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RESEARCH ARTICLE

Reporting Evidence of Resistance against Severe Metabolic Stress in Human Lung Carcinoma

Maryam NAKHJAVANI[*°], Farshad H. SHIRAZI [**]

Reporting Evidence of Resistance against Severe Metabolic Stress in Human Lung Carcinoma

SUMMARY

Many cancer patients suffer from failure in the treatment and cancer recurrence, usually with a more resistant character. It is because a tumor is a heterogeneous tissue and cells within a tumor are in different metabolic stages. The aim of this study was to investigate the effect of long-term metabolic stress on human lung cancer, A549 cell line. The cells were seeded at the density of 4000 cells/cm^2. Upon reaching to plateau state, the cells were exposed to serum-free media for 6 days. At 24-hour intervals, the cells were tested for morphology, live cell count, cell cycle distribution, cell size, mitochondrial function and protein content. Also, after each day of starvation, starved cells were re-exposed to media containing 10% serum, for 24 hours. Then the above tests were performed on the cells. All experiments were performed triplicate. Results showed that, within the study period, live cell count was less than control cells. However, the cell count was kept at a plateau-like state with an increasing trend on the final day of the study. Up to 73.5% of the cells accumulated in the G1 phase. Upon releasing the cells, the G1 population decreased and the cells entered S and G2/M phases. However, cell size did not change significantly during the study. The mitochondrial function and protein content of the cells had an increasing trend in both starvation and release states. The results are suggestive of a highly resistant characteristic of this cell line when exposed to long-term serum-free metabolic stress.

Key Words: *: Cancer, A549, serum-free, metabolic stress, long-term, resistance*

İnsan Akciğer Karsinomunda Şiddetli Metabolik Strese Karşı Direnç Kanıtlarını Rapor Etme

ÖZ

Birçok kanser hastası tedavideki başarısızlık dolayısıyla genellikle daha dirençli bir karakter ile kanserin tekrarlamasından mağdur durumdadır. Çünkü bir tümör heterojen bir dokudur ve içindeki hücreler farklı metabolik aşamalardadır. Bu çalışmanın amacı uzun dönem metabolik stresin insan akciğer kanseri üzerindeki etkisini A549 hücrelerinde araştırmaktır. Hücreler, 4000 hücre / cm2 yoğunlukta ekilmiştir. Plato durumuna ulaşana kadar 6 gün boyunca hücreler serumsuz medyuma maruz bırakılmıştır. 24 saatlik aralıklarla, hücreler morfoloji, canlı hücre sayımı, hücre döngüsü dağılımı, hücre büyüklüğü, mitokondriyal fonksiyon ve protein içeriği açısından test edilmiştir. Ayrıca, hücreler her gün açlıktan sonra 24 saat boyunca % 10 serum içeren ortamlara tekrar maruz bırakılmıştır. Daha sonra yukarıdaki testler hücreler üzerinde yapılmıştır. Tüm deneyler 3 kez tekrarlanmıştır. Sonuçlar, çalışma süresi içinde canlı hücre sayısının kontrol hücrelerinden daha az olduğunu göstermiştir. Ancak, hücre sayısı son gün yükselen bir trend ile plato benzeri bir durumda tutulmuştur. G1 fazında hücrelerin % 73,5'i kadarı akümüle olmuştur. Hücreler salındıktan sonra, G1 popülasyonu azalmıştır ve hücreler S ve G2 / M fazlarına girmiştir. Ancak, hücre boyutu çalışma sırasında önemli ölçüde değişmemiştir. Mitokondriyal fonksiyon ve hücrelerin protein içeriği açlık ve salım durumunlarının her ikisinde de artış eğiliminde olmuştur. Sonuçlar, uzun süreli serumsuz metabolik strese maruz kaldığında bu hücre hattının oldukça dirençli karakteristiği işaret etmektedir.

Anahtar Kelimeler: *Kanser, A549, serumsuz, metabolik stres, uzun dönem, rezistans*

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[*] ORCİD:- Department of Toxico/Pharmacology, Shahid Beheshti School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[**] ORCİD:- Sciences Research Center, Shahid Beheshti Medical University, Tehran, Iran.

° Corresponding Author; Maryam Nakhjavani
Tel: +61405350736, email: mn.nakhjavani@gmail.com

INTRODUCTION

A key challenge in cancer treatment is the cancer resistance against chemotherapeutics and cancer recurrence (Miller, et al., 2016; Rebucci, et al., 2013). Many cancer researchers around the world are still trying to find novel treatments for the disease. These include, but not limited to, looking into new chemical structures and synthesizing and testing them (Aryanpour, et al., 2012; Nakhjavani, et al., 2014), investigating novel effective natural compounds, either in herbal products or toxins (Ebrahim, et al., 2016; Nakhjavani, et al., 2019; Nakhjavani, Vatanpour, et al., 2016; Vakili, et al., 2012), novel approaches towards nano-technology and looking for nano-medicines (Chauhan, et al., 2013; Park, et al., 2008; M. Sarafraz, 2013; M. Sarafraz, et al., 2017; M. Sarafraz, et al., 2012; M. M. Sarafraz, et al., 2014). It seems that the best outcome in cancer treatment is only gained when early enough detection of the tumor is guaranteed. Because most of the patients at the late stages of the disease die as a result of unsuccessful treatment procedure or suffer from tumor recurrence, eventually (Crabtree, et al., 2014). Therefore, besides so many available novel and traditional treatment methods, the necessity of deeper investigations in the biology of tumors is still felt.

