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Differential Iron Regulatory Genetics in 2D & 3D Culture of Breast Cancer Cells

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Differential Iron Regulatory Genetics in 2D & 3D Culture of Breast Cancer Cells

Abstract

The iron regulatory axis has consistently been shown to be perturbed in cancer cell lines relative to non-cancerous cell lines. As cancer cells rapidly divide and grow, they require iron to fuel many intracellular processes, including DNA replication and protein synthesis. Three-dimensional cell culture is an increasingly popular method of culture that purportedly more accurately mimics the *in vivo* microenvironment of cancers over traditional two-dimensional culture. This project was prompted by previous lab results to investigate differential iron regulatory gene expression in 2D and 3D spheroid culture models. We replicated the findings that the gene hepcidin is induced in 3D culture. Furthermore we found that the iron storage gene ferritin was significantly induced in 3D culture. We hypothesized that this induction was related to increased oxidative stress in the spheroids and supported this theory with an experiment that induced hypoxia in 2D and 3D models.

Introduction

The iron regulatory pathway has been well characterized. Serum iron circulates bound to a protein called transferrin. Transferrin delivers iron to cells by binding to a membrane protein called transferrin receptor 1. Once iron enters the cell it either enters the labile iron pool, where it can be utilized for various cellular functions, or is safely stored in the iron storage protein ferritin, which is composed of both heavy and light chain forms. While free iron has been shown to generate reactive oxygen species, iron stored in ferritin does not. Iron can also be exported from the cell via a membrane protein termed ferroportin. Ferroportin is further regulated by an extracellular protein hepcidin, which binds ferroportin and triggers its degradation. Many cancers are shown to upregulate hepcidin expression; thus, lowering the amount of ferroportin available to remove iron from cells and increasing the available iron the cell has available for cellular processes (e.g. DNA synthesis & oxidative phosphorylation). (Torti 2013).

In this research we compared traditional 2D plate culture to 3D spheroid culture of MCF-7 breast cancer cells. In the 3D model, cells are cultured in ultra-low adherent wells to form spheroids rather than in traditional petri dishes. Theoretically, these spheroids are a more accurate representation of the true breast cancer physiology as the spheroids mimic the nutrient and oxygen gradients and loss of basoapical polarity found in actual breast cancer (Vidi, Bissell, & Lelièvre, 2012).

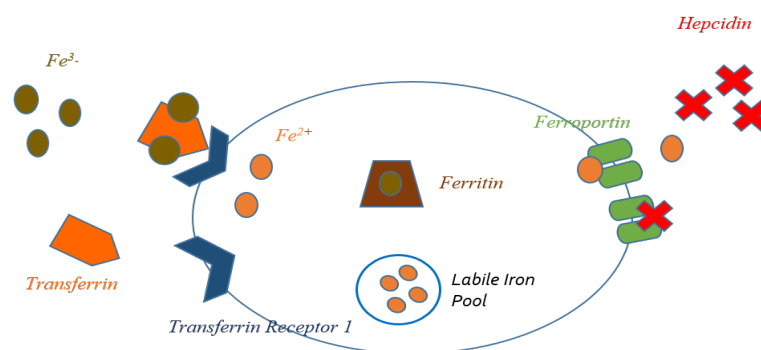


Figure 1

Results

Throughout my research, I investigated differences in gene expression of various iron regulatory genes between 2 dimensional and 3 dimensional MCF-7 breast cancer cell culture models. An image of my cultured MCF-7 spheroids can be seen in Figure 1.

