The Effect of Nuclear and Cytoplasmic hnRNPA2B1 Isoforms on the Proliferation and Migration of Breast Cancer Cells

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Plain Language Summary

Breast cancer has the second highest mortality rate among diagnosed malignant tumors worldwide. It can arise from many mechanisms at the molecular level like splicing, which is the transformation of pre-mRNA to mature mRNA. The process of splicing involves splicing factors which are proteins; one of them is hnRNPA2B1. Previous research shows that high levels of hnRNPA2B1 correlate with one feature of breast cancer: high rates of cell growth. However, for another feature—metastasis or migration of cancer cells from the primary site to a secondary site such as the lungs—the reports are contradictory, with some showing positive while others show negative correlation between expression of hnRNPA2B1 and metastasis. The hnRNPA2B1 protein has two forms (called isoforms) that are generated by a process called alternative splicing. One isoform is a protein that localizes in the nucleus of cells, while the other localizes to the cytoplasm. Since previous research did not differentiate between the nuclear vs. cytoplasmic protein, we investigate the effect of each isoform of hnRNPA2B1 on both breast cancer cell growth and migration. Our results show that the cytoplasmic hnRNPA2B1 causes reduced cell proliferation and cell migration of breast cancer cells.

Abstract

Breast cancer has the highest incidence rate and the second highest mortality rate among diagnosed malignant tumors worldwide. One of the many molecular mechanisms involved in cancer is the regulation of slicing by proteins called splicing factors. The expression of these proteins (high or low) has been shown to contribute to the aggressiveness of breast cancer. One such protein is hnRNPA2B1, whose high expression has been previously shown to have a positive correlation with breast cancer cell proliferation. However, for another hallmark of cancer, namely breast cancer metastasis, previous research has reported both negative and positive correlations, making it hard to conclude on hnRNPA2B1's contribution to breast cancer. While hnRNPA2B1 is a splicing factor, its own expression is regulated by alternative splicing. Specifically, exon 2 of hnRNPA2B1 could either be included in the isoform, which ultimately produces a nuclear protein. Whereas an isoform that excludes exon 2 produces a cytoplasmic protein. The localization of hnRNPA2B1 in the nucleus vs. cytoplasm has profound consequences on its ability to function since its main role is to regulate splicing of other genes, a process that is strictly nuclear. Interestingly, previous research did not differentiate between the two isoforms but rather focused on hnRNPA2B1 as one protein. Here, we investigate the effect of each isoform of hnRNPA2B1 on breast cancer cell proliferation and migration. Our results show that inducing the cells to only produce the cytoplasmic hnRNPA2B1 caused lesser cell proliferation and cell migration of breast cancer cells.

Publication Category

Student-Faculty Collaboration

Academic Context

This project was part of the College Honors thesis research of Tajammul under the supervision of Younis. College Honors in Biological Sciences require, among other things, students to complete an extended research project and to disseminate the project.

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Introduction

Breast cancer has the highest incidence rate worldwide and is the second highest cause of female mortality globally [GLOBOCAN, 2020]. While breast cancer prevalence is higher in the USA and Europe compared to countries in the Middle East, the latter have been experiencing an alarming increase in the incidence rate of breast cancer (Donnelly et al., 2011). Moreover, breast cancer patients in the Middle East, including Qatar, are diagnosed at more advanced stages of the disease when they first present with significantly greater size of tumors (Donnelly et al., 2011). Like all cancers, uncontrolled division of cells is one of the hallmarks of breast cancer (Thu et al., 2018) and is tightly controlled by many cellular players and processes, including altered gene expression and abnormal alternative splicing of pre-mRNA. Alternative splicing is a process that allows genes to express different isoforms that ultimately produce proteins of various functions from the same DNA sequence (El Marabti et al., 2018). This process is regulated by RNA binding proteins (RBPs) called splicing factors. One such protein is heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), which belongs to the hnRNP A/B family that have proteins with closely related sequences and a conserved modular structure (Yin et al., 2020).