Tumor heterogeneity is nowadays a big area of concern for cancer researchers (Easwaran, et al., 2014; Jamal-Hanjani, et al., 2015; Martelotto, et al., 2014). Metabolic heterogeneity, defined as diverse and dissimilar metabolic phenotype of cancer cells inside a tumor, is now a matter of focus for many researchers and is gaining more and more popularity (Martinez-Outschoorn, et al., 2017). The cells in the inner parts of a tumor do not have sufficient access to nutrition and oxygen and they suffer from physical stress. The cells on the outer layers, on the contrary, have a favorable growth condition. They have sufficient access to nutrition and oxygen, discard waste more readily and bear less physical stress (Nakhjavani, Nikounezhad, Ashtarinezhad, et al., 2016). Hence, they can proliferate more easily. Likewise, their response to chemotherapeutics is different from the other cells. Chemotherapeutics usually cause a better response in rapidly proliferating cells (Brunton, et al., 2005), especially when there is sufficient angiogenesis around the cells (Moserle, et al., 2014). In other words, limited angiogenesis may result in the limited efficacy of the chemotherapeutics. There is evidence indicating that in some tumors, the tumor regrows rapidly after the cessation of the therapy or in the periods between the cycles of therapy (Stewart, 2007). It seems that as the tumor shrinks, the quiescence cells, which have escaped death caused by cytotoxic agents, are once again exposed to favorable growth conditions. If the cells have acquired a resistance characteristic, they can develop further resistance towards the chemotherapy regimen (Nikounezhad, et al., 2016; Stewart, 2007; Tannock, 1987; Zhang, et al., 2006). This shows that the need for more effective chemotherapeutic agents is still felt. But, before that, a better understanding of the biology of different types of cells within a tumor is required.

Lung cancer is still one of the major causes of cancer mortality (Torre, et al., 2016). Previously, we developed an *in vitro* model to study the effect of metabolic stress on different cancer cells (Nakhjavani, Nikounezhad, & Shirazi, 2016; Nakhjavani, Stewart, et al., 2017; Nikounezhad, et al., 2016). We performed some studies on human lung carcinoma and evaluated cancer cells' reactions in low-serum concentrations in a long-term study. The interesting results of those studies made us put another step ahead and check these cells' reactions in a serum-free media. Hence, in the current study, we aimed to study the reaction of human adenocarcinoma cells against severe metabolic stress; a long-term exposure to serum-free media. To the best of authors' knowledge, this is the first study to explore the effects of long-term metabolic stress in a serum-free media on human lung cancer. This study was performed to gain primary results about how these cells react to such harsh conditions, in the hope of providing more insights for cancer researchers about the characteristics and biology of this type of cancer.

MATERIALS AND METHODS

Materials

All chemicals, reagents and cell culture materials used in this study were from Sigma-Aldrich (St. Louis, Missouri, USA), except Fetal Bovine Serum (Gibco, Grand Island, NY, USA) and Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY). Human lung adenocarcinoma cell Line A549 (IBRCC10080) was purchased from Iranian Biological Resource Center.

Cell culture and preparation

A549 cells were thawed and underwent three passages before the experiment. The cells were seeded at the density of 4000 cells/cm^2 in culture dishes (Nakhjavani & H Shirazi, 2017). In order to use a model of cells, which mimics the cells of the central parts of a tumor, the experiment started when cells were at the plateau phase of their growth curve. Figure 1 shows a schematic flow chart of the experiment

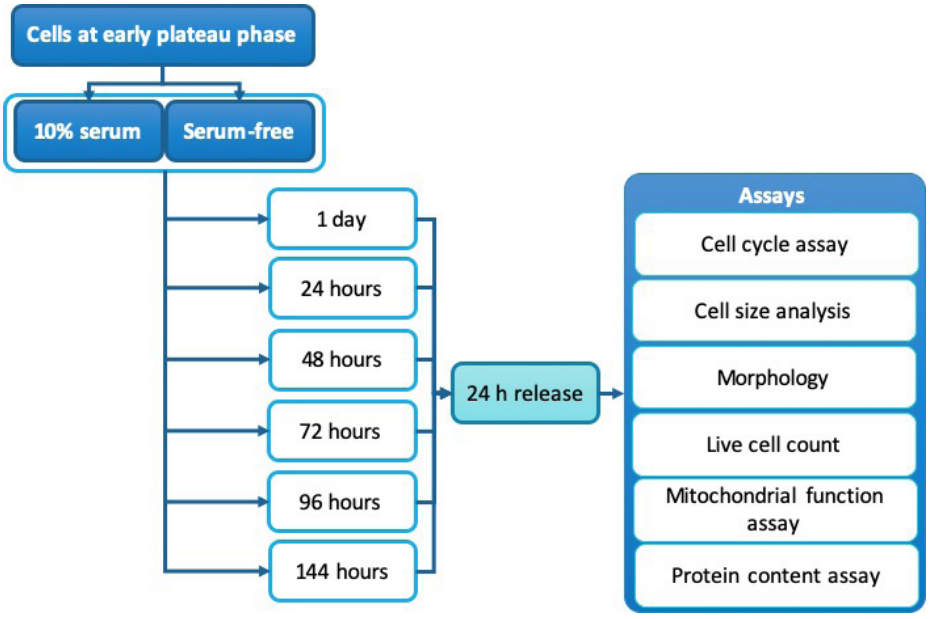

Figure 1. Schematic flow chart of different stages of the experiment design of this study.

design of this study. At the start of the experiment, the media over the cells was changed with a serum-free media to expose the cells with severe metabolic stress. Then at 24-hour intervals, the following tests were performed on the starved cells.

In addition, following 24, 48, 72, 96, 120 and 144 hours of starvation, the cells were re-exposed to optimum media containing 10% serum. This re-exposure to complete optimum media is referred to as "release" throughout this paper. The release period for different groups of starved cells lasted for 24 hours. Then, the released cells were tested as described below.

Flow cytometry and cell cycle analysis

Flow cytometry was used to determine the stages of the cell cycle, as previously described (Nakhjavani & Shirazi, 2017). Briefly, 1×10^6 cells in 1 ml of Ca^{2+}/Mg^{2+} free phosphate buffer saline were centrifuged (700 g, 4 °C for 10 min) and fixed with ice-cold ethanol for 2 hours at -20°C. Then the cells were stained with the staining solution including RNase A, triton X-100 and Propidium Iodide. Finally, cell cycle was measured with a BD FACSCalibur™ (Becton Dickinson, USA) and the final data were analyzed in FlowJo® software, version 7.6.1.

Cell size analysis

The results from flow cytometry were analyzed for mean size measurement. Geometrical mean distribution of forward scattering based on the method described by Rathmell *et al.* was calculated in FlowJo® software, version 7.6.1. (Rathmell, et al., 2000).