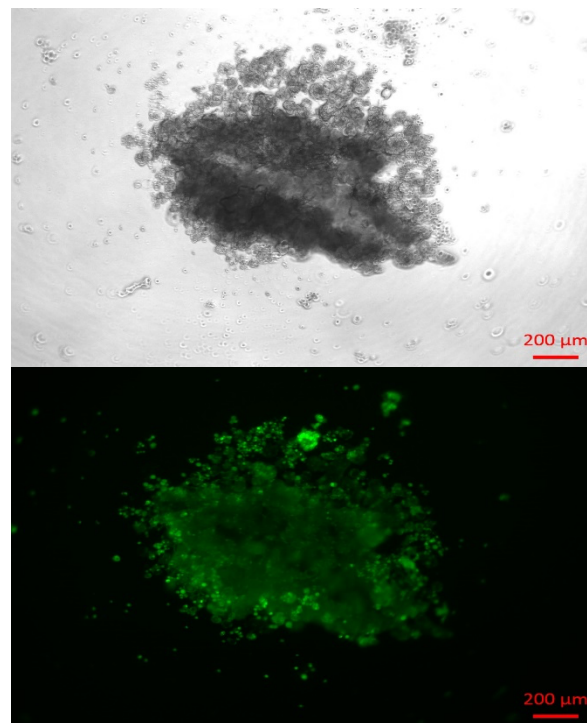


Figure 2 (Hanna, 2016)

MCF-7 spheroids under microscope. Bottom image is stained with calcein with green fluorescence indicates cell viability.

The initial experiment for this project aimed to replicate previously seen upregulation of hepcidin in the 3D model via RT-qPCR. These results are visible in Figure 3.

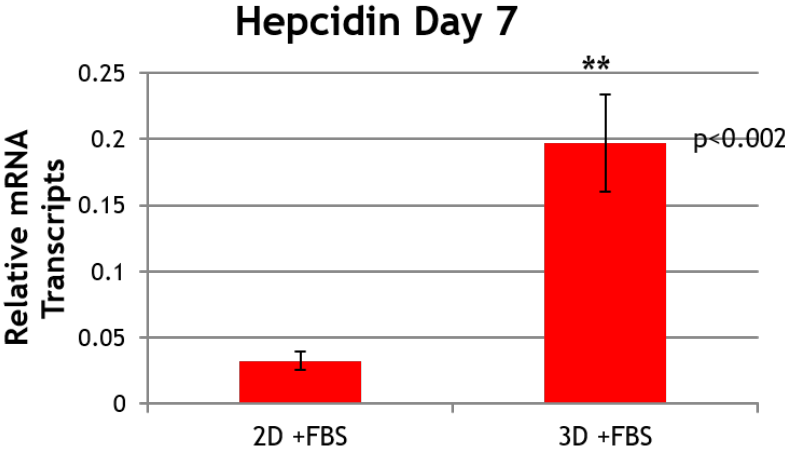


Figure 3 (Hanna, 2016)

Here +FBS indicates that the cells were cultured with fetal bovine serum, a solution containing various nutrients, including iron, to promote cell growth. Only +FBS data is shown here because without FBS cells did not survive until day 7.

We investigated other iron regulatory genes, including ferritin L & H, ferroportin, transferrin receptor 1 (TFR1), and iron regulatory protein 2 (IRP2). These results are summarized in Figure 4.

