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Key cytoskeletal proteins are upregulated in 2D cell culture in comparison to 3D culture

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Key cytoskeletal proteins are upregulated in 2D cell culture in comparison to 3D culture

by

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ABSTRACT

The cytoskeleton is the structural framework of the cell, and plays an important role in cell motility, signalling pathways, and cell behaviour. It is composed of various proteins that form a dynamic network of filaments and tubules to mechanically support the cell. Commonly, cytoskeletal proteins such as β-actin are used as controls or references in many laboratory techniques because they are assumed to remain stable across various conditions. However, most of the research to date has been performed on 2D monolayers of cells adhered to plastic. Recently, 3D cell culture techniques have become more feasible and increasingly popular as they better represent the physiological microenvironment found in the body. As more research shifts from 2D to 3D culture, the changes in cytoskeleton due to culture conditions need to be better characterized. To study gene expression, eleven human cell lines were cultured in 2D and 3D, and reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed. For analysis, ten control genes were evaluated for stability, and the geometric mean of the three most stable genes was used for normalization. The results show a significant downregulation of several cytoskeletal genes such as β-actin, vimentin, and keratin in 3D compared to 2D. Similarly, key cytoskeletal proteins were also found to be downregulated in 3D using Western blots normalized to total protein. These results demonstrate that the microenvironmental differences between culture conditions can considerably influence cytoskeletal component levels across various cell types. Integrating these findings with the published literature, we suspect that the supra-physiological stiff surface in plastic-adherent culture leads to overexpression of cytoskeletal proteins – presumably also impacting many other pathways and cell behaviour. These findings challenge the assumption that β-actin provides a consistently stable reference gene/protein, emphasize the importance of physiologically relevant culture systems, and highlight the need to select appropriate controls for specific experimental conditions.

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List of Symbols, Abbreviations, & Nomenclature

Symbol/Abbreviation	Definition
α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
ζ	zeta
λ	lambda
μ	mu or micro
π	pi
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
cDNA	Complimentary DNA
DAPI	4'6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dPCR	Digital PCR
ef1α	elongation factor 1-α
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
hESCs	human embryonic stem cells
mESCs	mouse embryonic stem cells
mRNA	Messenger RNA
MSCs	mesenchymal stem cells
NF	Normalization Factor
nm	nanometers
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
relData	Relative Data
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcriptase quantitative PCR
tRNA	Transfer RNA
SDS-PAGE	sodium dodecyl (lauryl) sulfate polyacrylamide gel electrophoresis
hPSCs	human pluripotent stem cells
NGS	next generation sequencing
RNA-Seq	RNA-Sequencing
ECM	extracellular matrix

Symbol/Abbreviation	Definition
2D	Two-dimensional
3D	Three-dimensional
18S	18 Svedberg Units

CHAPTER ONE: INTRODUCTION

1.1 Tissue Engineering

In Canada, over 1,600 people are added to the organ donation list every year and there are currently 4,500 individuals waiting for an organ donation [1]. Just Ontario spends roughly $100 million dollars every year supporting the care for people waiting for a kidney donation [1]. Moreover, treating patients for an organ or tissue failure accounts for 50% of the total annual healthcare cost in the United States [2]. The field of tissue engineering involves generating tissues and organs for the human body that are functional and free of immune rejection [3]. In the short term, it can also help by providing relevant tissue model systems to study disease physiology, pathology, and/or perform drug discovery [3]. Thus, tissue engineering research holds a tremendous potential to revolutionize the field of medicine.

The three fundamentals of tissue engineering involve a cell source, scaffold for the cell, and growth factors to support the cell [4]. The cell source can greatly influence the success of tissue engineering. Commonly, commercial cell lines are used in research to study instead of primary cells from humans. This is because most commercial cell lines are cost effective, easy to use, characterized, reproducible, provide an unlimited supply of material, and have less ethical concerns in comparison to using primary human tissue [4]. The scaffold for the cell is also referred to as the template or substrate or extracellular matrix (ECM). The ECM functions to recreate the microenvironment that the cell was originally found in. ECM also provides support to the cell to allow for survival after transplantation.

Although there has been significant research progress in the field, there are still significant hurdles in translating advances in tissue engineering from bench to bedside [5]. The

main hurdle is being able to control cell and tissue behaviour to create functional engineered tissues. In order to study cell behaviour, cells are first cultured and then molecular biology techniques are used to observe their components. Cells can then be manipulated with external factors and conditions and the change in behaviour can be observed. This introduction will expand on common cell culture and laboratory techniques and how they can be used to observe the inner components of the cell such as the cytoskeleton.

1.2 Cell Culture Techniques

In order to study the fundamental function and behaviour of cells, the first step is to grow the cells in large enough quantities to study them. Cell culture is an important and widely used practice where cells are grown outside their natural environment (*in vitro*). For over a century, cell culture has been performed in a two-dimensional (2D) layer adhered to polystyrene plastic or glass. This has been widely accepted and has significantly advanced the understanding of cell behaviour [6]. Plainly, this is not an accurate representation of the microenvironment or niche found in the native tissue or organ. Most cells exist in a three-dimensional (3D) environment within the human body. Indeed, when comparing the 2D model behaviour to the behaviour in body, many complex biological responses such as receptor expression, transcriptional expression, cellular migration, and apoptosis were found to be different [7]. For instance, important characteristics of cancer cells cannot be modelled in 2D cultures at all [6]. There have been advancements in 2D culture such as co-culture of multiple cell types, sandwich culture where cells are placed between two layers of substrate, micro-patterning where the surface is engineered to certain shapes, and alteration of substrate stiffness. To date, these advancements

have not solved all the issues that come with the simplicity of 2D culture [6]. The advantages and disadvantages of both 2D and 3D culture are compared in Table 1 below.

Table 1. Advantages and Disadvantages of 2D and 3D Cell Culture Techniques

Cell Culture	Advantages	Disadvantages
2D	– conventional – more previous fundamental research to be found – easy handling – ideal for high throughput screening – easy downstream processing – cost-effective	– less biologically relevant – limited cell-to-cell and cell-to-matrix interactions – rigid, inert system with no cell/tissue architecture – no diffusion gradients present – gene expression is less representative – drug screening is less predictive
3D	– represents more *in vivo* microenvironment – physiological cell-to-cell contact – cell interactions with extracellular matrix – similar morphology and polarity – diffusion gradients present (drugs, oxygen, nutrients, waste) – maintains *in vivo* gene expression patterns – better model for drug resistance	– more complex system – higher cost – further complex downstream processing – challenges for adoption – hard to compare across various methods of 3D culture

To overcome these issues, various models of 3D cultures are being created. 3D cell culture is used to refer to any model in which the cells are able to grow and interact with their environment in all three dimensions. 3D cell culture can be categorized into scaffold techniques and scaffold-free techniques. Scaffold techniques consist of using a scaffold, hydrogel, and/or

other materials. Scaffold free techniques can include spheroid culture where spheres of cells are made through various methods including embryoid bodies, hanging drop, bioreactors, and microwells. Microfluidics or microchips are another way 3D culture can be studied at a small scale. Overall, these 3D models allow us to study better proliferation, differentiation, and gene expression. They also allow us to study motion and migration which is not possible in 2D models. These 3D models better represent the conditions found in the human body and allow cells to reconstruct their own environment with improved cell-to-cell and cell-to-matrix interactions (Figure 1-1) [6]. As 3D culture advances in technology and becomes more feasible, more researchers are adapting 3D model systems. Importantly, 3D culture allows cells to reconstruct the microenvironment or niche around them. [6], [7]

However, 3D culture can come with its own set of challenges. Since there are many different methods of 3D culture, they lack a standard system and it is difficult to compare results between different studies. In addition, many of the 3D cell culture techniques do not typically allow control over the number of cells that form the 3D structure and can produce heterogenous results. To resolve this issue, Ungrin et al. [8] developed the pyramidal microwell system (commercialized as AggreWell) that allows us to make cell aggregates of consistent shape and size. These microwell plates will be the main 3D cell culture method explored in this thesis. In summary, these 3D models greatly advance our ability to study cell behaviour, but it is difficult to relate this behaviour back to the 2D culture. Currently, there is not much research on the characterization of the differences between 2D and 3D culture. Given that 3D culture is relatively new to the field, a lot more research and characterization needs to be performed. Overall, 3D culture is found to be more physiologically relevant. [6], [7]