Morphological studies

At the defined time of the experiments, the morphology of the cells was studied using an inverted light microscope and a camera (Mitocam, Motic®, Wetzlar, Germany).

Trypan blue assay-live cell counting

Trypan blue dye exclusion assay is mostly known as one of the methods for assessing cell viability. However, in a long-term study when different time-points are studied, it can show the trends in cell proliferation, based on the increase or decrease in live cell count (Nakhjavani, et al.; Nakhjavani, Stewart, et al., 2017; Nikounezhad, et al., 2016; Shirazi, et al., 2011). Briefly, A549 cells were trypsinized, diluted with complete media and mixed with trypan blue dye at the ratio of 1:1. Finally, 15 µL of the mixture was used for counting of live cells under an inverted light microscope.

Evaluation of cell mitochondrial function

To measure mitochondrial function, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used (Jamali, et al., 2015). At the time of the experiment, sterile-filtered MTT solution was added to the media above the cells (10% V/V). The cells were kept in dark for 4 hours and later, the supernatant was gently removed. 200 µL of DMSO was added to the cells and following a 30-minute shaking, the absorbance of each well was recorded at 570 nm.

Evaluation of cell protein content

A549 cells' protein content was measured using Sulforhodamine B (SRB) assay (Nakhjavani, et al., 2014). This method is based on the stoichiometrically binding of the dye sulforhodamine B, as a fluorescent dye, to proteins of live fixed cells, to quantify cellular protein (Kuete, et al., 2017). Briefly, at the time of the experiment, the cells were fixed to the wells of the 96-well plate using cold trichloroacetic acid. Following fixing the live cells, excessive chemicals and dead bodies were washed twice using deionized distilled water, air dried and exposed to sulforhodamine B dye (50 µL) for 30 minutes. The wells were again washed twice with deionized distilled water. Then tris buffer (10 mM, pH 10) was added to wells and the absorbance of each well was read at 540 nm.

Statistical analysis

Two-way repeated-measures analysis of variance (ANOVA) was used to compare the response of starved cells to metabolic stress exposed to them. All the comparisons were performed in GraphPad PRISM® software, version 5, and *p*-value of 0.05 was considered as the statistically significant limit.

Results

Morphology, cell size and proliferation patterns

Figure 2, comparatively shows the number of live cell count in the control and test cells. As it is shown, the count of live cells following starvation in a serum-free medium is significantly less than control cells in the first 3 days of the study. After that, the cells reach a steady state at days 3 and 4 and amazingly, cell count increases after that (Figure 2A). It seems like the cells are resisting further cell death. In control cells, however, the cells show a decreasing trend in live cell count (Figure 2B).

Releasing the cells in media containing 10% serum for 24 hours does not cause a statistically significant increase in cell count in either of the test or control cells. However, a relatively (but not statistically significant) increased cell count is observed in the test cells.

Figure 2C shows the percent of changes in cell count of test cells compared to control cells, in percentage. This comparison is gained by dividing the live cell count in the test group by their relative number in the control group. This figure shows that there are increased cell counts in test cells. This

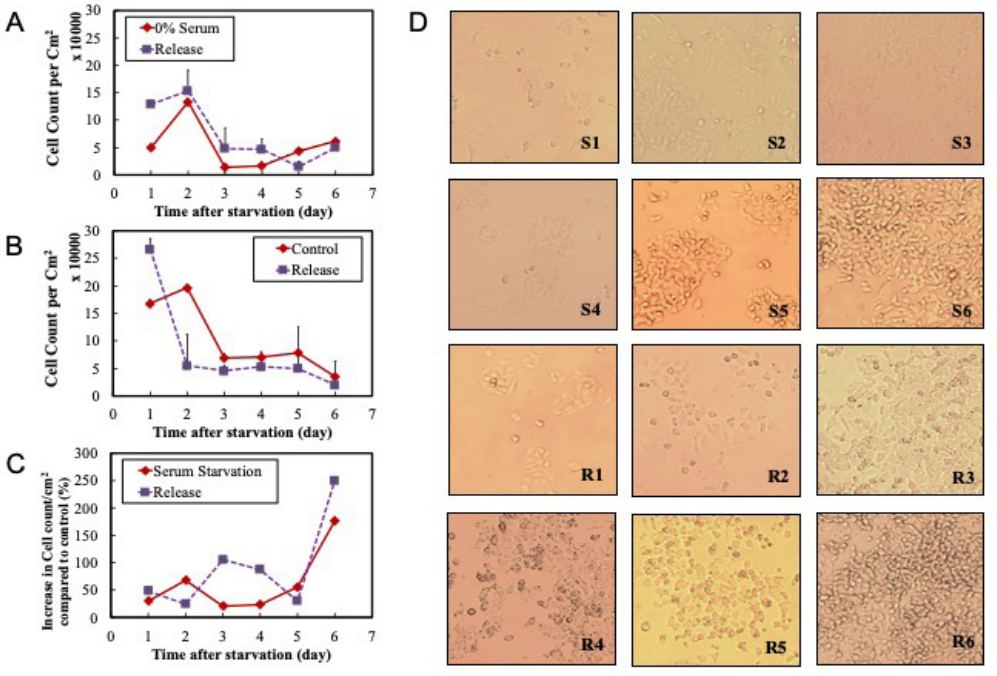

Figure 2. Live cell count of A549 cell line following starvation in serum free media (A) or releases in 10% serum supplemented media (B), and the relative cell count of starved cells (C) by trypan blue method. The experiment was done three times and data are shown as mean ± standard deviation. D) Morphological status of A549 cell line (10X), following 1 to 6 days of starvation in a serum free media. Following each starvation day, the cells were released in a complete media containing 10% serum for 24 hours. Starvation days from day 1 to 6 are demonstrated with S1 to S6 and release days are shown with R1 to R6.

Table 1. Cell size distribution of A549 cells in a serum free media for 5 days. Cell size is presented in either starvation or release mode. To release the cells, media with 10% serum was exposed to cells for 24 hours. The test was repeated 3 times and data are presented as mean ± standard deviation.