As a splicing factor, hnRNPA2B1 impacts RNA processing and splicing of many genes that eventually affects the translation of these RNAs to proteins. While hnRNPA2B1 is a splicing factor that regulates other genes, its own pre-mRNA undergoes alternative splicing that generates several isoforms. Two of these isoforms are based on alternative splicing of exon 2 of hnRNPA2B1. Exon 2 encodes 12 amino acids at the N terminus that function as a nuclear localization signal. Thus, when they are included in the translated protein, hnRNPA2B1 localizes to the nucleus and can regulate nuclear processes such as splicing of other genes. Isoform B1 of hnRNPA2B1 retains exon 2 and is shown to be the predominantly expressed isoform. On the other hand, isoform A1 lacks exon 2 and is a cytoplasmic protein. In the cytoplasm hnRNPA2B1 would not be able to regulate splicing, which is a process that occurs strictly in the nucleus.

The hnRNP A/B family of proteins have been studied extensively, especially their potential role in the cancer growth, progression, and metastasis (Hu et al., 2017). This includes hnRNPA2B1 whose expression has been studied in relation to various hallmarks of breast cancer (Hu et al., 2017). However, the role of hnRNPA2B1 in breast cancer is still not clear (Gao et al., 2021). The previous research on hnRNPA2B1 has generated contradictory reports. For example, Hu et al. (2017) showed that high hnRNPA2B1 expression promotes breast cancer development and is positively correlated with breast cancer metastasis, leading to poor prognosis and lower chances of survival for breast cancer patients. On the other hand, Liu et al. (2020) concluded that high expression of hnRNPA2B1 negatively regulates breast cancer metastasis and is correlated with better prognosis of breast cancer patients. A more detailed analysis of these contradicting studies shows that while the role of hnRNPA2B1 in metastasis is inconclusive and outright contradictory, the role of hnRNPA2B1 with regards to the positive contribution of high hnRNPA2B1 to cancer cell proliferation seems to be agreed upon. More specifically, higher expression of hnRNPA2B1 results in more cell growth, and knockout of hnRNPA2B1 leads to tumor growth inhibition by inducing cell cycle arrest at the S phase (DNA synthesis phase) and programmed cell death or apoptosis (Hu, 2017). This effect is generalized to other cancers including glioblastoma (Golan-Gerstl, 2011).

While hnRNPA2B1 plays an important role in breast cancer progression, it is still not clear what is the role of each of the two isoforms. The prior studies in breast cancer did not differentiate between the A2 and the B1 isoforms, but rather grouped them together as one protein. This creates a gap in our understanding of how each isoform contributes to tumorigenesis given that their functions could be drastically different based on their localization in cancer cells. Interestingly, a cytoplasmic hnRNPA2B1 has been previously shown to positively correlate with the aggressiveness of liver cancer (Shilo et al., 2014). Together, these observations provide a compelling reason to study the hnRNPA2B1 isoforms separately. Thus, the objective of the study was to test the effect of each of the isoforms corresponding to nuclear and cytoplasmic hnRNPA2B1 on cell proliferation and cell migration of breast cancer cells. To study the effect of both isoforms on breast cancer aggressiveness, we used a triple negative breast cancer cell line, MDAMB231, to induce skipping of exon 2 after which we tested its effect on breast cancer cell proliferation and migration. Given the earlier reports in liver cancer, our hypothesis was that the cytoplasmic hnRNPA2B1 would cause greater breast cancer cell aggressiveness.

Methodology

Cell Culture

The triple negative cell line, MDAMB231, was used in this study. Cells were grown on plastic flasks and submerged in complete medium that consisted of Dulbecco's Modified

Eagle Medium (DMEM) supplemented with 10% FBS, 1% Penicillin and Streptomycin, and 1% non-essential amino acids. Cells were grown to around 90% confluency before they were passaged as such: Cells were washed with phosphate buffered Saline (PBS), detached with trypsin, and spun down at 1000 rpm for 5 minutes. The cell pellet was resuspended in complete medium to dilute it at an appropriate ratio before replating the cells on new flasks. All cell culture reagents were purchased from Thermo-Fisher. The cells were kept in a humidified incubator at 37°C and 5% CO_2.