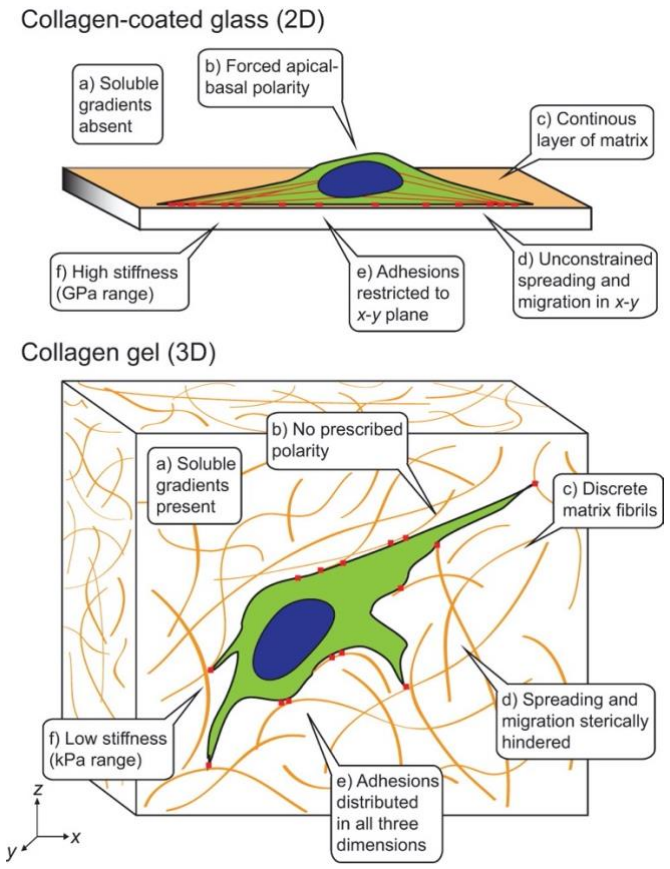


Figure 1-1. Cell microenvironment differences between 2D and 3D. This schematic shows how cells in 2D and 3D microenvironment interact differently with their surroundings due to mechanical and chemical cues. Please note that this image represents only one kind of 3D culture (gels) and is representative of cell types that show polarity (such as epithelial). Image was directly taken from [9] *with permission from The Company of Biologists (Publisher) provided by Copyright Clearance Centre (CCC).*

After cells are grown, common molecular biology techniques are used to observe cell behaviour. The central dogma of molecular biology describes the process in which the information flows in a sequential order from DNA to RNA to protein [10]. The two main types of nucleic acids are DNA and RNA which stand for deoxyribonucleic acid and ribonucleic acid, respectively. The double stranded DNA is missing the reactive hydroxyl group that makes it more stable compared to single stranded RNA. The synthesis process from DNA to RNA is called transcription and from RNA to protein is called translation [10]. Proteins are the workhorses of

the cells; they are involved in structure and carry out virtually every biochemical reaction that occurs in living things [10]. DNA and RNA are composed of nucleotides and proteins are composed of amino acids [10]. To analyze these components, common choices could include polymerase chain reaction (PCR) for DNA expression, reverse-transcriptase quantitative PCR (RT-qPCR) for messenger RNA (mRNA) expression, and western blots for protein expression.

1.2.1 Gene Expression

The gene expression can be measured through various techniques. The thesis will mainly focus on the common reverse-transcriptase quantitative PCR (RT-qPCR). The quantitative means that it gives a relative amount of expression based on the amount of amplified DNA [11]–[13]. As such, qPCR is used when the expression of a few known target genes needs to be analyzed. RT-qPCR is the gold standard for expression analysis and is usually the first test for gene expression. Microarrays are another common technique used to study differentially expressed genes. Microarrays are a robust and affordable solution and they can be useful for when the target genes are not known or to study a whole transcriptome level analysis [14]. More recently, next generation sequencing (NGS) technologies have revolutionized genomic research. An example of this is RNA-Sequencing (RNA-Seq) which can observe differential expressions of RNA at a much broader dynamic range compared to microarrays [14].

1.2.2 Protein Expression

The proteome consists of all the proteins that are produced or modified by an organism or system. In order to study the proteome, many proteomic techniques exist. Western blot is a semi-quantitative technique that detects specific proteins in a given sample [15]. In a Western blot, the proteins are denatured and separated based on their size to allow for detection of

target proteins [15]. Flow cytometry can be used to measure intracellular protein levels in a quantitative manner. Other techniques could include mass spectrometry which allows for high-throughput identification of proteins and sequencing of peptides [16]. Similarly, to microarrays for nucleotide analysis, protein microarrays can be used to detect relative levels of the proteins present in the cell. In addition, immunocytochemistry techniques can be used to visualize the proteins within the cell. This can give insight into the localization and polymerization states of the proteins.

1.2.3 Normalization in Gene Expression Studies

To ensure that the changes in the gene or protein expression levels in a cell are due to the experimental conditions and not due to other factors, normalization is performed. Normalization for gene expression in RT-qPCR can control for variations between samples during multiple steps within the protocol. It could control for the amount of starting material, extraction yield, enzymatic efficiencies, and transcriptional activity [17], [18]. Additional controls could include no reverse transcription control, positive controls from previous experiments, negative controls, and more. Yet, these controls are not always enough. For example, the total RNA mass is not sufficient for normalization because it consists predominately of rRNA molecules and is not representative of mRNA fraction [18].

Due to these issues, the most common normalization method is to use an endogenous (internal) control that remains stable across various experimental conditions [17]. These internal controls are commonly referred to as reference or housekeeping genes because they are involved in the basic structure or function of the cells. This means that they are constitutively (continuously) expressed at a constant level, regardless of the needs of the cells. Typically, they

are also expressed at medium to high levels allowing for easy detection [19]. Internal reference genes should not vary in tissues or cells in response to experimental treatment. In literature, there are commonly accepted reference genes used in majority of the research. These include genes that are involved in basic cell structure such as common cytoskeleton markers including β-actin (*ACTB*), other actin proteins, and tubulin (*TUBB*) [17], [19], [20]. These also include genes that are involved in basic cell function such as: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), an enzyme used for breaking down glucose during glycolysis [17], eukaryotic initiation factor 2 B (*EIF2B1*), involved with translation initiation [21], ubiquitin (*UBC*), a small protein that regulates other proteins [17], and 18S rRNA, part of the ribosomal RNA [10]. To perform normalization, the ratios of the mRNA concentration between the gene of interest to the reference gene is then reported [18].

1.2.4 Normalization in Protein Expression Studies

Western blots are one of the most common proteomic techniques because they require relatively inexpensive equipment compared to some of the other techniques [22]. Western blots can also include controls based on the different parts of the procedure [15], [22]. Instead of normalizing the target protein relative to cell number, tissue volume, or total protein, the most common method involves normalizing to an internal protein. Internal controls for western blot also include the same housekeeping proteins as controls for gene expression studies such as β-actin, tubulin, and GAPDH [15], [23], [24].