Status	Serum%	Cell Size (FSC Geom. Mean) ± SD					
		Time after starvation (day)					
		1	2	3	4	5	6
Starvation	10	480±0.8	397.5±1.7	423.5±4.2	278.5±0.1	301±3.2	n.d.[a]
	0	451±4.2	377.5±17.7	453±5.6	445±2.8	480.5±9.2	n.d.
Release[b]	10	433±2.5	362.5±3.3	386.5±2.5	380±2.7	368.5±0.5	n.d.
	0	450.5±3.5	416.5±9.1	428±7.2	458±5.6	457±0.7	n.d.

[a] Not Determined

[b] exposing the cells to 10% serum for 24 hours

increase is mostly observed in the first and last days of the study.

Figure 2D shows the morphology of the cells. The figure shows that during the study period, not only the cell colonies become denser, but also the cells become rounder. This figure also confirms the results gained in the cell count experiment.

Changes in cell size were also studied through flow cytometry, as shown in Table 1. Statistical analysis demonstrated that, in a comparison of starved to released cells, no significant change in cell size happened in the studied cells. Small fluctuations in cell size could be as a result of cells passing through different phases of the cell cycle.

Cell cycle distribution

As it is shown in Figure 3, it seems that these cells wisely decide to accumulate in the G_1 phase of the cycle. As the cells are starved for a longer period of time, the percent of cells accumulated in the G_1

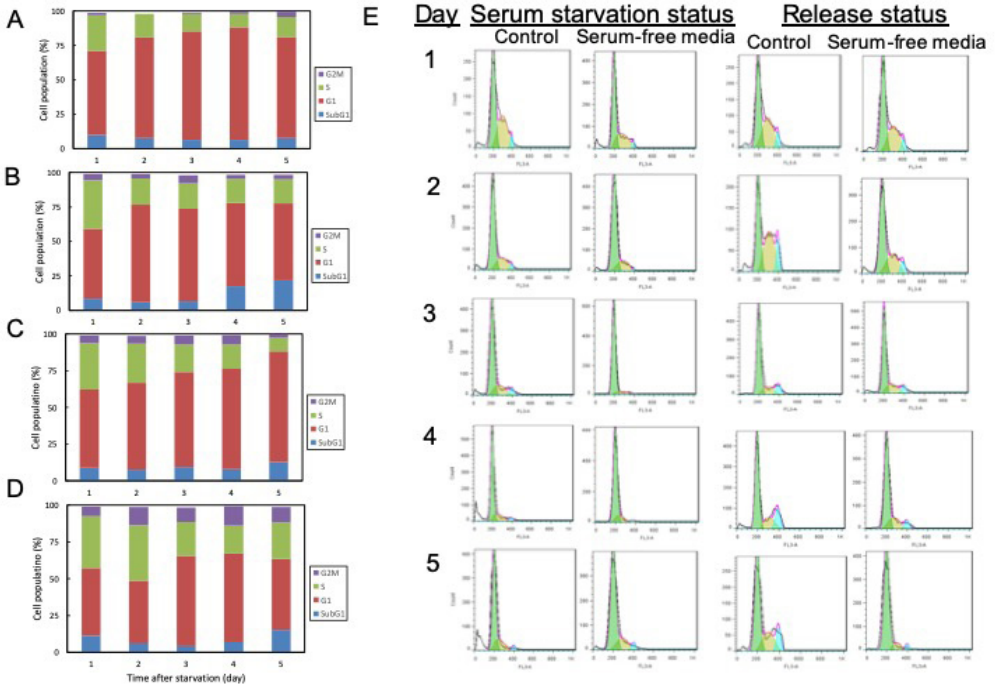

Figure 3. Cell cycle distribution (%) of A549 cell line, following 1 to 5 days of starvation in a serum free media compared to control cells. Cell cycle distribution of the cells following exposure to (A) serum-free media and (B) 10% serum. Following each starvation day, the cells were released in a complete media containing 10% serum. Cell cycle distribution of the cells released in 10% serum following serum starvation in serum-free media (C) or being kept in 10% serum (D) are shown. (E) Histograms of cell cycle distribution. White curves represent sub-G_1 fraction, and green, yellow and blue curves represent G_1, S and G_2/M fraction of the cells, respectively.

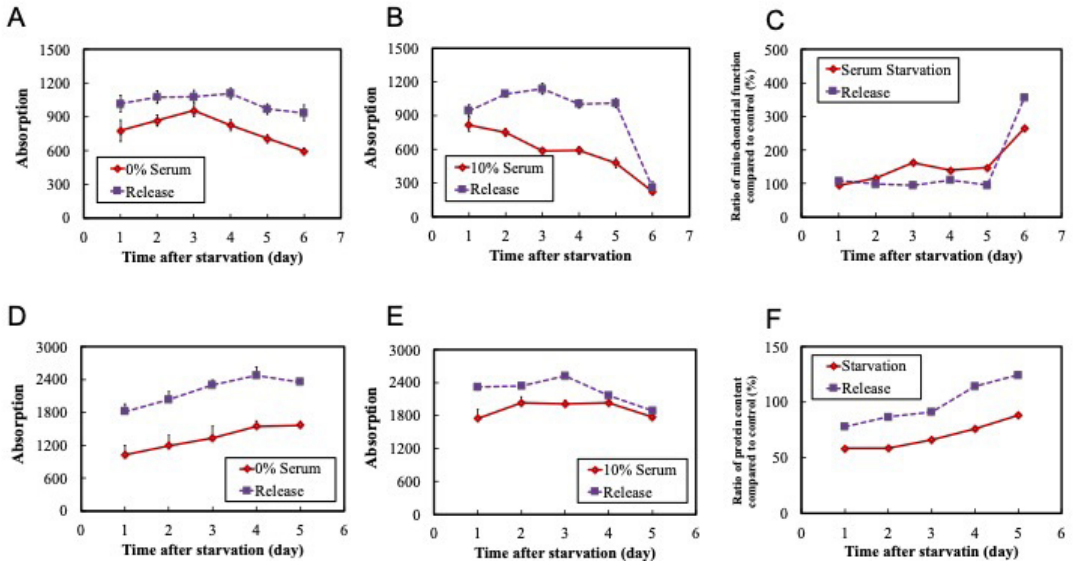

Figure 4. Mitochondrial function of A549 cells. Comparison of starvation at serum-free media and releasing the cells in 10% serum (A), control cells (B), and relative mitochondrial function of starved cells (C). Figures D, E and F show protein content of A549 cells. Comparison of starvation at serum-free media and releasing the cells in 10% serum (A), control cells (B), and relative Protein content of starved cells (C).

phase increases. For example, the G_1 cell population in starved cells reached from 61.26±9.58 (percent) on the first day of the study reached to 73.38±2.62 (percent). At the same time, the sub-G_1 population, which are indicative of apoptosis, decreased slightly (from 9.81±2.48 to 7.31±0.25).