Antisense Morpholino Oligonucleotide (AMO) Design

To induce skipping of hnRNPA2B1 exon 2, a 30 nucleotide AMO was designed that is antisense to the intron 1/exon 2 boundary. Upon binding of the AMO to hnRNPA2B1 pre-mRNA it would mask the 3' splice site, inducing skipping of exon 2. A scrambled AMO was used as a control.

Transfection of MDAMB231 cells

MDAMB231 cells were plated the day before transfection and allowed to reach 80-90% confluency. The next day, the cells were dissociated using the same protocol above for passaging, but instead of replating the detached cells, they were counted by mixing equal volume of cells and 0.4% trypan blue and counting live cells using a Countess automated cell counter (ThermoFisher). The appropriate number of cells (1 million per condition) was then centrifuged at 1000 rpm for 5 minutes, washed with PBS, centrifuged again, and the pellet was resuspended in 100 ul of resuspension buffer (provided in the transfection kit from ThermoFisher) per 1 million cells of the cells. To transfect the cells, the Neon electroporation system was used according to manufacturer's recommendations (ThermoFisher). After electroporation, the cells were diluted in complete medium lacking Penicillin and Streptomycin.

Cell Proliferation

To measure the effect of transfecting the various AMO on cell proliferation, 3000 transfected cells were plated per well in 100 ul complete medium lacking Penicillin and Streptomycin in an opaque 96-well plate. Five wells per condition per measurement were plated. At the time of the measurement, the CellTiter-Glo Luminescent assay (Promega) was used. The CellTiter-Glo buffer was thawed and equilibrated to room temperature. The buffer was added to the CellTiter-Glo substrate which formed the CellTiter-Glo Reagent. It was then mixed by gently vortexing, swirling or inverting contents to obtain a homogeneous solution. 100 ul of that solution was added per well and equilibrated at room temperature for 30 minutes. Luminescence was then recorded using the GloMax instrument (Promega). Measurements were made immediately after plating (Day 0) and daily for four days after transfection.

RNA Extraction

To check whether transfecting the cells with the hnRNPA2B1 AMO did induce skipping of exon 2, total RNA was extracted from cells 48 hours after transfection using RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. Briefly, the media was removed and the cells were washed once with PBS. RLT buffer (350 ul) plus 3.5 ul of beta mercaptoethanol were then added per well. The cells were then scraped and transferred to a QIAshredder column and spun. The flow-through was transferred to gDNA eliminator spin column and centrifuged. The flow-through was collected and mixed with 350 ul of 70% ethanol (freshly made). Up to 700ul of this mixture was transferred to RNeasy spin column and centrifuged. The flow-through was discarded and the column was washed with 700ul of buffer RWI followed by two washes with 500ul of buffer RPE. The column was then placed into a new tube and centrifugation was done without adding any buffer to get rid of all remnants of the wash buffers. The dry column was then placed in a new 1.5 ml tube and the RNA was eluted in 30 ul of RNase-free water. All centrifugation steps were done at 11,000 rpm for 30-60 seconds in a desktop centrifuge. The RNA quantity and quality were checked on a nanodrop spectrophotometer.

Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

To convert the RNA to cDNA, the Protoscript II first strand cDNA synthesis kit (New England Biolabs) was used according to the manufacturer's recommendations. Briefly, 1 ug of RNA was mixed with 1 ul oligo dT primer and 1 ul of random hexamer primer. It was then heated to 65°C for 5 minutes in PCR machine followed by a cooling step to 4°C. 10ul of reaction mix was then added followed by 2ul of the enzyme mix which made the total volume equal to 20ul. The following program was then run to synthesize cDNA: 25°C for 5 minutes, 42°C for 60 minutes, 85°C for 5 minutes followed by a 4°C hold. After the run was completed, 180 ul of nuclease-free water was added and then transferred to a microcentrifuge tube. The amount and quality of cDNA was then measured using nanodrop spectrophotometer.

