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BHLHE40-AS1 a Long-noncoding RNA Regulates DEC1 on Breast Cancer Progression

An honors thesis presented to the Department of Biological Sciences,

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In partial fulfillment of the requirements for graduation from The Honors College

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Research Mentor: Dr. Rebecca DeVaux

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Abstract:

Ductal Carcinoma in Situ (DCIS) is a precursor to breast cancer where abnormal cells have been found in the mammary duct but have not become invasive and spread into surrounding tissue. There has been an increase in early diagnosis of DCIS, but only around 40% of the cases ever progress to an invasive stage, and thus detection can lead to unnecessary surgeries. To identify which of these 40% progress, long noncoding RNAs were used. Through RNA sequencing of DCIS and invasive cells from real patient samples, BHLHE40-AS1 was discovered, a long-noncoding RNA, to be highly expressed in invasive breast cancer and low in DCIS, suggesting that BHLHE40-AS1 supports breast cancer progression. We hypothesize that BHLHE40-AS1 affects cellular protein expression and down regulates DEC1 protein expression in the cell. Reasons for protein loss could be through transcription, translation, and protein degradation; we are focusing on inhibiting transcription. To determine if BHLHE40-AS1 is altering DEC1 mRNA abundance, we treated our cells with small interfering RNA (siRNA). A qPCR was conducted to observe the knockdown of the expression of BHLHE40-AS1 and of DEC1. The siRNA was targeted against BHLHE40-AS1, and when we observed knockdown of BHLHE40-AS1 we also got knockdown of DEC1 mRNA. To determine the half-life of DEC1 we used Actinomycin D, which is known to inhibit transcription and measure mRNA abundance over time. When measuring the stability of DEC1 RNA with siRNA's, the change in rate between the siRNA's and the control is small leading to the conclusion that the effect on DEC1 is not due to transcript degradation, but may be due to a problem with transcription. To validate this hypothesis, we will next perform a nuclear run-on assay. In conclusion, we have identified a long noncoding RNA that may support breast cancer progression, through interaction with DEC1. After validating our observations, we will test BHLHE40-AS1 affect on progression an in vivo mouse model

Acknowledgements

Foremost, I would like to express my sincere gratitude to Dr. Jason Herschkowitz and Dr. Rebecca DeVaux for the support, patience, knowledge and mentoring that was kindly provided throughout the course of this research project. It was a pleasure and an honor to have worked alongside them.

Besides my advisor and mentor, I would also like to thank Rolando Valentin from the Biological Science department for his guidance, encouragement, and support. I also would like to thank The Collegiate Science and Technology Entry Program (CSTEP) for opening the doors to research, and constantly supporting me in any way possible. Because of people like you, I am where I am today. Thank you.

Last but not the least, I would like to thank my family members, and friends whose love and guidance are always with me in whatever I pursue.

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Introduction:

Breast cancer is the most common cause of death in women around the world. Risks factors for Breast cancer can include: age, genetic mutations, and family history. Breast cancer is characterized by different stages of the disease's progression, as well as by whether the cancer is invasive or non-invasive (Key, 2001). We are working with stage zero breast cancer or also known as Ductal Carcinoma in Situ (DCIS).

Ductal Carcinoma in Situ (DCIS) is a precursor to breast cancer where abnormal cells have been found in the mammary duct but have not become metastatic (Figure.1) (Van Cleef, et al, 2014). Metastasis is a process in which tumor cells can spread and invade surrounding tissues. This process is responsible for 90% of cancer related deaths, however mechanisms by which metastasis occurs are not known (Wang, 2010). Once the cells become invasive and escape the duct, we have a cancerous, potentially metastatic and life threatening disease, breast cancer. The risk in developing DCIS starts at the age of 40 and reaches a plateau after age of 60, most commonly among African American, Asian, and Hispanic Women (Van Cleef, et al, 2014). DCIS in most cases has no symptoms, and could be diagnosed through a mammography, or a biopsy (Mayo Clinic, 2014). DCIS usually cannot be detected by a clinical examination (Van Cleef, et al, 2014).

There has been an increase in early diagnosis of DCIS over the past 30 years, but only around 30% of the cases ever progress to an invasive stage, and thus tens of thousands of women each year undergo unnecessary surgery and radiation therapy (Morris E, et al, 2015). In our lab, we are studying to determine the 30% of these cases that do in fact progress to an invasive stage. To study these cases, we investigated the role of long-noncoding RNA in DCIS detection.