1.3 Cytoskeleton

The cytoskeleton components are a very common reference as they are responsible for the structural framework and shape of the cell. The cytoskeleton is a highly dynamic structure

with components and proteins that can quickly assemble and disassemble into networks of filaments and tubules. This allows the cytoskeleton to play an important role in cell movement, signalling pathways, and other cell behaviours. The cytoskeleton comprises of three main types of fibres that vary in size, protein composition, and function (Figure 1-2) [25]. The largest fibres are the microtubules with a diameter of 25 nanometers (nm) consisting mainly of the protein tubulin. The main function of microtubules is cell division and transport. [25]

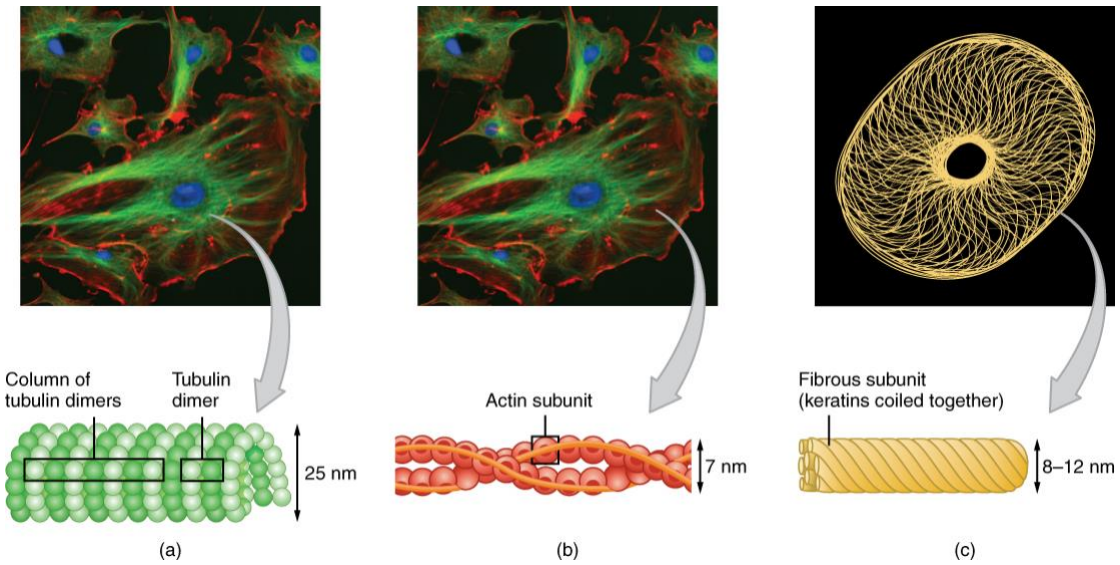


Figure 1-2. Cytoskeleton Proteins. Three *important structural fibres of cytoskeleton are shown in detail. Top image shows stained cell and bottom image shows fibre schematic. These structures play an important role in cell shape and movement. Image was directly taken from* [26] *under Creative Commons Attribution 4.0 International license.*

The middle fibres of cytoskeleton are called the intermediate filaments with a diameter of 8-12 nm and their main function is to provide structural support [25], [27]. The protein subunits of the intermediate filaments vary by the cell type. For example, keratins are mostly found in epithelial cells [27]. Vimentin filaments can be found in a wide range of cell types from the mesenchyme origin and often colocalize with microtubules [27]–[31]. Lamins are also found in all cell types and form a meshwork to support the inside of nuclear membrane [27].

The smallest fibres are the actin filaments with a diameter of 7 nm [32]. These actin

filaments are the major facilitator of movement inside the cell. Actin proteins are one of the most abundant protein, they are highly conserved and ubiquitously found in all eukaryotic cells [33], [34]. An actin monomer is ~43 kilodaltons (kDa) globular protein (G-Actin) that can polymerize to form a linear microfilament (F-Actin) made up of two actin monomers. Moreover, actin can form stress fibers composed of 10-30 actin filaments (F-actins) and myosin crosslinked with various proteins such as α-actinin [35]. Stress fibres form a highly regulated actomyosin structure in non-muscle cells, and have an important role in cell contractility to help with cell adhesion, migration, and morphogenesis [35]. There are six actin isoforms which be categorized into three α-actin isoforms ($\alpha_{\text{skeletal muscle}}$, $\alpha_{\text{cardiac muscle}}$ and α_{vascular}), one β-isoform ($\beta_{\text{cytoplasmic}}$), and two γ-isoforms ($\gamma_{\text{cytoplasmic}}$ and $\gamma_{\text{smooth muscle}}$) [34]. As implied by their name, $\alpha_{\text{skeletal muscle}}$ is the actin expressed in skeletal muscle, $\alpha_{\text{cardiac muscle}}$ is expressed in cardiac muscle, and $\gamma_{\text{smooth muscle}}$ is expressed in smooth muscle [36]. The remaining two actin isoforms, $\beta_{\text{cytoplasmic}}$ and $\gamma_{\text{cytoplasmic}}$ are ubiquitously expressed [36]. These isoforms are 93% identical in their amino acid sequence [36], [37]. As tubulin and β_{cyto}-actin are the proteins that are found in most cell types, they are the ones most commonly used as references.

1.4 Cell Types

This thesis will focus on different cell types found in the human body. The different types of human cells that can be divided into four different categories: epithelial, connective, muscle, and nervous [38]. Epithelial tissue (epithelium) are sheets of tissue that line structures of the body such as skin surface (epidermis), trachea, digestive tract, etc. [38]. Connective tissues bind cells and organs together and function to protect, support, and integrate all parts of the body. Connective tissues are derived from the mesenchyme and include cartilage, bone, and fibrous

connective tissue [38]. There are many differences seen when comparing epithelial cells to mesenchymal cells [38]. Epithelial cells are tightly held together by junctions and have a cuboidal or circular morphology. Epithelial cells are also polarized where the basal side is different than the apical side [38]. Mesenchyme cells are not polar and have a spindle shaped morphology [38]. They do not adhere to one another but instead adhere to the extracellular matrix [38]. As such, epithelial cells have strong cell-to-cell connections using cadherins and mesenchyme cells have strong cell-to-matrix connections using integrins [38]. Also, epithelial cells are bound to their basal lamina whereas mesenchymal cells are very mobile [38]. Epithelial to Mesenchymal transition is a process in which epithelial cells lose their polarity, gain mobility, and become mesenchymal cells [39], [40]. This occurs in early human development, during stem cell differentiation, and also during initiation of metastasis in cancer [40].

To summarize, this chapter introduces common cell culture techniques and compares across 2D and 3D culture. 3D culture allows for more physiologically relevant models of cells. Some common laboratory techniques were also introduced for gene expression and protein expression. These techniques use an endogenous reference to normalize across different samples and conditions. One of the most common endogenous reference is the cytoskeletal component β-actin. In addition, the three main cytoskeletal fibres were introduced along with their protein components and functions. The common cell types of the human body were also introduced.

CHAPTER TWO: LITERATURE REVIEW

2.1 Cytoskeletal Components as Controls

2.1.1 Single Reference Controls are Not Reliable

Many different laboratory techniques use similar genes/proteins as references for normalization. As previously mentioned, β-actin, GAPDH, tubulin, cyclophilin, ef1α, ubiquitin, and 18S rRNA are some of the common endogenous internal controls for normalization in gene expression studies. Above all, *GAPDH* and *ACTB* are used as a single control gene in more than 90% of the cases in high impact journals [41], [42]. The high impact journal articles reviewed were *Cell, Science, Nature, Proceedings of the National Academy of Sciences, Journal of Biological Chemistry* and *Molecular and Cellular Biology*. In particular, β-actin is one of the most common used in both gene and protein expression because it is expressed in all eukaryotic cell types [43]. However, there is a growing body of literature that suggests that β-actin is not as stable as previously thought. It has been found that β-actin changes its expression in response to various biomedical stimuli such as exposure to Matrigel [44], hormones [45], viruses [46], [47], serums [45], diet [48], exercise [49], hypoxia [50], and more. In addition, it varies during different cell stages such as growth and aging, differentiation, and developmental stage. The expression has also been seen to vary between different disease states and cancer [33]. In the majority of tumor cells and tissues, β-actin has been found to be upregulated. Overall, it has been found to be de-regulated in many different types of cancer including liver [51], [52], melanoma [53], renal [54], colorectal [52], [55], [56], gastric [52], pancreatic [52], esophageal [52], lung [57], breast [56], prostate [58], ovarian [59], leukemia [60], and lymphoma [61]. From those, it was found to be downregulated in colorectal, esophageal, some breast and lymphoma cancers. Furthermore, it

has been hypothesized that the β-actin polymerization and localization can promote cancer motility, invasiveness, and metastasis [62]. Majority of these findings were based on gene expression, but some also included protein analysis. In particular, breast and colorectal cancer were studied using both microarray data and Western blots [56]. The lymphoma study used RNA analysis along with 2D PAGE (polyacrylamide gel electrophoresis) [61].