To check for inclusion or skipping of exon 2 of hnRNPA2B1, PCR was performed using the cDNA as a template. The OneTaq 2X Master Mix with standard buffer (New England Biolabs) was used according to manufacturer's recommendation. Briefly, 12.5 ul of the OneTaq 2X Master Mix were mixed with 100 ng of cDNA, 1 ul each of forward and

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reverse primers, and nuclease-free water up to 25 ul. The thermocycling conditions for the PCR were as follows: initial denaturation at 94°C for 30 seconds, followed by 35 cycles of 94°C for 30 seconds, 51°C for 30 seconds and 68°C for 30 seconds. The final extension was done at 68°C for 2 minutes after which tubes were held at 10°C.

Agarose Gel Electrophoresis

To check whether the AMO was successful in inducing exon 2 skipping, the RT-PCR product was resolved on a 1.5% agarose gel. To make the gel, 0.75 g of agarose was added to 50 ml of 1x Tris base, acetic acid and EDTA buffer (TAE) and boiled in microwave for around a minute to dissolve the agarose. After that, 2.5ul of ethidium bromide was added to the flask which was then swirled to ensure proper mixing. The mixture was poured into a casting box and left for around 30 minutes for the gel to polymerize. The gel was then submerged in 1x TAE. In addition to the 100bp ladder, the RT-PCR products were mixed with loading dye to a final concentration of 1X and were then loaded into the wells. The gel was run at 100 Volt for 40 minutes. Imaging was done on a UV-box.

Protein Extraction and Western Blot

To verify that the AMO did not cause complete knock down of both isoforms but a protein is still being made in cells, we collected cell pellets from transfected MDAMB231 cells, which were washed once with PBS. To lyse the cells, 200ul of RIPA lysis buffer plus 2ul protease inhibitors were added. This was incubated on ice for 10-15 minutes and mixed occasionally. Centrifugation was done at 10,000rpm at 4°C for 10 mins. The supernatant was collected and stored at -80°C.

To prepare the protein extract for western blot, the lysates were mixed with 1 X loading buffer and denatured at 95°C for 5 minutes. A protein ladder (5 ul) and 10-20 ul of protein extract were loaded to each well of a 10% premade polyacrylamide gel (ThermoFisher). The gel was run for 1 hour at 120Volts to separate and resolve the proteins. The proteins were then transferred to the nitrocellulose membrane using a semi-dry blotter (ThermoFisher). Blocking was then done using the Licor Blocking buffer after which a solution constituting 1.5 ml of blocking buffer + 1.5 ml TBS + 3 ul of Triton + 3 ul of Rabbit hnRNPA2B1 and 3 ul of Mouse GAPDH was added to the membrane and incubation was done overnight. TBST wash buffer was then used to wash the membrane four times. Next day, secondary antibody (solution constituting 2.5ml of block-ing buffer + 2.5ml of TBS + 5ul of Triton + 1ul of mouse 2° antibody + 1ul of rabbit 2° antibody) incubation was done for 1 hour. Washing was then done with TBST four times after which imaging was done on a Licor imaging system (Licor).

Cell Migration

To check for cell migration, scratch assay was done. 12-well plate was prepared by adding 1ml of warmed media in each well. Cells transfected with control AMO and hnRNPA2B1 AMO were seeded into the culture plate such that after 24 hours of growth, they reached 70-80% confluency. Once confluent, the cell monolayer was scratched in a straight line using a pipette tip. After scratching, medium was removed, and washing was done with PBS to remove detached cells; fresh complete medium was then added. Imaging was then done after which cells were placed in 37°C incubator. After 6 hours, microscope imaging was done and results were compared.

Results

To delineate the impact of hnRNPA2B1 isoforms on breast cancer cell proliferation and migration, we designed an Antisense Morpholino Oligonucleotide (AMO) that would induce skipping of exon 2. Upon transfection of the AMO into MDAMB231 cells, it is expected that the expression of nuclear hnRNPA2B1 isoform will cease and only the cytoplasmic isoform would exist in these cells. A control AMO (scrambled sequence) was also transfected into MDAMB231 cells.

To confirm that the hnRNPA2B1 AMO knocked down the nuclear isoform, RNA was extracted after transfection after which reverse transcription was done. That was followed up by Polymerase Chain Reaction (PCR) to amplify each isoform and the samples were then run on 1.5% agarose gel. The sample in which control AMO was transfected showed two bands corresponding to the nuclear and cytoplasmic hnRNPA2B1 isoforms while the sample in which hnRNPA2B1 AMO was transfected showed only one band corresponding to the cytoplasmic hnRNPA2B1 isoform (Figure 1).