The story is different in control cells. On the second day of the study, there is evidence of cells trying to accumulate in the G_1 phase (reaching from 50.48±1.36 to 70.66±2.00). But this seems to be an unsuccessful effort, as from this day on, the percent of the cell population in G_1 decreases gradually to 50.99±4.71. On the other hand, the population of sub-G_1 gradually increases, from about 6% to about 22%. This explains the decreased population of G_1, G_2/M and S phases. In other words, these control cells are slowly dying. This is also evidenced by morphologic images and live cell count (Figures 2 and 3), as explained above.

Figure 3 also shows the results of cell cycle analysis for the released cells compared to starved cells. This data shows that after releasing the cells in media containing 10% serum for 24 hours, cells gradually leave the G_1 phase and enter S phase and G_2/M phase. For example, percent of cells in G_1 and S on the first day of the study are 61.26±9.58 and 25.97±4.90, respectively. These percentages are changed following releasing the cells in optimum media to 53.55±1.45 and 31.58±0.14 for G_1 and S phases, respectively.

Mitochondrial function and protein content

Figure 4 shows the results of MTT assay on these cells. While the mitochondrial function in control cells is decreasing gradually (Figure 4B), there are no significant differences between the mitochondrial function of the cells in the 6-day of the study (p<0.05) (Figure 4A). However, releasing the starved cells in an appropriate medium causes a significant increase in the mitochondrial function of the cells. Figure 4C shows that, in both starvation and release states, the relative function of mitochondria in starved cells in higher and in an increasing trend.

The same pattern is observable for cells protein content, as is shown in Figure 4D, 4E and 4F. While the protein content of cells in the control state is either stable or decreasing (Figure 4E), their protein content in the starvation state has an increasing trend (Figure 4D). Furthermore, these figures show that releasing the cells can increase cells' protein content considerably. Figure 4F shows the relative protein content of the starved cells. Obviously, the protein content in starvation and release modes is increasing compared to control cells.

DISCUSSION

A tumor has a heterogeneous tissue and cells inside a tumor experience different levels of metabolic state (Martinez-Outschoorn, et al., 2017; Stewart, 2007). For example, quiescence, a stage in the cell cycle represented as G_0, has been shown to be characteristic of human lung cancer cell lines,

such as A549 (Ho, et al., 2007). Accelerated cellular senescence (ACS) is also another stage of the cell cycle in which the cells are irreversibly accumulated in G_0/G_1 phase. These cells are usually formed as a result of exposure to chemotherapeutics or radiotherapy (Roberson, et al., 2005). Senescent and quiescent cells are two examples of cells within a tumor that acquire low metabolic state. Quiescent cells are able to return to the normal cell cycle, while senescent cells remain at the G_0/G_1 state and can even make the tumor outgrow (Saretzki, 2010). Furthermore, tumors include necrotic sections as well (Thomlinson, et al., 1955). Quiescence is a matter of tumor cells' access to nutritional and growth factors. Upon limitation of these factors or even exposure to toxic agents, cells choose to stay in a dormancy state and cause tumor recurrence or metastasis even after years of a supposedly successful cancer treatment (Moore, et al., 2011; Sosa, et al., 2014). Since nutrition is one of the key factors in such a process, and since angiogenesis is not sufficient in all sections of a tumor and the cells do not have equivalent access to nutritional and growth factors (Batchelor, et al., 2007), they have this opportunity to enter a low metabolic state. These cells bear more physical stress and have less access to sufficient oxygen and nutrients. In addition, their metabolic waste is hardly removed. This was the basis for designing our current study. Previously, we showed that A549 cells show resistance characteristics at some levels of metabolic stress (media containing 0.5 and 0.25% serum) (Nakhjavani, Nikounezhad, Ashtarinezhad, et al., 2016). In that study, we showed that the A549 cells could accumulate as high as 80% in the G_1 phase of the cell cycle. The intriguing results of that study made us put a step forward and study the cells' reactions in a more severe metabolic condition, serum-free media in a long-term study.

To mimic the physical stress that the cells bear in a tumor, the study was performed when the seeded cells reached their early plateau state (Nakhjavani & Shiraizi, 2018; Nikounezhad, et al., 2016). Control cells were grown in media containing 10% serum, while test cells were exposed to serum-free media for 6 consecutive days. At 24-hour intervals, cells reaction towards such harsh conditions was tested with different experiments.

The results showed that although serum starvation caused a significant decrease in live cell count with observably rounder and weaker features, the remaining live cells were somehow very resistant towards death. They highly accumulate in the G_1 phase of the cycle and upon exposure to favorable conditions, leave G_1 phase and enter S and G_2/M phases of the cycle. These findings were comparable to our previous results with this cell lines at 0.5 and 0.25% serum (Nakhjavani, Nikounezhad, Ashtarinezhad, et al., 2016). This result was also interesting because in a similar study on human ovary carcinoma, A780 cell line, the cells accumulated to about 50% in G_1 phase, with an increasing pattern in sub-G_1 population (Nakhjavani & Shirazi, 2018). This finding comparatively shows that human lung adenocarcinoma cell line, compared to human ovary carcinoma is more capable of resisting against severe metabolic stress, can accumulate in the G_1 phase without an increased Sub-G_1 population. The explanation behind this observation, accumulation in G_1 phase, could be behind a specific checkpoint in cells, known as restriction point (Blagosklonny, et al., 2002; Campisi, et al., 1982; Pardee, 1974). At this point, the cell starts to evaluate the environmental conditions. In favorable environment with sufficient nutrition and oxygen, the cells go on with the cycle and in low nutrient medium, the cells stop in G_1. This is known to be the case for normal cells. They lower their metabolic state to a basal level and enter a quiescence state (Pardee, 1989). Cancer cells, however, are known to have different characteristics from normal cells. They are said to know how to escape death signals, resist against apoptosis, be independent of growth signals, induce angiogenesis and escape from growth suppressor signals (Hanahan, et al., 2000, 2011; Lazebnik, 2010). In the current study, we observe that the cells actually accumulated in the G_1 phase of the cycle.