Long noncoding RNAs (lncRNA) were thought to play no vital roles when discovered that they are incapable of coding for proteins (Poting, et al, 2009). However, it was found these long noncoding RNAs could directly regulate the stability of mRNA, and a play a role in a number of other functions such as: regulating biological processes, gene expression, genomic imprinting, and playing important roles in tissue homeostasis conditions (Wang, Shan, Yao, 2016). In the lab, we have found evidence of, and are studying, the long noncoding RNAs that influence DCIS progression. In collaboration with Dr. Behbod at the University of Kansas Medical center, we took biopsies from patients with pre-invasive and invasive cells. These samples were subjected to RNA sequencing from where we identified long non-coding RNAs that were expressed on DCIS and invasive cells. We discovered a long noncoding RNA, BHLHE40-AS1, being expressed on the invasive cells (Figure 2).

We hypothesize that BHLHE40-AS1 affects cellular protein expression and down regulates DEC1 protein expression in the cell. To test our hypothesis, we used GapmeRs (or Antisense Oligonucleotides (ASOs)). ASOs function in the cytoplasm and knockdown the expression of RNA by attaching to a link and being cleaved by RNAse H leading to the down regulation of protein (Walder, 1988). The decrease in DEC1 protein could be through the process of transcription, translation, and/or protein degradation; the lab is focused on DEC1 protein loss in relation to the process of transcription.

To test our hypothesis we conducted a series of experiments: western blotting, and qPCR. The process of identifying and separating proteins is known as western blotting. Western blotting consists of three steps: A separation by size, transfer to a solid support, and marking target protein using a proper primary and secondary antibody to visualize (Mahmood, Yang, 2012). We used this technique to observe how much DEC1 protein is in each well when the ASOs/siRNA

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are present. We then used this information to design an experiment to test the transcript stability of BHLHE40-AS1. To do this, we conducted qPCR's. PCR stands for Polymerase Chain Reaction, a technique that allows rapid amplification of a specific segment of DNA. On the other hand, quantitative PCR (qPCR) provides information, that indicates how much specific DNA or gene is present in the sample.

The PCR process contains three steps: Denaturation, annealing, and extension. The reaction solution is first heated above the melting point, allowing the strands to separate, denaturation. In this step, the DNA is denaturing, unwinding, and breaking the hydrogen bonds that hold together the two strands of the DNA molecule, leaving us with a single stranded DNA. The temperature is then lowered, allowing the specific primers to bind to the target DNA. This provides a starting place for the DNA polymerase to bind and replicate the target DNA. Lastly, the temperature is raised again, which allows the DNA polymerase to extend the primers, adding nucleotides to the developing strand (Garibyan, 2013). For our experiment, conducting a qPCR is very important because we want to know how much DNA/RNA is present at a certain time point. This allows for the identification of whether the ASO's or the siRNA's are knocking down the expression of BHLHE40-AS1, leading to a loss of protein.

Materials and Methods:

Development of western blot

Using this technique, we can separate proteins based on mass (kDA), and we do this through gel electrophoresis. The samples are loaded; 10ul ladder, 10ul control, 10ul ASO 2, 10ul ASO 3, and 30ul ASO 4. The 12% tri-glycine gel runs at 100 volts. These results are then

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transferred to a membrane (PVDF), in transfer buffer and are run in the cold room at 100 volts for one hour. This produces a band for each protein present. An antibody is used to specifically detect its antigen. An antibody has a specific function, and its specificity of the antibody-antigen interaction enables a target protein to be identified (either labeled with an enzyme or fluorescent dye). In our case we used the primary antibody for rabbit, therefore the secondary antibody was anti-rabbit to detect the target antigen (Anti-Rabbit, ThermoFisher). We used a primary antibody that specifically binds to the protein, and was left in the cold room overnight on rotator. The membrane was then washed three times with tri-buffered saline (TBST) the following day. The main purpose of using TBST is to prevent non-specific binding of the antibody. After the three washes, the secondary antibody (rabbit), 1.5ul was added, and placed on rotator for one hour. The membrane was then rinsed an additional three times with TBST. To develop our western blot, we added 1 mL of each developing reagent (developing reagent must not be mix), and scanned it using the STORM machine.