To ensure that the hnRNPA2B1 AMO did not knock down both protein isoforms, a western blot was done. GAPDH was used as the loading control. Protein sample from non-transfected MCF7 was used as the positive control. All three lanes, namely the protein samples from non-transfected MCF7, MDAMB231 with control AMO transfection and MDAMB231 with hnRNPA2B1. AMO transfection, showed bands corresponding to the size of hnRNPA2B1 (Figure 2).

Next, we tested the effect of docking down the nuclear isoform of hnRNPA2B1 on breast cancer cell proliferation. For

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FIGURE 1. HnRNPA2B1 AMO is efficient in knocking down the nuclear isoform of hnRNPA2B1 without affecting the cytoplasmic isoform. RT-PCR of RNA extracted from MDAMB231 cells that were transfected with control and hnRNPA2B1 AMOs. To verify that the hnRNPA2B1 AMO was able to knock down one of the isoforms, the RT-PCR products were resolved on 1.5% agarose gel at 100Volts for 40 minutes. The first lane is loaded with 5ul of 100 bp ladder. Negative controls were samples to which reverse transcriptase was not added (minus sign on top.) Actin served as a loading control. The gel results show that the hnRNPA2B1 AMO was successful in inducing skipping of exon 2 leading to one band of hnRNPA2B1.

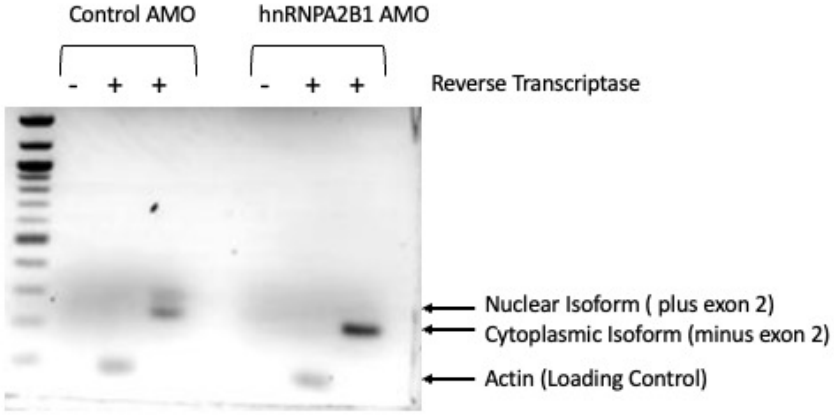
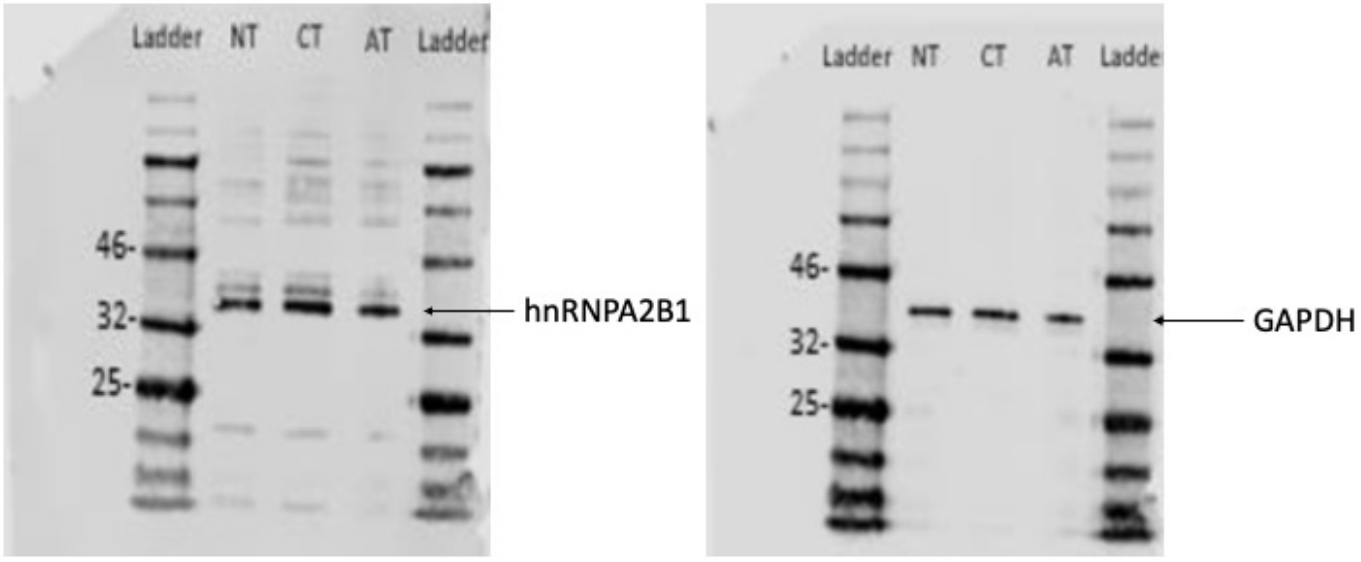