However, it should different from quiescence, because the cells are not living in their basic metabolic state. Our results showed that there is an increased number of live cell count in the latest days of the study was observed, which was really amazing. Moreover, mitochondrial function and protein content of the cells had an increasing pattern, which was mostly observable in the last days of the study. This increasing trends in live cell count, mitochondrial function and protein content are comparable to our other study on A549 cells exposed to 0.5% and 0.25% serum (Nakhjavan, et al., 2018). These observations show that the cells are actually experiencing some kind of metabolism, which allows them to show increased cell count, mitochondrial function and protein content. The reason behind that could be explained by autophagy, which can save the cells against death (Jin, et al., 2007). Even in normal cells, autophagy has a cell protective role against genetic instabilities (Mathew, et al., 2007). It can also remove destructive macromolecules and help the longevity of the cell (Alavez, et al., 2011; Madeo, et al., 2010). In the absence of anabolic signaling, autophagy is triggered. This can help the cell survive longer and also use the

extracellular sources of energy as much as possible (Amaravadi, et al., 2007; Lum, et al., 2005).

The observed cell survive could also be explained by the Warburg effect, glutaminolysis and glutamine addiction in cancer cells (Bhutia, et al., 2014; Vander Heiden, et al., 2009). These two phenomena are dependent on glucose and glutamine, which were both available in our experiments. Glucose and glutamine provide carbon, nitrogen and free energy for cellular processes. Glucose, through oxidative phosphorylation in mitochondria, can produce ATP. It can also provide required substrates for the production of acetyl-CoA, which can later provide ribose, fatty acids and unnecessary amino acids (DeBerardinis, et al., 2008). Glutamine can also be a source of nitrogen and NADPH. Glutamine can provide required nucleotides for the synthesis of DNA and it is also connected to the mTOR pathway and controls the cell cycle, anti-oxidant and nutritional status of the cells (Bhutia, et al., 2014; Dibble, et al., 2013; Jewell, et al., 2013).

The other section of the study was focused on the cells' ability to return to a normal life following starvation. This was done by exposing the starved cells to media containing 10% serum. The results showed that although the cells didn't show any significant increase in cell count or mitochondrial function, the cells showed significantly increased protein content. This is while for cells exposed to 0.25 and 0.5% serum, cell count and mitochondrial function also increased significantly following starvation and re-exposure to media containing 10% serum (Nakhjavan, et al., 2018). It seems that following a long-term starvation to serum-free media and then re-exposing to optimum serum concentration, the cells choose to take in as much protein as they can, to regenerate their internal damages, rather than increasing in cell count or increasing mitochondrial function. To find out whether these claims are correct requires further investigations. The findings with this cell line are also comparable with the results of another study on human breast adenocarcinoma cell line (MCF-7) (Nakhjavani, Stewart, et al., 2017). In that study, MCF-7 cells underwent steroid and serum starvation for a 6-day study. The huge metabolic stress exposed to the cells included omitting not only serum but also steroid stimulation. Re-exposure to favorable conditions resulted in mostly increase in cells' protein content. However, we now know that not all cancer cells react in this way. A2780 cells, under similar conditions, showed a mostly increased mitochondrial function rather than protein content (Nakhjavani & Shirazi, 2018). This is another proof that each cancer cell with a different origin has unique characteristics.

CONCLUSION

The current research was designed to, for the first time, study the resistance pattern of human lung adenocarcinoma, A549 cell line, against a long-term and extreme metabolic stress. In our previous works, we studied the effect of low levels of serum concentration on the cell cycle distribution of this cell line and we noticed that the cells show some levels of resistance characteristics against limited and low levels of serum accessibility. In that study, we focused on cells distribution in different stages of the cell cycle. We expanded our investigation to a higher level of stress (long-term stress in serum-free media) and evaluated A549 cells' reaction in other aspects including their size changes, morphological changes, and inner cell responses to stress such as mitochondrial reactions and protein content changes. Also, we evaluated cells' ability to self-repair and self-regeneration following metabolic stress. This was done by re-exposing the cells to optimum media, containing 10% serum. The results of our study showed that this cell line has a highly resistant characteristic against extreme metabolic stress. Long-term metabolic stress does not kill all the cells. The selective population of the cells remain, accumulate in G_1 phase, maintain their live cell count, and tend to increase their mitochondrial function and protein content. Also, upon releasing the cells in optimum media, they start to slightly proliferate and increase cells' internal functions. All of these results demonstrate cells resistant characteristics against such a harsh metabolic stress. Although the results of this study could open a new door to the biology of cancer cells and many researchers could use this data for their further investigations in designing new cytotoxic structures, this study lacks sufficient investigation about the underlying mechanisms that led to such reactions in this cell line. The results of this study show only a big picture of this cancer cell line's reaction towards severe metabolic stress. Hence, further studies are required to investigate the inter- and intra-cellular mechanisms in this cancer cell line which lead to resistant characteristics.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

REFERENCES

Alavez, S., Vantipalli, M. C., Zucker, D. J., Klang, I. M., & Lithgow, G. J. (2011). Amyloid-binding compounds maintain protein homeostasis during ageing and extend lifespan. *Nature, 472*(7342), 226-229.

Amaravadi, R. K., & Thompson, C. B. (2007). The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clinical Cancer Research, 13*(24), 7271-7279.

Aryanpour, N., Mansouri-Torshizi, H., Nakhjavan, M., & H. Shirazi, F. (2012). Cytotoxicity of diimine palladium (II) complexes of alkyldithiocarbamate derivatives on human lung, ovary and liver cells. *Iranian Journal of Pharmaceutical Research, 11*(2), 689-695.