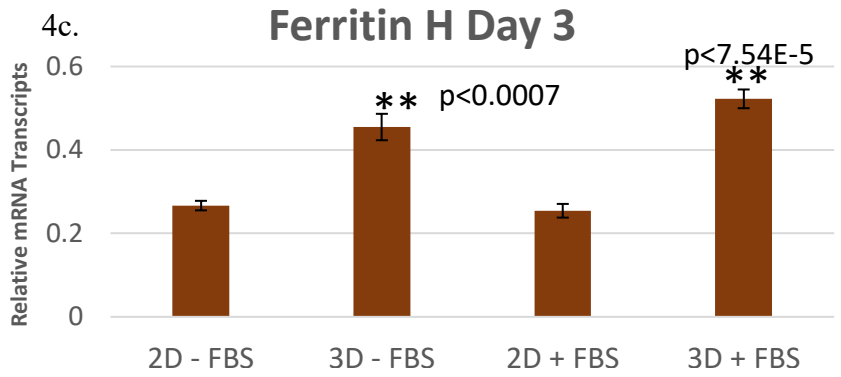
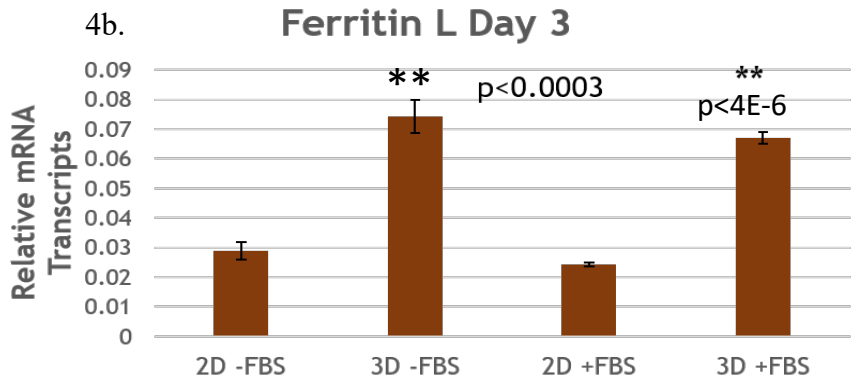
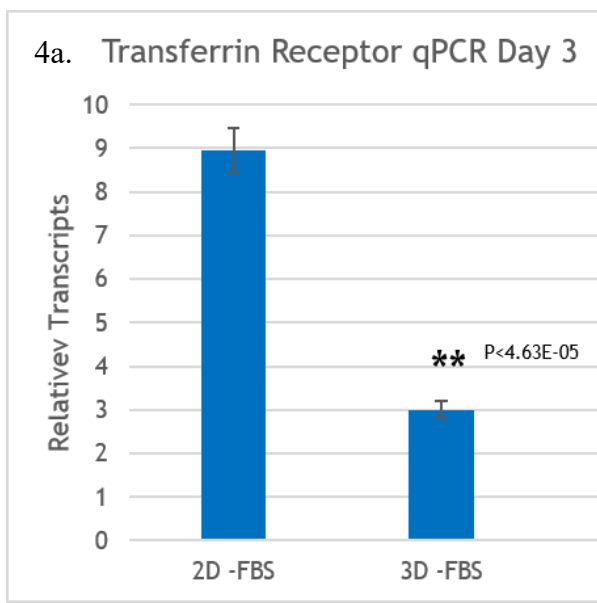


Figure 4 (Hanna, 2016)
 Figure 4a only displays -FBS data as this trend was not observed when cultured with FBS.

Figure 4 prompted further investigation the cause of increased transcription of these genes. We hypothesized that hypoxia may play a role in the regulation of these genes as the 3D spheroids experience hypoxia towards their centers due to lack of contact with an oxygenated atmosphere, a phenomenon that 2D cells do not experience.

In accordance with this hypothesis we repeated the RT-qPCRs, but this time placed a sample of each model's cells in a hypoxic environment.

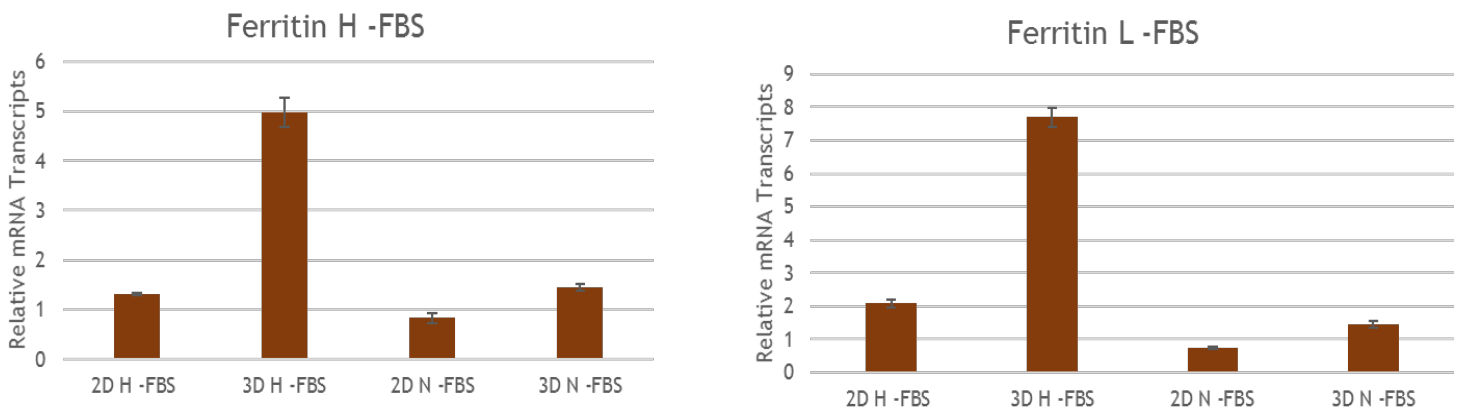


Figure 5 (Hanna, 2016)

H indicates the hypoxic condition while N represents the normoxic condition.