FIGURE 2. HnRNPA2B1 AMO does not cause complete loss of both protein isoforms. To verify that inducing exon 2 skipping does not affect the expression of cytoplasmic hnRNPA2B1 protein a western blot was carried out. The membrane to the left was incubated with anti-hnRNPA2B1 antibody, whereas that on the right was incubated with anti-GAPDH antibody, which served as the loading control. NT = non-transfected MCF7 cell lysates (positive control). CT = MDAMB231 cells transfected with control AMO. AT = MDAMB231 cells transfected with hnRNPA2B1 AMO. The results confirmed that while the AMO does cause loss of the nuclear isoform at the RNA level, the hnRNPA2B1 protein was still expressed suggesting that the cytoplasmic isoform was not affected.



that, CellTiter-Glo assay was performed. This assy uses luminescence as a measure of metabolically active (live) cells. As shown in Figure 3, as early as 24 hours after transfection and until 3 days later, the MDAMB231 cells that were transfected with hnRNPA2B1 AMO showed less luminescence compared to cells transfected with a control AMO. The readings of normalized luminescence for the sample of control AMO ranged from ~1.00 to ~1.75 while the readings for that of hnRNPA2B1 AMO ranged from ~1.00 to ~1.15 (Figure 3).

Given that the main discrepancy in the literature is with regards to the role of hnRNPA2B1 in metastasis, we tested the effect of knocking down the nuclear protein on cell migration as this process is required for metastasis. For that, a scratch assay was used in which a monolayer of AMO-transfected cells was scratched and the migration of cells into the empty space was assessed six hours later (Figure 4).

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FIGURE 3. Knocking down the nuclear isoform of hnRNPA2B1 results in reduced Cell Proliferation in MDAMB231 cells. To check for cell proliferation of MDAMB231 cells (growth of the cells), CellTiter-Glo assay was done for the cells transfected with both control AMOs and the hnRNPA2B1 AMOs. 100 ul of the CellTiter-Glo Reagent was added to each well containing 100 ul of the cell mixture. The readings were then taken over four days. For each condition 5 replicate wells were used per day. CellTiter-Glo Assay results show that when transfected with hnRNPA2B1 AMO, the cell proliferation was less than when transfection with the control AMO was done.