Batchelor, T. T., Sorensen, A. G., di Tomaso, E., Zhang, W.-T., Duda, D. G., Cohen, K. S., ... Zhu, M. (2007). AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients. *Cancer Cell, 11*(1), 83-95.

Bhutia, Y. D., Babu, E., Prasad, P. D., & Ganapathy, V. (2014). The amino acid transporter SLC6A14 in cancer and its potential use in chemotherapy. *Asian Journal Of Pharmaceutical Sciences, 9*(6), 293-303.

Blagosklonny, M. V., & Pardee, A. B. (2002). The restriction point of the cell cycle. *Cell cycle, 1*(2), 102-109.

Brunton, L., Lazo, J., & Parker, K. G. (2005). Gilman's The Pharmacological Basis of Therapeutics. 11th. *New York: Mc Graw-Hill*.

Campisi, J., Medrano, E. E., Morreo, G., & Pardee, A. B. (1982). Restriction point control of cell growth by a labile protein: evidence for increased stability in transformed cells. *Proceedings of the National Academy of Sciences, 79*(2), 436-440.

Chauhan, V. P., & Jain, R. K. (2013). Strategies for advancing cancer nanomedicine. *Nature materials, 12*(11), 958.

Crabtree, T. D., Puri, V., Robinson, C., Bradley, J., Broderick, S., Patterson, G. A., ... Yang, M. (2014). Analysis of first recurrence and survival in patients with stage I non–small cell lung cancer treated with surgical resection or stereotactic radiation therapy. *The Journal of Thoracic and Cardiovascular Surgery, 147*(4), 1183-1192.

DeBerardinis, R. J., Lum, J. J., Hatzivassiliou, G., & Thompson, C. B. (2008). The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metabolism, 7*(1), 11-20.

Dibble, C. C., & Manning, B. D. (2013). Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nature Cell Biology, 15*(6), 555-564.

Easwaran, H., Tsai, H.-C., & Baylin, S. B. (2014). Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. *Molecular Cell, 54*(5), 716-727.

Ebrahim, K., Vatanpour, H., Zare, A., Shirazi, F. H., & Nakhjavani, M. (2016). Anticancer activity a of Caspian cobra (Naja Naja oxiana) snake venom in human cancer cell lines via induction of apoptosis. *Iranian Journal of Pharmaceutical Research: IJPR, 15*(Suppl), 101.

Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57-70.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674.

Ho, M. M., Ng, A. V., Lam, S., & Hung, J. Y. (2007). Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Research, 67*(10), 4827-4833.

Jamal-Hanjani, M., Quezada, S. A., Larkin, J., & Swanton, C. (2015). Translational implications of tumor heterogeneity. *Clinical Cancer Research, 21*(6), 1258-1266.

Jamali, B., Nakhjavani, M., Hosseinzadeh, L., Amidi, S., Nikounezhad, N., & Shirazi, F. H. (2015). Intracellular GSH alterations and its relationship to level of resistance following exposure to cisplatin in cancer cells. *Iranian Journal of Pharmaceutical Research: IJPR, 14*(2), 513.

Jewell, J. L., & Guan, K.-L. (2013). Nutrient signaling to mTOR and cell growth. *Trends in Biochemical Sciences, 38*(5), 233-242.

Jin, S., DiPaola, R. S., Mathew, R., & White, E. (2007). Metabolic catastrophe as a means to cancer cell death. *Journal of Cell Science, 120*(3), 379-383.

Kuete, V. Anticancer Activities of African Medicinal Spices and Vegetables. https://kundoc.com/pdf-anticancer-activities-of-african-medicinal-spices-and-vegetables-.html. Access date: 26 July 2019.

Lazebnik, Y. (2010). What are the hallmarks of cancer? *Nature Reviews Cancer, 10*(4), 232-233.

Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T., & Thompson, C. B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell, 120*(2), 237-248.

Madeo, F., Tavernarakis, N., & Kroemer, G. (2010). Can autophagy promote longevity? *Nature Cell Biology, 12*(9), 842-846.

Martelotto, L. G., Ng, C. K., Piscuoglio, S., Weigelt, B., & Reis-Filho, J. S. (2014). Breast cancer intra-tumor heterogeneity. *Breast Cancer Research, 16*(3), 210.

Martinez-Outschoorn, U. E., Peiris-Pagés, M., Pestell, R. G., Sotgia, F., & Lisanti, M. P. (2017). Cancer metabolism: a therapeutic perspective. *Nature Reviews Clinical Oncology, 14*(1), 11-31.

Mathew, R., Kongara, S., Beaudoin, B., Karp, C. M., Bray, K., Degenhardt, K., . . . White, E. (2007). Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes & Development, 21*(11), 1367-1381.

Miller, K. D., Siegel, R. L., Lin, C. C., Mariotto, A. B., Kramer, J. L., Rowland, J. H., . . . Jemal, A. (2016). Cancer treatment and survivorship statistics, 2016. *CA: A Cancer Journal for Clinicians, 66*(4), 271-289.

Moore, N., & Lyle, S. (2011). Quiescent, slow-cycling stem cell populations in cancer: a review of the evidence and discussion of significance. *Journal of Oncology, 2011*.

Moserle, L., Jiménez-Valerio, G., & Casanovas, O. (2014). Antiangiogenic therapies: going beyond their limits. *Cancer Discovery, 4*(1), 31-41.

Nakhjavan, M., & Shirazi, F. H. (2018). How human lung adenocarcinoma cells react towards long-term metabolic stress; a follow up. *Indian Journal of Pharmaceutical Education and Research, 52*(1), In Press.

Nakhjavani, M., & H Shirazi, F. (2017). Reporting the effect of cell seeding density on growth pattern of cancer cell lines. *Iranian Journal of Pharmaceutical Sciences, 13*(2), 87-94.

Nakhjavani, M., Hardingham, J. E., Palethorpe, H. M., Tomita, Y., Smith, E., Price, T. J., & Townsend, A. R. J. M. (2019). Ginsenoside Rg3: potential molecular targets and therapeutic indication in metastatic breast cancer. *Medicines (Basel), 6*(1), 17.