Discussion

Our results were able to further support differential expression of iron regulatory genes between tradition 2D and 3D spheroid culture of MCF-7 breast cancer spheroids. The generated spheroids were analyzed via RT-qPCR had significantly induced (more than seven times induction) hepcidin transcripts relative to the 2D cultures as seen in Figure 3.

Subsequently, we began investigating possible perturbations in the other iron regulatory genes, including ferroportin, transferrin receptor, and the ferritin heavy and light chains. While ferroportin was shown to have no significant difference in transcripts, differences can be seen for transferrin receptor and the ferritin heavy and light chains in Figure 4. We found that transferrin receptor was downregulated in –FBS spheroid cultures, while ferritin H and L were both upregulated significantly in –FBS and +FBS spheroid cultures.

The upregulation in ferritin in spheroid culture prompted inquiry into the mechanism behind the induction. We theorized that since spheroids undergo increased oxidative stress, cells were upregulating ferritin expression to prevent iron from generating more reactive oxygen species. This experiment supported our hypothesis as both ferritin proteins were found to be induced in hypoxic environments when compared to the normoxic condition, depicted in Figure 5. TFR1 was not found to be consistently impacted by the hypoxic condition.

Future goals for this project include performing protein expression analysis on ferritin L and H chains to investigate whether transcript upregulation in the spheroids is affecting actual protein expression.

Materials and Methods

Real-time Quantitative qPCR

High Pure RNA Isolation Kit from Roche Diagnostics was used to purify RNA from cells. Using the kit's instructions, Oligo(dT) primer was used to synthesize cDNA with up to 80 nanograms of RNA being reverse transcribed in a total volume of 50 microliters. We performed serial dilutions of RNA for reverse transcription to generate a standard curve. 2 microliter aliquots of produced cDNA were combined with 18 microliters of qPCR reaction mixture with 10 microliters of 2X SYBR Green PCR Master Mix and primers. Each sample had 3 replicates. Negative controls without cDNA and without primers were used to rule out DNA contamination.

Spheroid Cell Culture

We coated U-bottom 96-Well Polystyrene Round Bottom Microwell Plates from Thermo Fischer Scientific with Poly(2-hydroxyethyl methacrylate) (polyHEMA) 24 hours before spheroid plating. 2.4 grams of polyHEMA were dissolved in 70% ethanol initially to make a 10X stock. This stock was diluted to 1X with 70% ethanol and 30 microliters of this solution were left in a sterile laminar flow hood overnight. The next day MCF-7 cells were trypsinized with 0.05% trypsin from monolayer plates and 8,000 cells per well were placed in appropriate growth medium (DMEM with or without FBS). Plates were grown in 37-degree Celsius environment with 5% CO₂ and 20% O₂. Spheroid viability and images were generated using 2 uM calcein-AM from Life Technologies and imaged using Zeiss Axio Vert.A1 microscope.

Traditional Cell Culture

MCF-7 breast cancer cells were acquired from the Wake Forest University Comprehensive Cancer Center Tissue Culture Core facility. For traditional 2D cell culture experiments, cells were cultured in Dulbecco's minimal essential medium (DMEM) either with

or without 10% Fetal Bovine Serum (FBS). Cells were grown in a 37-degree Celsius environment with 5% CO₂ and 20% O₂.

Hypoxia Experiment

To induce hypoxic condition on cells, both tumor spheroids and 2D cultured cells were incubated at 5% O₂ while normoxic condition was kept at 20% O₂. VEGF induction was used to confirm hypoxic stress.

References

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