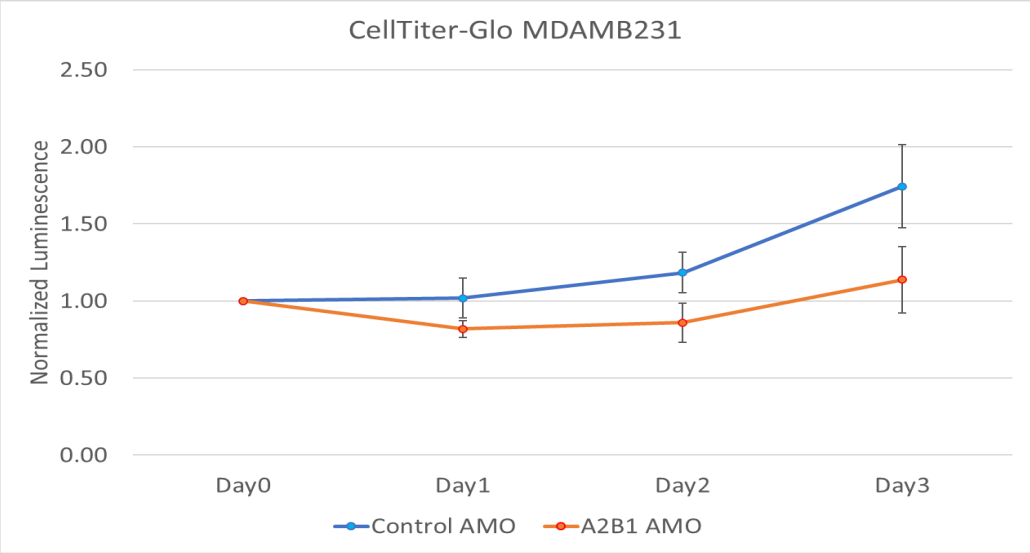
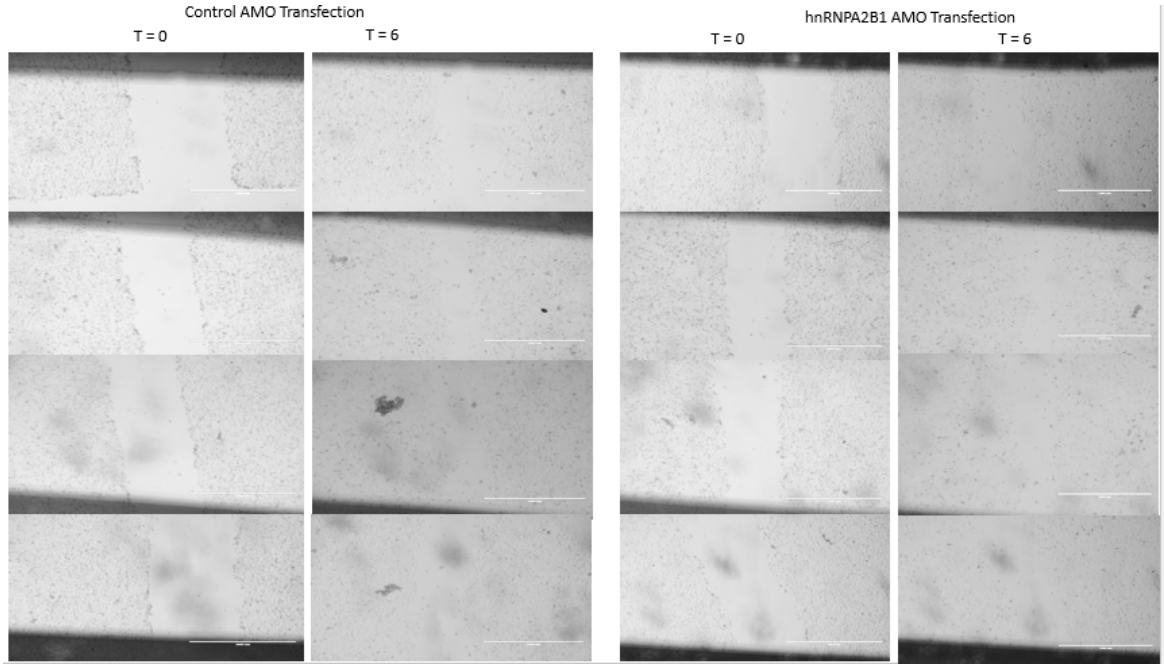


FIGURE 4. Cell Migration is reduced in breast cancer cells that do not express nuclear hnRNPA2B1 isoform. To check for the migration of MDAMB231 cells after transfection of AMOs, scratch assay was performed. Using a pipette tip, 4 scratches were made, and cells were imaged under the microscope. After 6 hours, the same regions were again imaged, and cell migration was observed and compared.