2.1.2 Alternative Methods for Normalization

Other internal controls have also been shown to vary under different conditions. To date, no single control has been identified to serve as a gold standard. Due to this, it has been suggested to use two or more controls for a proper normalization. Multiple programs exist to allow for easy assessment of multiple reference genes through excel. Three examples of these include GeNorm, BestKeeper, and Norm-Finder [63]. GeNorm was developed by Vandesompele et al. in order to identify the most stable control genes in a given set of tissues and the minimum number of genes to use [17]. They evaluated ten reference genes from different functional classes in multiple tissues and demonstrated that a single gene is not enough. The geometric mean of multiple genes was determined to be an accurate normalization factor [17]. This strategy allows the user to determine the number of control genes needed and rank them based on stability. The software is freely available at https://genorm.cmgg.be/. The second program, BestKeeper, also selects least variable gene using geometric mean by using raw data instead of data converted to copy number [60]. It is also freely available at https://www.gene-quantification.de/bestkeeper.html. A third program, Norm-Finder, is freely available by request. This program measures the gene variation but also ranks the potential reference genes by how much they differ between the experiment conditions studied in the experiment [64]. These

strategies were brought forward in 2002-2004, yet it is not clear if they are being used at a large scale yet. However, GeNorm website does indicate that the excel version from 2002 has been downloaded more than 15,000 times as of September 2018.

2.1.3 Controls in Protein Expression Studies

Similar to gene expression studies, the most popular internal controls in western blot are β-actin, tubulin, and GAPDH. β-actin is common because it is found at concentrations greater than 100 μM and due to its highly conserved sequence, most antibodies to β-actin can be used for many different species [24]. But as highlighted above, β-actin is not a stable reference gene or protein as it can be affected by many different cellular processes [23], [24]. In addition, it has been shown that the concentrations of protein loading controls are too high when they being used to quantify low abundant proteins [15], [24], [65]. This prevents them from being able to show loading differences above the relatively small total protein amounts of 0.5-10 μg [15]. As such, β-actin concentrations can fall outside the linear range of optical densitometry detection and become uncorrelated with target protein concentration amounts [65]. Instead of relying on a single protein as the loading control, total protein staining (TPS) methods have been introduced [15], [23], [24], [65]. Currently, a variety of methods and dyes for TPS exist. These include Ponceau S, stain-free techniques, Sypro Ruby, Epicocconone, Coomassie R-350, Amido Black, and Cy5 [15]. TPS allows for quantification of the protein similar to the internal control using densitometry measures. The current consensus in the field is that using any of these total protein staining methods are better than using a single internal protein as a control [15], [23], [24], [65]. However, it is unclear if TPS methods are known or accepted by most labs yet.

2.2 Substrate Stiffness

2.2.1 Human Cells and Stiffness

Cells in the human body are embedded in tissues that vary greatly in their softness and rigidity depending on the organ and location. The elastic moduli can span a wide range from ~100 Pascals (Pa) for very soft tissues (fat or brain) to > 10,000 Pa for muscle, and only reaching high values of around ~20 GPa (gigapascals) for cortical bone [66] [67]. In contrast, cells grown on plastic surfaces (such as polystyrene) or glass have an elastic moduli of 3-3.5 GPa or 50-90 GPa, respectively [68]. Different cell types have different responses to the substrate stiffness based on the type of the cell and the tissue they originated from [69]. For instance, neurons branch more on softer substrates (<0.5 kPa) [70], fibroblasts achieve maximal spread at surface stiffness of ~10 kPa [71], and chondrocytes (cartilage) only begin to spread at 10 kPa [72]. A current hypothesis that explains increased spreading on stiffer surfaces is that by pulling on the matrix at focal adhesions, the cell creates tension within its membrane and in the underlying cortical actin mesh [73]. Based on this, the magnitude of the tension depends on the material properties of the matrix: a stiff matrix will resist more cellular force than a soft matrix [73].

2.2.2 Actin Stress Fibers

Focal adhesions are large structures of proteins that transmit mechanical force and regulatory signals between the extracellular matrix (ECM) and the interacting cell. They allow the cytoskeleton inside the cell to connect and communicate to the ECM outside the cell. Actin stress fibers generate contractile forces that regulate the assembly and dynamics of focal adhesions [35]. As such, actin stress fibers have an important role in mechanosensing [35]. The ability of cells to sense the mechanical properties of the local environment such as viscosity,

microstructure, and the stiffness is important for the cellular functions [35], [74]. This mechanosensing can be important for stress fiber organization, cell differentiation, and fate determination, particularly in stem cells [75]. Cells assemble stress fibers only when they encounter mechanical force (stress). As such, human cells that are grown on rigid substrates like glass or plastic display thick stress fibers but those fibers are absent or very thin on the same cells grown on softer substrates [35]. Moreover, stress fibers and focal adhesions are aligned along the major cell axis when cells are grown on a stiff substrate. They become smaller and are poorly aligned on softer substrates [69], [76]. For example, when fibroblasts are undergoing development to myofibroblasts in dermal wound tissue, they develop prominent stress fibers to allow wound closure [77]. To note, the stress fibers of myofibroblasts are composed of α-actin compared to β-actin of non-muscle cells [78]. Similarly, epithelial cells differentiate into myoepithelial cells during wound closure with stress fiber assembly [79]. These studies illustrate that stress fibres are formed under extreme mechanical stress.

2.3 Mechanosensing

2.3.1 Effect of Stiffness on Cytoskeleton in 2D Conditions

The mechanical environment around the cell can greatly impact the cell's structure and function. The passive mechanical properties of the local environment can include viscosity, microstructure, and stiffness of the substrate [74]. It has been shown that cells grown on stiff substrates assemble actin stress fibers [80], display a larger spread phenotype [81], upregulate integrin expression [71], modify their substrate adhesions [81]–[83], and activate signaling pathways specific to contractility [82], [84], [85]. For instance, substrate stiffness can influence the differentiation of mesenchymal stem cells with soft, medium, and stiff substrates leading to

neurogenic, myogenic, and osteogenic, respectively [75]. Moreover, it was shown that mesenchymal stem cells, C2C12 myoblasts, and hFOB cells (human bone osteoblasts) cultured on collagen-coated polyacrylamide (PA) gels with increasing substrate stiffness showed a progressive increase in cell stiffness [75]. In fibroblasts, substrate stiffness can affect the stress fiber formation, along with rate and direction of cell migration, as well as focal adhesion [76]. A study with fibroblasts grown on fibronectin-coated PA gels visualized the cytoskeleton structure and studied the fibroblast area and stiffness. In this study, it was shown that gels between 1-5 kPa shows actin distributed evenly over the cell volume with not organized into stress fibres. On 10 kPa, bundled actin structures are typical of stress fibres [86]. Finally, on glass (1 GPa), the actin cytoskeleton is organized into large stress fibres [86]. Between 1-5 kPa, fibroblasts adjust their stiffness to match the substrate. After 5 kPa, the cells remain softer than their substrate presumably because they reach the limit of mechanism to reinforce cytoskeleton [86]. Majority of these studies used protein-coated PA gels and were able to show how substrate stiffness can impact cells in a 2D manner. This system allows for excellent control of substrate stiffness but does not allow for cells to be cultured in a 3D manner [74].

2.3.1 Effect of Stiffness on Cytoskeleton in 3D Conditions

As previously mentioned, these cells can also be cultured in a 3D matrix gel to allow for conditions that are more representative of their native environment. When looking at studies with these 3D gels, it was shown that the stiffness of collagen gels regulates the morphology of embedded fibroblast cells where softer gels allow for elaborate dendritic extensions. Similarly, the proliferation and migration of epithelial cells is regulated by the stiffness of the gel [87]. To summarize, there is ample evidence that supports that substrate stiffness can affect cell function

in various cell types. It has been shown that stiffer substrates lead to stiffer cells with more stress fibres. However, very little is known about how 2D substrate stiffness correlates with 3D substrate stiffness as most studies focus on one cell type in either 2D or 3D context. This gap was highlighted in a 2007 review about cellular responses to cellular stiffness, yet there has still not been much progress to resolve this issue [3].

