized that additional inhibition of STAT3 signaling may augment the effects of SRC targeting and result in more robust cancer cell death.

After effectively knocking down STAT3 levels with siRNA, the effect of simultaneous inhibition on apoptotic effects was investigated by blotting for PARP-1 cleavage, a well-known marker of apoptosis. A MTT assay had already shown that the inhibition of SRC by PP2 decreased the number of DU145 cancer cells over time, but it was unclear whether this decrease was due to apoptosis or decreased cell proliferation. With western blot analysis, it was shown that DU145 cells that had both SRC and STAT3 inhibited exhibited the most defined levels of PARP-1 cleavage; this indicates that apoptosis, and not decreased cell proliferation, is responsible for the decreased number of cells.

With evidence from the western blot indicating a stronger apoptotic response in DU145 cells treated with both PP2 and STAT3 siRNA, further investigation into the STAT3 pathway was needed to clarify the mechanisms behind the interactions between SRC and STAT3. To accomplish this, levels of MCL-1 expression were investigated after inhibiting SRC and knocking down STAT3. From the qRT-PCR analysis, we see that simultaneous inhibition leads to a 3-fold and 2-fold decreases when compared to exclusive treatments of STAT3, and SRC, respectively. The observed differences are in agreement with results demonstrating that inhibition of SRC is ineffective in inhibiting activity of STAT3 and, hence, decreasing its downstream targets such as MCL-1. Furthermore, the clear decrease in levels of the anti-apoptotic MCL-1 correlates with a stronger apoptotic response in cells treated with PP2 and STAT3 siRNA. Taken together, this data suggests that combination of SRC inhibitors with STAT3 siRNA may be a more effective treatment for inducing the apoptosis of prostate cancer cells than either treatment alone.

Future experiments could include combining these treatments in xenograph and syngeneic mouse models to investigate the advantages of this novel treatment in preclinical trials. Also, this study suggests advantages in developing a small-molecule inhibitor for STAT3 that can be used instead of STAT3 siRNAs to increase the effectiveness of targeting such a pivotal gene in apoptosis.

In summary, the simultaneous inhibition of both SRC and STAT3 denotes a novel way to treat prostate cancer cells that is more effective and more efficient in inducing apoptosis than either treatment alone. The findings in this study may prove to be very useful in the development of future therapeutic approaches in the treatment of prostate cancer patients.

References

1. Brawer MK. Prostate cancer: where have we been, where we are, where we are going. World J Surg. 2000; 24;1200-4

2. Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics. CA Cancer J Clinical 1999;49;8-31

3. Tatorov, O., Mitchell, T. J., & Seywright, M. (2009, May 15). Src Family Kinase Activity Is Up-Regulated in Hormone-Refractory Cancer. Clinical Cancer Research.

4. Fizazi, K. (n.d.). The Role of Src in Prostate Cancer. Annals of Oncology. Bromberg, J. (2002, May). Stat Proteins and Oncogenesis. Journal of Clinical Investigation.

5. Cao, X., Tay, A., Guy, G., & Tan, Y. H. (1996, April). Activation and association of Stat3 with Src in v-Src-transformed cell lines. Molecular and Cellular Biology, 16(4), 1595-1603.

6. Bromberg, J. (2002, May). Stat Proteins and Oncogenesis. Journal of Clinical Investigations.

7. Turkson, J., Bowman, T., Garcia, R., Caldenhoven, E., De Groot, R. P., & Jove, R. (1998, January 30). Stat3 Activation by Src Induces Specific Gene Regulation and Is Required for Cell Transformation. Molecular and Cellular Biology, 18(5), 2545–2552.

8. Lee, S. O., Lou, W., Qureshi, K. M., Mehraein-Ghomi, F., Trump, D. L., & Gao, A.C. (n.d.). RNA Interference Targeting Stat3 Inhibits Growth and Induces Apoptosis of Human Prostate Cancer Cells. The Prostate, 60, 303-309.

9. Nam, J.-S., Ino, Y., Sakamoto, M., & Hirohashi, S. (2002, July). Src Family Kinase Inhibitor PP2 Restores the E-Cadherin/Catenin Cell Adhesion System in Human Cancer Cells and Reduces Cancer Metastasis. Clinical Cancer Research, 8, 2430 –2436.

10. Boulares, A. H., Yakovlev, A. G., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S., & Smulson, M. (1999, August 13). The Role of Poly(ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis. The Journal of Biological Chemistry.

11. Nijhawan, D., Traer, E., Fang, M., Zhong, Q., Gao, W., Du, F., & Wang, X. (2003, June 3). Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes & Development.

12. Minagawa, N., Kruglov, E. A., Dranoff, J. A., Robert, M. E., Gores, G. J., & Nathanson, M. H. (2005, July). The Anti-Apoptotic Protein MCL-1 Inhibits Mitochondrial Ca2+ Signals. The Journal of Biological Chemistry, 280(39).

13. Qiagen. (n.d.). Purification of Total RNA from Animal Cells Using Vacuum/Spin Technology. In RNEasy Mini Handbook (4th ed., pp. 31-35).

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