Analysis

HnRNPA2B1 AMO was used to block the expression of nuclear isoform of hnRNPA2B1 while ensuring that the cytoplasmic hnRNPA2B1 protein was not knocked down. First, we confirmed that hnRNPA2B1 AMO knocked down the RNA corresponding to the nuclear isoform of hnRNPA2B1 without affecting the cytoplasmic isoform.

After RNA extraction of the cell lines in which AMOs were transfected, RT-PCR was done. The product was then run on 1.5% agarose gel. The negative control in which no reverse transcriptase was added showed no bands (as expected). The positive controls showed bands for actin (as expected). The control AMO showed two bands corresponding to each of the two isoforms, as expected (Figure

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1). The sample in which hnRNPA2B1 AMO was used showed just one band corresponding to the cytoplasmic isoform of hnRNPA2B1 meaning that it successfully knocked down the nuclear isoform.

On western blot, which is used to detect hnRNPA2B1 protein, MCF7 cells were used as a positive control and showed a band corresponding to hnRNPA2B1 (at around 37kDa). Another control used was the MDAMB231 cell line in which control AMO was transfected (not meant to knock down the protein or any of its isoforms). Lastly, hnRNPA2B1 AMO was transfected and on western blot, it showed bands similar to those of non-transfected MCF7 and the MDAMB231 cell line in which control AMO was transfected (Figure 2). That meant that the hnRNPA2B1 AMO did not knock down the cytoplasmic hnRNPA2B1 protein.

After confirming that the hnRNPA2B1 AMO did knock down the nuclear protein (Figure 1) but not the cytoplasmic protein (Figures 1 and 2), cell proliferation analysis was done. For cell proliferation, CellTiter-Glo assay was done which measures luminescence which is reliant on amount of ATP that is produced in only metabolically active and thus live cells. The readings of luminescence were taken over a span of three days and it showed that luminescence in the case of cytoplasmic hnRNPA2B1 was greater than that of hnRNPA2B1 having both isoforms which meant that cytoplasmic hnRNPA2B1 results in lesser cell proliferation relative to that of nuclear hnRNPA2B1 (Figure 3).

The final phenotype to be tested with respect to aggressiveness of breast cancer was cell migration. Scratch assay was done in which a pipette was used to make the scratch. Images were taken at that time and after 6 hours (incubated at 37°C). The cell coverage after six hours in the case of cytoplasmic hnRNPA2B1 was less compared to the hnRNPA2B1 having both isoforms meaning that the cell migration in the case of cytoplasmic hnRNPA2B1 is less than that of nuclear hnRNPA2B1 (Figure 4).

Conclusion

The fundamental buttress of the project was the Antisense Morpholino oligonucleotides (AMOs) producing the required isoform. The AMO was required to knock down the nuclear hnRNPA2B1 while ensuring that the cytoplasmic hnRNPA2B1 protein did not get knocked down. The first aim of the AMO was to knock down the nuclear hnRNPA2B1. The agarose gel result obtained after RNA extraction of breast cancer cells transfected with AMO followed by RT-PCR showed one band corresponding to the cytoplasmic hnRNPA2B1, meaning that the nuclear hnRNPA2B1 was successfully knocked down. Then, western blot result confirmed that the AMO did not knock down the whole hnRNPA2B1 protein as bands corresponding to hnRNPA2B1 could be seen on the western blot results. Therefore, the main conclusion regarding AMO is that it did not knock down the whole hnRNPA2B1 protein while knocking down the nuclear hnRNPA2B1.

Now that it was confirmed that AMO knocked down the nuclear hnRNPA2B1, the next step was to test it for cell proliferation and cell migration. CellTiter-Glo assay for cell proliferation confirmed that cytoplasmic hnRNPA2B1 resulted in less cell proliferation. Similarly, scratch assay to measure cell migration confirmed that cytoplasmic hnRNPA2B1 resulted in less cell migration. Therefore, while the previous research studies unanimously revealed that hnRNPA2B1 expression is positively correlated with cell proliferation while differed with respect to cancer cell migration, the main conclusion regarding hnRNPA2B1 in this study was that the cytoplasmic isoform of hnRNPA2B1 (i.e., the A2 isoform) leads to less cell proliferation and less cell migration relative to the nuclear isoform of hnRNPA2B1 (i.e., the B1 isoform).

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