For example, Byfield et al. [74] investigated the effects of endothelial cells in 2D and 3D stiffness. Endothelial cells *in vivo* reside in both types of environments: endothelial monolayers lining the conduit vessels are 2D, and capillaries surrounded by basement membrane are 3D *in vitro*. This was done by culturing endothelial cells on top of (2D) and within (3D) collagen gels of two different stiffnesses. The soft gel was made of 1.0–1.5 mg/ml of type-I collagen (124±8 to 202±27Pa) and stiff gel was made of 3.0 mg/ml of collagen (502±48Pa). They found that cells in stiffer gels showed more pronounced stress fibers and about 1.5-fold increase in staining for actin. As actin plays an important role in cell's mechanical properties, they measured the cell stiffness using micropipette aspiration and atomic force microscopy. They also found that cells from softer gels were about two times more complaint compared to the cells from stiffer gels in both the 2D and 3D conditions. Although they were able to compare the soft and stiff gels within the 2D and 3D conditions separately, they did not perform any comparison across the different conditions. [74]

2.4 Relevant Studies

2.4.1 Incorrect Normalization

In some cases, these studies use cytoskeleton markers as a reference gene/protein during normalization which could lead to a vast misinterpretation of results. For instance, if the

results are normalized to β-actin and β-actin shows significant downregulation in target condition compared to control condition. Then the normalized results would indicate that the target genes are significantly upregulated in the target condition. However, after proper normalization with stable controls, those genes could show no significant change. This is of key importance, particularly if the subsequent experiments are based upon the initial RT-qPCR results. As RT-qPCR is typically used as an earlier exploratory technique for new ideas or targets, this could save valuable time and resources down the road.

The effect of incorrect normalization were shown by the study by Glare et al. [88]. They showed that β-Actin and GAPDH were very variable when used as controls in gene expression for asthmatic airways. As mentioned above, there has been growing evidence that the mRNA expression and rate of transcription for both β-actin and GAPDH is influenced by many factors. β-actin and GAPDH expression levels vary with cell proliferation, and their transcription is upregulated quickly in response to mitogenic stimuli by growth factors including epidermal growth factor, transforming growth factor-β (TGF-β), and platelet derived growth factor [88]. However, researchers still use β-actin and GAPDH as control references even when normalizing mRNA levels of growth factors in asthma studies [88]. Glare et al. [88] measured the mRNA levels for β-actin, GAPDH, and interleukin-2 (IL-2) by competitive RT-PCR in cross sectional study of 26 healthy controls and 92 asthmatic subjects. The competitive RT-PCR involves a co-amplification of a competitive template along with the target template [89]. The competitive template can be a mutant cDNA that is amplified by the same primers but contains a new restriction site [89]. Target cDNA is co-amplified with a dilution series of competitor DNA of known concentration [89]. The competitive cDNA be distinguished from the target cDNA by restriction enzyme digest

after PCR [89]. The products are run on a gel and the initial concentration of target cDNA can be determined [89]. This study showed that asthmatics have reduced expression of both β-actin and GAPDH. However, IL-2 mRNA levels did not differ between subjects or treatment groups but when they were expressed as a ratio with β-actin, significant differences were seen. This research shows how using an inconsistent reference control for normalization can lead to significantly false results. This was shown only in asthmatic studies but can be broadly applied to different fields as β-actin and GAPDH are used as controls across many research fields. In fact, when "β-actin is not suitable for normalising mRNA expression" is searched on Google Scholar, 18,800 results as of December 28th, 2019 are shown [90]. Many of these different articles demonstrate how β-actin was an inconsistent control in human studies but also other species such as crustaceans, chickens, rats, mice, and many more. Most of the studies are similar to one illustrated above where they demonstrate instability of β-actin in one cell line and under one disease conditions. There were not many studies found that focus on a systematic review across multiple cell lines.

2.4.2 Cytoskeletal Changes in 2D and 3D Conditions

Previous studies that compare across 2D and 3D condition do not focus on the changes in the cytoskeleton. There was one study that was based on observing differentiation changes in both 2D and 3D conditions in mouse embryonic stem cells (mESCs) by Pineda et al. [91]. This study looked at differentiation markers, but also studied expression of germ lineage markers and cytoskeletal proteins. The mESCs were grown in a 2D monolayer on collagen coated surfaces or two types of 3D culture: embedded in collagen gels or suspended as embryoid bodies [91]. They studied multiple cytoskeletal proteins (actin α 1&2, keratin 8, lamin, tubulin, vimentin), an ECM

protein (fibronectin), and differentiation markers (Oct4 and GATA4). They found that the cells grown in 2D expressed higher levels of cytoskeletal elements and extracellular matrix proteins early on with the exception of tubulin [91]. In addition, both 2D and 3D cultures upregulated the expression of cytoskeletal markers overtime from day two to twelve of culture [91]. This research highlights the possibility that 2D culture promotes overexpression of cytoskeleton that is uncharacteristic of the conditions found in the body. It also shows the ability of 3D cultures to create their own cytoskeleton proteins and niche overtime. Nevertheless, this research is limited to only one mouse cell line. The research on human cell lines is typically found to be less abundant compared to mice. Moreover, there is a lack of research on general cell culture practices that can be expanded to a variety of cell types and culturing methods.

2.5 Thesis Rationale

As presented above, the current literature supports the contention that commonly used references (especially cytoskeletal components) vary considerably between cell types and conditions. The literature also shows that the external environment around the cell can greatly impact the cytoskeleton. Of particular concern, stiff surfaces can lead to an overexpression of cytoskeleton proteins such as actin and increase stress fibre formation. Thus, we hypothesized that the plastic adherent stiff 2D culture will show overexpression of cytoskeletal proteins in comparison to 3D culture, rendering these gene unsuitable for use as expression-level controls between 2D and 3D conditions. Overall, this thesis will explore the role of β-actin as a consistently stable reference gene or protein and highlight the need to select appropriate controls specific to the experimental conditions. It will also explore the changes in the amount of

cytoskeleton depending on the culture conditions thus emphasizing the importance of using physiologically relevant culture systems.

2.5.1 Hypothesis and Aims

Hypothesis:

Cytoskeletal proteins are not reliable reference markers – particularly when comparing across different culture conditions – as they are upregulated in 2D cell culture when compared to 3D culture.

Aim 1: To profile the ability of various cell types to form size-controlled microtissues

Aim 2: Assess whether mRNA expression levels of cytoskeleton genes are downregulated in 3D microtissues

Aim 3: Determine changes in cytoskeleton protein levels transitioning from 2D culture to 3D microtissues

CHAPTER THREE: MATERIALS AND METHODS

3.1 Cell Culture

This thesis will focus on human cell lines. For the initial experiment: to determine the minimum number of genes needed for stability, four cell lines were analyzed. These four cell lines were: fibroblasts, MCF7, Ishikawa, and HeLa. In the concurrent experiments, additional seven human cell lines listed were cultured to ~85% confluency (typically 4-5 days) on tissue-culture (TC) adherent plates or flasks. The media used for all the cell lines along with the source of the cells can be found in Table S 1 in the APPENDIX. Please note that the hPSCs cannot be grown directly on tissue culture treated plates and as such were grown on top of a layer of Matrigel™ Matrix (Corning, Cat#. CACB354230). It is important to note that the other conditions were kept consistent for each cell type including the passage number, media, oxygen conditions, incubator conditions, etc.

After that, the cells were passaged to grow in 2D adherent culture and 3D microwells. The same number of cells were seeded in both conditions in a 24 well adherent plate and 24 well microwell plate. The 2D Culture 24 well tissue culture (TC) treated plate was called CELLSTAR® Cell Culture Multiwell Plates and made of Polystyrene (Greiner Bio-One, Cat# 82050-892). The microwell plate is centrifuged with Pluronic F127 (5%, w/v, in sterile H2O) (Sigma-Aldrich P2443) at 2,000 g for 2 minutes. The plate is coated with Pluronic for 20 minutes at room temperature to ensure that the cells do not attach to the plate directly. The plates are washed carefully 3x with PBS with centrifuging 2,000 g for 2 minutes each wash. As one well of a 24 well microwell plate contains 1200 microwells, 120,000 cells were seeded per one well to make aggregates of 100 cell/aggregate. The suspended cells are seeded in the plate and then centrifuged at 200 g

for 3 minutes. The methodology can be found in video format in [92]. The cells in both conditions were supplemented with 1 mL of their corresponding media. The media change was carried out every 24 hours.