Transfection

The siRNA's plus RNAimax and serum free media were added to the plates and collected the cells to observe the knockdown expression. We added 10 microliters of the siRNA and 7.5 microliters of the RNAimax. RNAimax is a lipid that forms a bubble around the siRNA and can incorporate with the cell membrane to deliver its target. 1mL serum-free media was added to the cells and was left to complex for 10 minutes. Serum free media is used because serum has growth factors to allow the cell to grow; proteins in the serum would interfere with the bubble formation, and possible contamination with RNAses is avoided (Factors Influencing Transfection, ThermoFisher). At 10 minutes start incubating trypsin on cells, by removing the media and rinsing with 5mL of Phosphate-buffered saline (PBS). PBS was removed, and 1mL of

trypsin was added. PBS washing is needed to remove serum of media to allow trypsin to detach the cells from the plate; trypsin is a protease that cuts the proteins between the cell and the ECM (PRSS1 gene, 2012). The cells were incubated and checked after two minutes. The cells were collected and 4mLs of 10A media was added. The total 5mLs was added to a 15mL conical tube. 1mL of cells from the 15mL conical tube was added into three wells in a six well plate, for three plates. 10uL of the cells from the 15mL conical tube were counted to determine the amount of cell/mL

Math:

100,00 cell/mL (1/35 x 10^4 cells) = .2857= ~. 29 .71 x 10= 7.1mL media and 2.9mL cells.

We collected the cell plate after 72hrs and performed RNA extraction and then a qPCR. This transfection was conducted for the siRNA control, 12, and 15. This method could also be performed for the ASO's.

Actinomycin D

The transfected cells were removed from the incubator (37° Celsius) and placed under the hood. An aspirator was used to remove the media that was already inside on top of the cells (Do not remove media until master mix is made to avoid cells being dried). 2mL of Actinomycin D was added to each plate. We observed the plated cells for a total of 5 hours (0,1,2,3,5). Each plate had its RNA extracted using the complete RNA kit.

Math:

1mM actinomycin D = 1.2 ug/ul, we want 5ug/mL in 2 mL. (1.2ug/mL) x V1 = 5ug/mL x 2mL.

(1.2ug/mL) x V2 = 5ug/mL x 8mL. V1= 33.3ul. 45ugh/mL ÷ 1.2ug/mL = 37.5uL. 5ug x 20mL =

100uL/1.2ug = 83.3 uL

E.Z.N.A Total RNA Kit I

The cells were disrupted using TRK lysis buffer. 700ul of TRK lysis buffer plus 20uL B-me per mL of lysis buffer was added into each well. The cells were then scrapped and collected in a 1.5 mL centrifuge tube. One volume of ethanol (700uL) was added. Ethanol is added to provide appropriate binding conditions. A HiBind Mini Column is inserted into a 2 mL collection tube. 700uL of the sample is transferred into the HiBind RNA Mini Column, and centrifuge at 10,000-x g for 1 minute. The filtrate was discarded and this step was repeated until the entire sample had been transferred. 500uL of RNA Wash buffer I was added to the HiBind Mini Column, and centrifuged at 10,000 x g for 30 seconds. The filtrate was discarded. 500uL of RNA Wash Buffer II was added to HiBind Mini Column and centrifuged at 10,000-x g for one minute, discarding filtrate and repeating this step one more time. The RNA Wash Buffer II contains 100% ethanol. This step is crucial because it forces the precipitation of nucleic acid out of solution. The HiBind Mini Column is centrifuged at maximum speed for two minutes to completely dry. It is important to dry the HiBind Mini Column because residual ethanol may interfere with downstream applications. The HiBind Mini Column is transferred to a 1.5 mL centrifuge tube and treated with 40uL of DEPC water, and centrifuged at maximum speed for two minutes.

cDNA Reverse Transcriptase

The RNA samples are spec using a spectrometer (used for measuring the amount of RNA present). After the amount of RNA to be reverse is determined, it is added to a PCR tube. The RNA sample is diluted to 10uL with RNAse free water. A master mix is created using: 10x RT buffer, 25x dNTP, 10x RT random primers, multiscribe RT (added last), and RNAse free water. 10uL of the master mix is added to the 10uL of RNA and placed in a thermocycler. The resulting complementary DNA (cDNA) is ready for dilution and to be used in qPCR. cDNA is the product of the synthesis of DNA from an RNA template. DNA is a lot more stable than RNA, and PCR amplification only works on DNA.