Nakhjavani, M., Nikounezhad, N., Ashtarinezhad, A., & Shirazi, F. H. (2016). Human lung carcinoma reaction against metabolic serum deficiency stress. *Iranian Journal of Pharmaceutical Research*, *15*(4), 817-823.

Nakhjavani, M., Nikounezhad, N., & Shirazi, F. H. (2016). The effects parabens on the estrogenic receptors behavior in human breast adenocarcinoma MCF-7 cell line. *American Journal of Pharmtech Research, 5*(5), 119-128.

Nakhjavani, M., & Shiraizi, F. H. (2018). Reporting the effect of cell seeding density on growth pattern of cancer cell lines. *Iranian Journal of Pharmaceutical Sciences, In Press*.

Nakhjavani, M., & Shirazi, F. H. (2017). How human lung adenocarcinoma cells react towards long-term metabolic stress; a follow up. *Indian Journal of Pharmaceutical Education and Research, 51*(4), S667-S674.

Nakhjavani, M., & Shirazi, F. H. (2018). Reporting long-term effects of metabolic stress on human ovary carcinoma. *Marmara Pharmaceutical Journal, 22*(1), In Press.

Nakhjavani, M., Stewart, D. J., & Shirazi, F. H. (2017). Effect of steroid and serum starvation on a human breast cancer adenocarcinoma cell line. *Journal of Experimental Therapeutics & Oncology, 12*(1).

Nakhjavani, M., Vatanpour, H., Abootorabi, A., Shahriari, F., Mohamadzadehasl, B., Bovand, T., & Vatanpour, S. (2016). In vivo effect of lidocaine on mouse exposed to odontobuthos doriae scorpion venom. *International Journal of Medical Research & Health Sciences, 5*(11), 368-375.

Nakhjavani, M., Zarghi, A., & Shirazi, F. H. (2014). Cytotoxicity of selected novel chalcone derivatives on human breast, lung and hepatic carcinoma cell lines. *Iranian Journal of Pharmaceutical Research: IJPR, 13*(3), 953.

Nikounezhad, N., Nakhjavani, M., & Shirazi, F. H. (2016). Generation of cisplatin-resistant ovarian cancer cell lines. *Iranian Journal of Pharmaceutical Sciences, 12*(1), 11-20.

Pardee, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proceedings of the National Academy of Sciences, 71*(4), 1286-1290.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. *Science, 246*(4930), 603-608.

Park, J. H., Lee, S., Kim, J.-H., Park, K., Kim, K., & Kwon, I. C. (2008). Polymeric nanomedicine for cancer therapy. *Progress in Polymer Science, 33*(1), 113-137.

Rathmell, J. C., Vander Heiden, M. G., Harris, M. H., Frauwirth, K. A., & Thompson, C. B. (2000). In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Molecular Cell, 6*(3), 683-692.

Rebucci, M., & Michiels, C. (2013). Molecular aspects of cancer cell resistance to chemotherapy. *Biochemical Pharmacology, 85*(9), 1219-1226.

Roberson, R. S., Kussick, S. J., Vallieres, E., Chen, S.-Y. J., & Wu, D. Y. (2005). Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Research, 65*(7), 2795-2803.

Sarafraz, M. (2013). Experimental investigation on pool boiling heat transfer to formic acid, propanol and 2-butanol pure liquids under the atmospheric pressure. *Jounal of Fluid Mechanics, 6*(1), 73-79.

Sarafraz, M., Nikkhah, V., Nakhjavani, M., & Arya, A. (2017). Fouling formation and thermal performance of aqueous carbon nanotube nanofluid in a heat sink with rectangular parallel microchannel. *Applied Thermal Engineering, 123*, 29-39.

Sarafraz, M., Peyghambarzadeh, S., & Alavifazel, S. (2012). Enhancement of nucleate pool boiling heat transfer to dilute binary mixtures using endothermic chemical reactions around the smoothed horizontal cylinder. *Heat and Mass Transfer, 48*(10), 1755-1765.

Sarafraz, M. M., & Hormozi, F. (2014). Forced convective and nucleate flow boiling heat transfer to alumnia nanofluids. *Periodica Polytechnica Chemical Engineering, 58*(1), 37-46.

Saretzki, G. (2010). Cellular senescence in the development and treatment of cancer. *Current Pharmaceutical Design, 16*(1), 79-100.

Shirazi, F. H., Zarghi, A., Kobarfard, F., Zendehdel, R., Nakhjavani, M., Arfaiee, S., . . . Ashtarinezhad, A. (2011). Remarks in successful cellular investigations for fighting breast cancer using novel synthetic compounds *Breast Cancer-Focusing Tumor Microenvironment, Stem Cells and Metastasis*: InTech.

Sosa, M. S., Bragado, P., & Aguirre-Ghiso, J. A. (2014). Mechanisms of disseminated cancer cell dormancy: an awakening field. *Nature Reviews Cancer, 14*(9), 611-622.

Stewart, D. J. (2007). Saturable passive resistance in chemotherapy of epithelial maligancies. In R. A. Parsons (Ed.), *Progress in Cancer Drug Resistance Research.* (pp. 29-41). New York: Nova Science Publishers,Inc.

Tannock, I. F. (1987). Biological properties of anticancer drugs. *The Basic Science of Oncology*, 278-291.

Thomlinson, R., & Gray, L. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. *British Journal of Cancer, 9*(4), 539.

Torre, L. A., Siegel, R. L., & Jemal, A. (2016). Lung cancer statistics *Lung Cancer and Personalized Medicine* (pp. 1-19): Springer.

Vakili, N., Nakhjavani, M., Mirzayi, H. R., & Shirazi, F. H. (2012). *Studying silibinin effect on human endothelial and hepatocarcinoma cell lines.* Paper presented at the The 13th International Pharmaceutical Sciences Congress.

Vander Heiden, M. G., Cantley, L. C., & Thompson, C. B. (2009). Understanding the warburg effect: the metabolic requirements of cell proliferation. *Science, 324*(5930), 1029-1033.

Zhang, M., & Rosen, J. M. (2006). Stem cells in the etiology and treatment of cancer. *Current Opinion in Genetics & Development, 16*(1), 60-64.