To note, the same number of cells are seeded the TC and microwell plate and both conditions are supplemented with the same amount of media (1 mL). This ensures that the oxygen experienced by the cells remains the same in both conditions. Previous research by the Ungrin lab showed that the oxygenation (O_2) values in culture are often not reported [93]. These O_2 values are of key importance for reproducibility of results and can have a significant effect on the cell phenotype and behaviour [93]. These O_2 values can depend on many factors such as the plate and the surface growth area of cells, the height of the media above the cells, the type of cell and their metabolism rate, the density of cells seeded, the concentration of oxygen in the incubator/atmosphere around the cell, the temperature, and the location and altitude of the city [93]. To calculate these, Ungrin lab also built an Oxygen Diffusion Calculator that can be accessed at http://136.159.176.176:3838/o2/. For example, the HeLa cells were predicted to experience an oxygen level on the order of 0.14 mM at the cell culture surface in both 2D and 3D conditions. The full calculation result can be seen in Figure S1 in the APPENDIX. This oxygen levels at the cell culture surface would differ based on cell type and the calculations for each cell type can be seen in Table S2. These calculations were performed based on the initial seeding density and oxygen levels will decrease further as cells become more confluent. It is important to note that the 3D aggregates will experience heterogeneity, as the cells in the periphery region of the aggregate will have better access to the media, factors, and oxygen compared to cells near the core.

The cells in both 2D and 3D conditions were taken out at 48 hours. Cells in 2D were dissociated into single cell using TryplE or trypsin for 5 minutes in incubator. The cell aggregates in 3D were taken out intact. The media from both cell culture conditions was removed. At this point, the cells were frozen intact or lysed or fixed. The frozen cells or lysed cells were used for either DNA assay, RT-qPCR, or Western Blotting and the fixed cells were used for immunocytochemistry. To freeze, cells were stored as a pellet at -80°C until needed and the cells were lysed with the lysis buffer based on the technique. For fixation, the cells were fixed before or after dissociation into single cells by adding 4% PFA (paraformaldehyde) for 5 minutes. This was followed by three washes with PBS (phosphate buffered saline) and cells were stored in the fridge at 4°C until needed.

3.2 RT-qPCR

3.2.1 Background of RT-qPCR

RT-qPCR allows the quantification of mRNA expression which serves as a template for proteins. In this method, the RNA is first transcribed into complimentary DNA (cDNA) using reverse transcriptase. This cDNA is then used as a template for the quantitative PCR reaction. This is done by using a fluorescent dye such as SYBR Green that binds newly formed double stranded DNA molecules. This fluorescence can then be measured at the end of each amplification cycle to determine how much DNA has been amplified [11]–[13].

3.2.2 Detailed Methodology for RT-qPCR

To perform this, the cells were lysed, and the RNA was extracted following the protocol directly from Total RNA Purification Kit from Norgen Biotek (Cat# 37500). Following this, the RNA was quantified using a spectrophotometer, NanoDrop, which measures the absorbance of

nucleic acid amounts [94]. All nucleic acids absorb UV light at 260 nm, which is then compared in a 260/280 ratio for purity and a ratio of ~2.0 is accepted as “pure” for RNA [95]. Reverse transcription (RT) into cDNA is performed right away. The amount of RNA is normalized so all samples are at RNA amounts of 1 μg in the reverse transcription step. The full reaction has a volume of 20 μl composed of 4 μl of iScript™ RT Supermix and remaining 16 μl is a combination of the RNA template and Nuclease-Free Water. To normalize, the amount of RNA added is between 1-16 μL based on the measured amount and the remaining is nuclease free water to a total volume of 16 μL. The remaining RNA was stored at -80°C. The RT was performed using iScript™ Reverse Transcription Supermix for RT-qPCR from BIO-RAD (Cat# 1708840/1). The cDNA can be used directly for RT-qPCR or stored at 4°C. The initial gene panel is listed below in Table 2. The primers sequence listed in APPENDIX: Table S 3. PCR reactions were assembled using PowerUp SYBR Green Master Mix (Applied Biosystems, Cat# A25742). In order to save cost, BrightGreen super mix was used instead of SYBR green which has been found comparable in results.

For high throughput, the lab has an Eppendorf pipetting robot which was used for 384 well plates and is more time-efficient and accurate than hand-pipetting. For analysis of 96 well plates, StepOnePlus Real-Time PCR System from Thermo Fisher Scientific was used. For analysis of 384 well plates, Dr. Roman Krawetz’s QuantStudio™ 6 Flex System by Thermo Fisher was used. cDNA was tested using known reference primers before being used for quantitative experiments. Primers were also tested with previous positive controls before being used for quantitative experiments.

Table 2. List and Function of Genes Evaluated

Gene	Protein	Function
ACTA	α-actin	Cytoskeletal structural protein
ACTB	β-actin	Cytoskeletal structural protein
ACTR2	Actin related protein 2	Mediates actin polymerization
ACTR3	Actin related protein 3	Mediates actin polymerization
FN1	Fibronectin 1	Extracellular matrix
KRT8	Keratin 8 (type II)	Intermediate filament
KRT18	Keratin 18 (type I)	Intermediate filament
LMNA	Lamin A/C	Part of the nuclear lamina
TUBB	β-tubulin	Forms microtubules
VIM	Vimentin	Supports and anchors organelles in cytosol
EIF2B1	Eukaryotic translation initiation factor 2B α	Involved in initiation phase of eukaryotic translation
G6PD	Glucose-6-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis
POLR2A	DNA-directed RNA polymerase II subunit RPB1	Largest subunit of RNA polymerase II
PPIA	Peptidylprolyl Isomerase A	Accelerates protein folding
TBP	TATA-binding protein	General RNA polymerase II transcription factor
UBC	Ubiquitin C	Protein degradation
YWHAZ	14-3-3 protein ζ/δ	Signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules

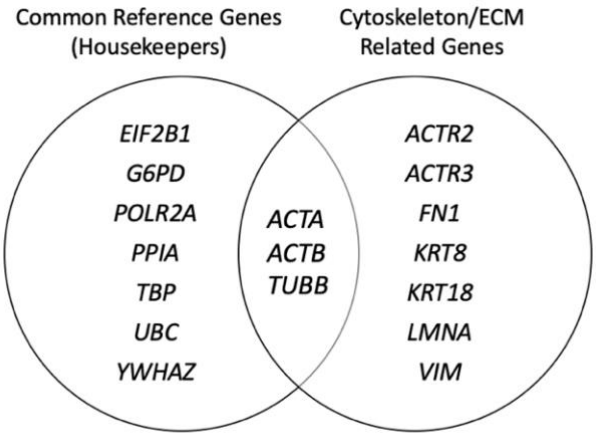


Figure 3-1. Venn Diagram of Gene Classification

3.3 Stability Analysis for RT-qPCR

Author's Note: These RT-qPCR experiments were performed by Jarin Thundathil and Bilal Hafeez under the supervision of Dr. Abdullah Al-Ani. All of the analysis was performed by the author, Saba Aslam using RStudio and Microsoft Excel.

3.3.1 Methods for Stability Analysis

Typically, gene expression levels are normalized to one stable internal control gene, but it is difficult to validate the stable expression of that control gene. To address this problem, Vandesompele et al. [17] developed a gene-stability measure to figure out the expression stability of control genes based on expression levels prior to any normalization. Based on this paper, initial RT-qPCR were performed to determine the proper controls based on specific experimental conditions. These experimental conditions included analyzing four different human cell lines in both 2D and 3D across seventeen different genes. The four cell lines were fibroblasts, MCF7, Ishikawa, and HeLa. The seventeen genes along with their functions can be found in Table 2 and are summarized below in Figure 3-1.