Quantitative Polymerase Chain Reaction (qPCR).

The cDNA samples are diluted with 180uL of RNAse free water. A master mix was created for DEC1, 18s, and ABI. 5uL of the cDNA samples were plated in a 384 well plate, following 5uL of the taqmam. The plate is placed into the qPCR machine and ran for 1 hour and 35 minutes. Once this is done, we obtain our results.

Results:

Our hypothesis was that BHLHE40-AS1 affects cellular protein expression and down regulates DEC1 protein expression in the cell. To test this hypothesis, cells were treated with small interfering RNA (siRNA), ASOs, and a qPCR was conducted to observe the knockdown of both BHLHE40-AS1 and of DEC1 expression. The siRNA was targeted against BHLHE40-AS1, and when we observe knockdown of BHLHE40-AS1 we also get knockdown of DEC1 mRNA.

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Loss of DEC1 expression

We performed a western blot to observe if there was a change in DEC1 protein. However, when we scanned our membrane using the STORM machine, we did not obtain the results that we were hoping for; the bands did not match our predictions of change in DEC1 protein. The membrane containing the protein was washed three times with tri-buffered saline (TBST) and the secondary antibody was added once more. After re-adding the secondary antibody for a second time, we still did not get the results that were expected. Therefore, we re-added the primary antibody to the membrane and left it overnight. We re-developed, and observed that when the ASOs are in the cell, we also see a loss of DEC1 protein (Figure.3). We do see a change in DEC1 protein, suggesting that the mRNA change is sufficient to reduce protein accumulation.

ASOs knockdown expression of BHLHE40-AS1

After the cells were transfected with the ASOS, a qPCR was conducted to observe the knockdown of the expression of the long noncoding RNA BHLHE40-AS1. When the ASOs are added into the cell, they knockdown the expression of BHLHE40-AS1 (Figure.4)

Measuring the stability of BHLHE40-AS1

To measure the half-life of BHLHE40-AS1, and DEC1 and compare it to hour zero, we inhibited transcription using Actinomycin D. Actinomycin D prevents RNA Polymerase progression by different concentrations; there are three classes of gene transcription, each with a different concentration. By hour 5, we lost 60% of our mRNA for DEC1 under control conditions (Figure.5). This indicates that DEC1 has a short mRNA half-life.

Knock down of DEC1 using Actinomycin D

We repeated the experiment using ASO 2 and ASO 3 to test the stability of the mRNA. With ASO 2 we lost transcript at a much faster rate, suggesting that BHLHE40-AS1 helps with the production and/or stability of the mRNA of DEC1 (Figure.6). However with ASO 3, we saw something different; we saw that relative to the control, there was more mRNA stating that it is more stable (Figure.7). These results were then compared to our previous experiment which were done using siRNAs.

siRNA Knockdown of BHLHE40-AS1

siRNAs were added to observe the knockdown of BHLHE40-AS1. We noticed that we have knocked down expression of the RNA, meaning that they do work (Figure.8). siRNA mediated Knockdown of BHLHE40-AS1 attenuates DEC1 expression. Because we knocked down expressions of the RNA it means that we got rid of DEC1 expression (when we have knock down of BHLHE4-AS1, we also have knockdown of DEC1 expression) (Figure.9).

To summarize our results, when we have knockdown of BHLHE40-AS1 we also see a lost of DEC1 protein, leading to our next hypothesis is the effect on DEC1 due to transcript degradation?

Discussion/Conclussion:

We have identified a long noncoding RNA that may support breast cancer progression, through interaction with DEC1. Knockdown of BHLHE40-AS1 throgh ASOs and siRNAs, results in loss of DEC1 protein. If BHLHE40-AS1 is indeed linked to breast cancer progression,

it can be utilized to create better diagnostic tests to improve patient treatment options and outcomes.

To discuss our next hypothesis; is the effect on DEC1 due to transcript stability (Figure.10)? And is the change in (Figutr.9) enough to result in (Figure.8)? We measured the effect on DEC1, and determined that the transcript degradation could be because we started with less RNA. In comparison to the control, the change in loss of transcript between the blue bar and the green bar is very small. We are interpreting that the transcript is degrading at the same rate of the control; this together means that when we conduct our experiment we could be starting with less RNA.

To further validate our hypothesis, we will next perform a nuclear run-on assay (NOA). Taking our results from our first hypothesis into consideration, what can we learn from the nuclear run-on-assay?

- o If we are starting off with less RNA, could we see why? What is going on that is resulting in the loss of RNA?

- o Nuclear run-on-assay measures genes that are actively being transcribed

In order to perform a nuclear run on assay we will need to optimize some conditions. The condition that needs to be optimized is: finding a time point that would work best at knocking down the expression of DEC1. We designed an experiment using 24, 48, 72 hour time points, but unfortunately there were some technical errors.

Our hypothesis for the nuclear run on assay is, that we may be starting with the less RNA in the first place, which may mean, that something is acting to get rid of the transcript. We
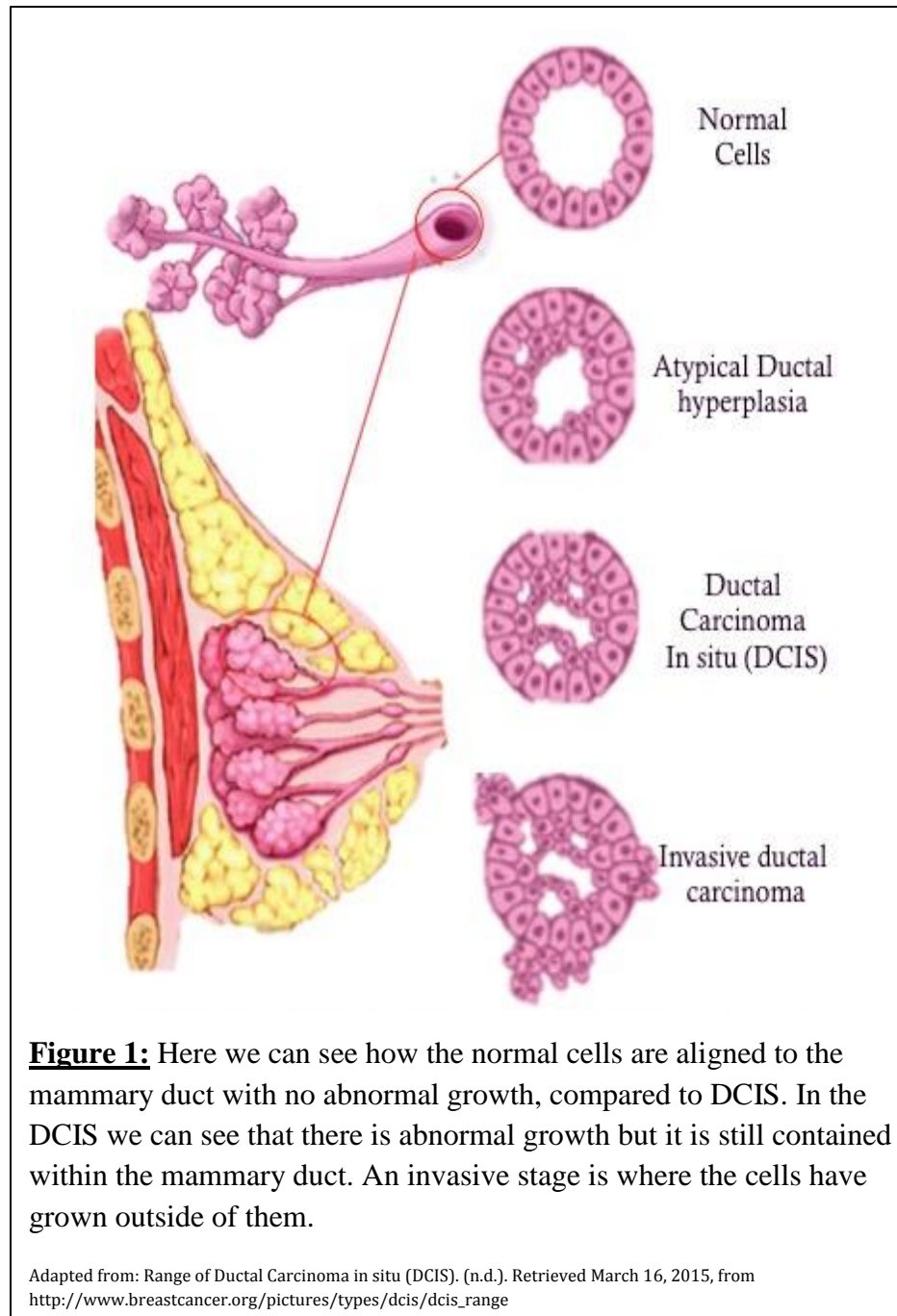
determined that compared to the control, we are losing transcript for siRNA 15 at a similar rate. With the nuclear run on assay it will tell us if something is going on at the beginning of the process, however it will not tell us why. If we are able to see the abundance of the RNA at the beginning of transcription, then we can confidently say that we are losing the RNA.