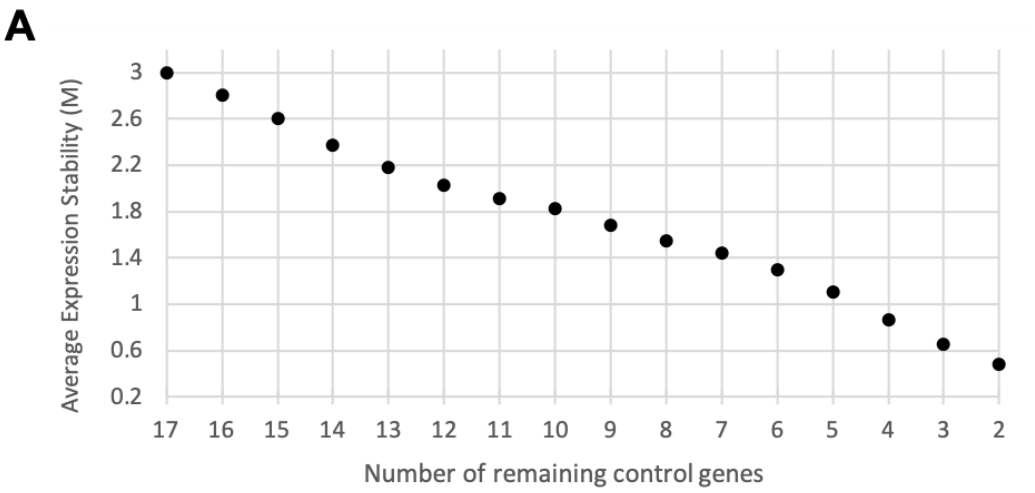
The gene-stability measure is based on the principle that the ratio of two ideal control genes expression is consistent through all samples, not dependent on experimental condition or cell type. Thus, if there is variation in the expression ratio of two control genes, it means that either one or both of these genes are not consistently expressed. To assess this, for every control gene, they determined the pairwise variation with all other control genes as the standard deviation of logarithmically transformed expression ratios. This was defined as the internal control gene-stability measure, M, as the average pair-wise variation of one gene with all other control genes. Genes with the lowest M values have the most stable expression. Assuming that

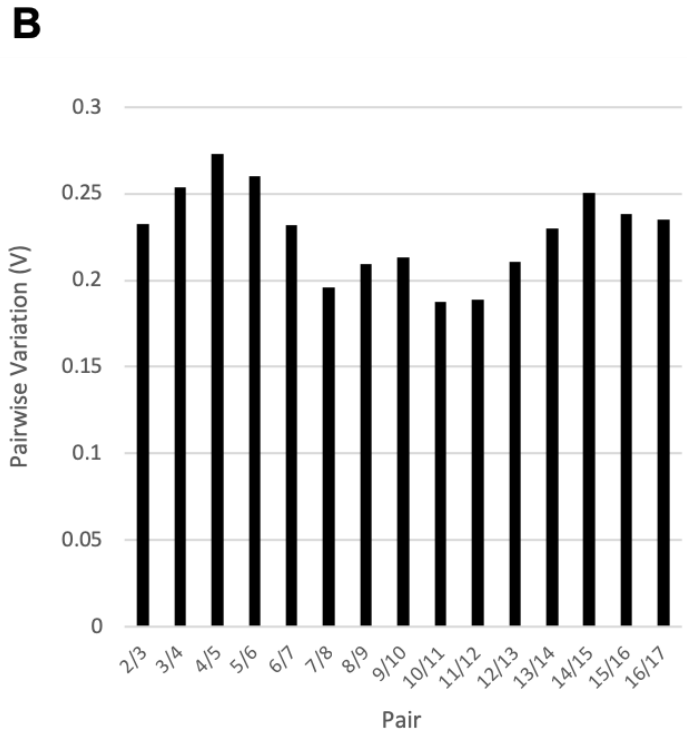
these genes are not co-regulated, stepwise exclusion of the gene with the highest M value leads to a combination of two constantly expressed control genes with the most stable expression.

3.3.2 Stability Analysis Calculations

To perform the calculations, the C_T values were converted to a matrix of expression values named relData using the equation: $relData = 2^{\min(C_T)-C_T}$. This converted the C_T values into a number between 0 to 1 to allow for better comparison within values. After this, the Normalization Factor (NF) was calculated by using the geometric Mean on relData for each gene. The systematic variation between repeated experiments for the same gene was defined as the pairwise variation, V, to account for the inherent machine, enzymatic, and pipet variation. The NFs (geometric means) were used to calculate $V_{j/k}$ using equation: $V_{jk} = sd(\log_2(\frac{NF_j}{NF_k}))$ where j would be gene of interest and k would be any other gene. The M value for each gene was calculated as the arithmetic mean of pairwise variation of that gene (j) with the rest of the 16 genes. This M value was used to put the genes in the ranking order of most consistent expression between samples to least consistent. After the genes were ranked, the gene of interest would be considered n and the gene right below in ranking would be n+1. The pairwise variation for one gene to the gene below it ($V_{n/n+1}$) was calculated using equation:

$V_{n/(n+1)} = sd(\log_2(\frac{NF_{(n+1)}}{NF_n}))$.





C

Rank	Gene
1*	EIF2B1
1*	YWHAZ
3	PolR2A
4	TUBB
5	ACTA
6	UBC
7	ACTR3
8	PPIA
9	KRT18
10	ACTR2
11	LMNA
12	TBP
13	G6PD
14	KRT8
15	ACTB
16	FN1
17	VIM

Figure 3-2. Stability of control genes measured across four cell lines. The four cell lines were fibroblasts, MCF7, Ishikawa, and HeLa. A: Average expression stability values (M) of remaining control genes after stepwise exclusion of the least stable control gene. B: Pairwise Variation (V) shown. C. Genes ranked in order of their expression stability. Rank 1 being the most stable. 1 denotes that the two most stable control genes cannot be ranked in order because of required use of gene ratios for stability measures. These calculations were performed using R package named SLqPCR (developed by Matthias Kohl based on GeNorm)*

3.3.1 Stability Analysis Results

Figure 3-2A shows that as more genes are excluded, the lower the M value thus the higher the stability expression. The genes are removed in the order of the least consistent to most consistent expression. This is used to determine the ranking shown in C. Figure 3-2B shows that the pairwise variation between two different ranked genes ($V_{n/n+1}$) where low variation values of V correspond to high correlation coefficients. It can be seen that pairwise variation is lower when the 3rd gene is included compared to the 4th gene as the value for $V_{2/3}$ is 0.23 and value for $V_{3/4}$ is 0.25. This shows that adding the fourth gene does not lead to lower variation. However, the next lowest value is at $V_{6/7}$, with the absolute lowest V value at 10/11 of 0.18. It is not feasible to run seven or eleven control genes for each experiment. The original paper used 0.15 as an arbitrary cut-off for V value but the V values observed in this data were of higher range. However, the original paper also highlighted that minimum number of three control genes is suitable. Three genes are also useful in the manner that if only two are used where one is variable, it would be hard to tell which one is more accurate. But if three controls are included where one is variable with the other two being consistent, the variable gene will be set apart and can be looked into with further experiments. Figure 3-2C shows the ranking of these genes. *EIF2B1* and *YWHAZ* were found to be the most consistent genes. In addition, when the correlation between normalization factors was performed, it was found that top three ranked genes (*EIF2B1, YWHAZ*, and *POLR2A)* correlated strongly ($R^2 > 0.92$) with each other. But when *POLR2A (Rank 3)* was observed against *TUBB (Rank 4)*, there was lower correlation ($R^2 = 0.87$). Subsequently, *TUBB* (Rank 4) and *ACTA* (Rank 5) showed a strong correlation ($R^2 = 0.98$). This indicates that the control genes correlate well with each other, and the cytoskeletal genes

correlate well with each other. But when comparing across the two groups, there is low correlation. This solidified the selection of top three ranked genes as controls for future studies. As such, the geometric mean of the top three control genes, *EIF2B1, YWHAZ*, and *POLR2A* will be used as the reference genes for further experiments. To note, these reference genes perform diverse functions such as translation initiation, signal transduction, and subunit of RNA polymerase II, respectively. This reduces the chances that these genes are co-regulated. Further experiments will involve analysis of additional cell lines listed in Table 3.

3.4 Microarrays

3.4.1 Background of Microarrays

A microarray is a multiplex lab-on-a-chip technology that involves an array on a solid surface that can assay a large amount of biological material at once. There are different types of microarrays that depend on the type of target material. These could include nucleic acid, proteins, tissues, cells, chemicals, antibodies, and more. This thesis will focus on nucleic acid microarrays that allow studying gene expression of cDNA or mRNA. These types of microarrays are made of a microchip that contains an array with microscopic DNA spots that the target cDNA can attach to. These spots can be a short section of a gene or other DNA element that are used to hybridize the target cDNA sample. The hybridized cDNA can then be detected and quantified by the amount of fluorophore or chemiluminescence measured. Their disadvantages could include the low dynamic range of the assay and the need for probe design sequences. Overall, microarrays allow high-throughput screening in a miniaturized, multiplexed, and parallel way for both processing and detection of large amounts of cDNA.