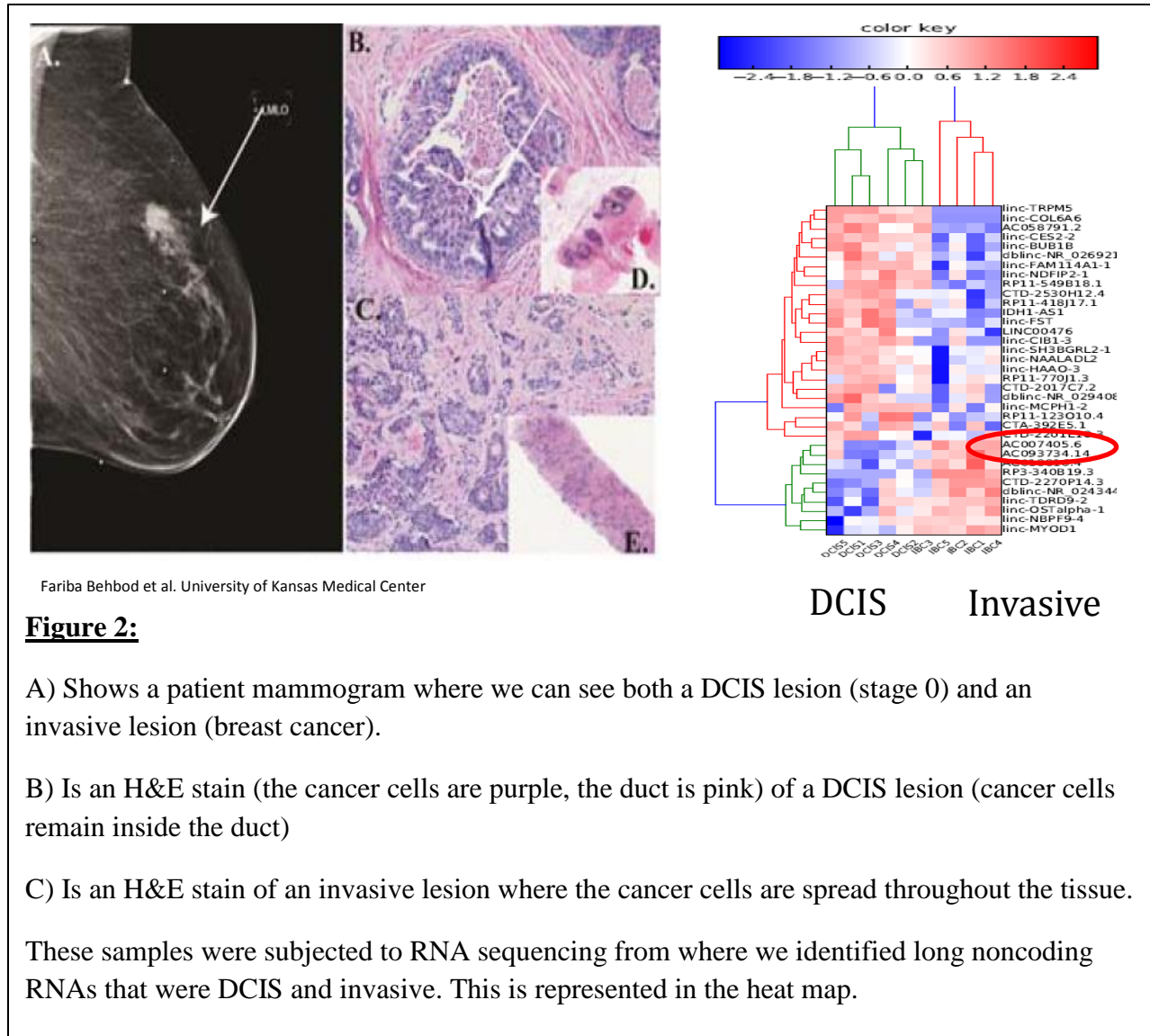
During transcription, if we have knockdown of cells and we see that they are all stuck in mitosis, the cell is not transcribing anything. If the knockdown is producing other effects like on cell cycles, we can see an effect on transcription as well. In order to perform the nuclear run on assay, we would need at least 6 million cells, and primers that should be 10-22 nucleotides in length, have GC content of 40-60%, and produce short (50-120 base pair) amplicon (Roberts, et al, 2015). However, we do not want to do a nuclear run on assay because it is a very technical, challenging experiment to do. Nuclear run on assay uses phosphate 32, which is radioactive; radiation could isolate RNA, but in order to use radiation we have to be certified. Instead we are using a click-it kit because it offers, an easier, faster, alternative way. We essentially want to do the NOA but through the other kit. The click-it kit captures the newly made RNA; the newly made is composed of introns and exons. After validating our observations, we will test BHLHE40-AS1 effect on progression an in vivo mouse model.

Our hopes are to be able to apply these findings to humans. By applying these findings to humans we could avoid: unnecessary treatments, therapies, and target the breast cancer cases that will progress to an aggressive, invasive, stage.

Figure 1: Here we can see how the normal cells are aligned to the mammary duct with no abnormal growth, compared to DCIS. In the DCIS we can see that there is abnormal growth but it is still contained within the mammary duct. An invasive stage is where the cells have grown outside of them.

Adapted from: Range of Ductal Carcinoma in situ (DCIS). (n.d.). Retrieved March 16, 2015, from http://www.breastcancer.org/pictures/types/dcis/dcis_range

Fariba Behbod et al. University of Kansas Medical Center

Figure 2:

A) Shows a patient mammogram where we can see both a DCIS lesion (stage 0) and an invasive lesion (breast cancer).

B) Is an H&E stain (the cancer cells are purple, the duct is pink) of a DCIS lesion (cancer cells remain inside the duct)

C) Is an H&E stain of an invasive lesion where the cancer cells are spread throughout the tissue.

These samples were subjected to RNA sequencing from where we identified long noncoding RNAs that were DCIS and invasive. This is represented in the heat map.

Figure 3: Loss of DEC1 protein. Samples were treated with ASOs (control, 2, and, 3). Samples were run on a 12% tris-glycine gel, at 100 volts. GAPDH was used as a loading control.

Figure 4: Knockdown of BHLHE40-AS1 expression. A qPCR was conducted using the ASOs on the long noncoding RNA BHLHE40-AS1 at 72 hours.

Figure 5: **Knockdown of DEC1 under control conditions.** Using Actinomycin D to inhibit transcription, a qPCR was conducted to observe the RNA expression of DEC1. By hour 5, we lost 60% of mRNA.

Figure 6: **Knockdown of DEC1.** Using Actinomycin D to inhibit transcription, a qPCR was conducted to observe the RNA expression of DEC1. With ASO 2 the transcript was lost at a much faster rate.

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Figure 7: **Knocking down of ASO's of DEC1.** Using Actinomycin D to inhibit transcription, a qPCR was conducted to observe the RNA expression of DEC1. With ASO 3 the transcript was loss at a much slower rate than the control.

Figure 8: **siRNA knockdowns of BHLHE40-AS1.** Using Actinomycin D to inhibit transcription, a qPCR was conducted to observe the RNA expression of BHLHE40-AS1.

Figure 9: **a siRNA knockdown of BHLHE40-AS1 attenuates DEC1 expression.** Using Actinomycin D to inhibit transcription, a qPCR was conducted to observe the RNA expression of BHLHE40-AS1 affect on DEC1.

Figure 10: **The effect on DEC1 due to transcript stability.** Using Actinomycin D to inhibit transcription, a qPCR was conducted to observe the RNA expression of BHLHE40-AS1 affect on DEC1 mRNA. Relative to the control, the loss of transcript is very small.

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