3.4.2 Microarray Databases

Good bioinformatics and statistics practices for microarrays are well established and easy-to-use free software and pipelines are readily available [17], [60], [64]. After detection, microarray gene expression data can be stored in microarray databases. These databases allow storage of measured data, manage a searchable index, and make the data available to other application for analysis and interpretations. There are two categories of microarray databases:

1. A peer reviewed, public repository that adheres to academic or industry standards. This is designed to be used by many analysis applications and groups. An example of this could include the gene expression omnibus (GEO) from National Center for Biotechnology Information (NCBI) or ArrayExpress from European Bioinformatics Institute (EBI). These typically follow the Minimum Information About a Microarray Experiment (MIAME) guidelines that outline the minimum information that needs to be included for each experiment.
2. A privatized repository that is associated with a specific group such as lab, company, university, etc. or a specific application or analysis method. These could be commercial, non-profit, or academic. These databases may be harder to access.

3.4.3 Microarray Analysis using ReceptoR

In order to analyze publicly available microarray data, an accessible software tool, ReceptoR, developed in the Ungrin lab [96]. This tool allows mining of publicly available transcriptome data to study specific gene sets. Initially, it was designed to study receptor expression and predict active signaling pathways by pooling multiple arrays [96]. But it also served as a great tool to study gene expression of a specific group such as cytoskeletal genes

within one array. Currently, ReceptoR is limited to data from two specific types of arrays: mouse data from the GeneChip Mouse Genome 430 2.0 Array (GPL1260) platform, and human data from the Affymetrix Human Genome U133 Plus 2.0 Array (GPL 570). However, these two arrays contain an abundant amount of data which includes 144,134 sample records for the human data as of December 2018 [96]. The Affymetrix Human 133_2.0 Plus high-density oligonucleotide array currently queries >47,000 transcripts and variants, >38,500 genes, and >54,000 probe sets [97]. Each gene on this array is represented by 11 probe pairs of 25-mer oligonucleotides that span the coding region for the genes and expressed sequence tags (ESTs) represented [97]. At first, the list of cytoskeleton related genes was obtained from Gene Ontology and was found under cytoskeletal organization (GO:0007010). This list was incorporated into the ReceptoR Platform by Dr. Derek Toms. Then the steps from Figure 3-3 were followed to perform data analysis. Please note that the Affymetrix Human 133_2 + array does include housekeeping/control gene sequences which consist of GAPDH, β-actin, ISGF3 (interferon-stimulated gene factor 3) [97]. These controls were not used during data analysis performed in this thesis. ReceptoR performs data normalization on raw microarray data.

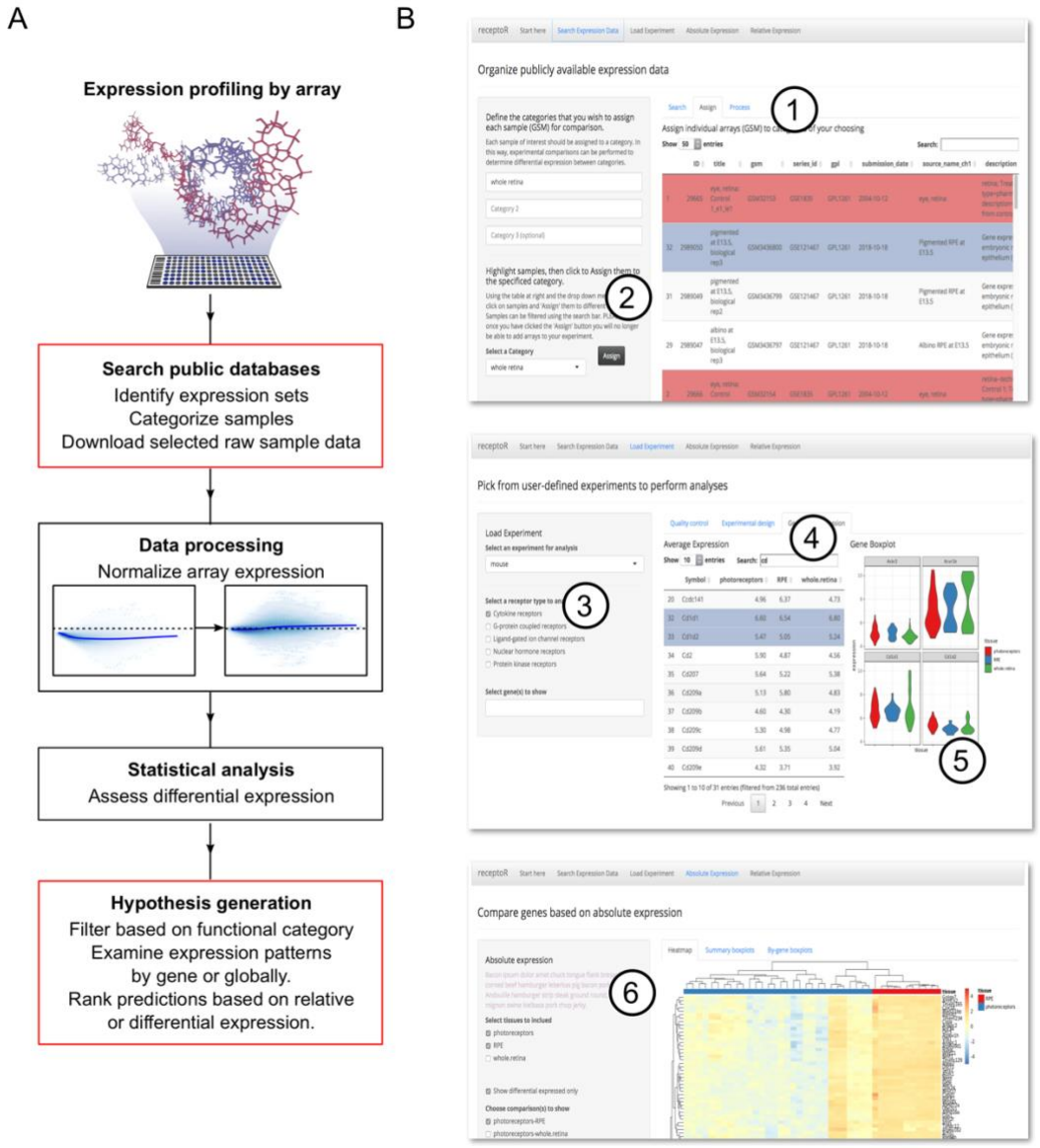


Figure 3-3. Data Analysis of Publicly Available Microarray Data using ReceptoR. A. The pipeline for mining, categorizing, and analyzing the microarray data. User interaction steps are shown in red. B. ReceptoR allows users to analyze microarray experiments by searching public experimental series for specific sample expression data (1) and categorizing each sample according to their experimental design (2). After retrieving and processing the expression data, predictions can be filtered by genes coding for specific receptor types (3), individual gene data can be sorted (4), and visualized (5). Absolute expression levels of receptor-coding genes can be clustered based on assigned categories and filtered based on differential expression between groups (6). This figure and method details were taken directly from [98]

3.5 Western Blot

3.5.1 Background of Western Blot

The samples are loaded onto an SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) gel. This gel is passed through a voltage where the proteins charged negatively with SDS go towards the positively charged anode. Smaller proteins travel faster thus allowing the separation by size. After completion, these protein samples are transferred onto a membrane. This membrane can then be stained with antibodies towards the specific protein of interest. After this, a secondary antibody can be used with a detection method to visualize the proteins of interest. [10]

3.5.2 Detailed Methods for Sample Preparation

The frozen cell pellets from the full well were used. Samples were lysed using 100 μl Laemmli buffer for western blot. Laemmli buffer was composed of 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8. Then, the lysed cell extract was transferred to 1.5 ml Eppendorf tubes and boiled at 90°C for 10 minutes. Protein concentrations of supernatants were determined using the DC Protein Assay (Bio-Rad Laboratories, Cat# 500-0114) using Bovine Serum Albumin (BSA) as a standard (Bio-Rad). Samples were mixed with 6X loading dye (5% SDS, 30% glycerol, 250 mM Tris-HCl pH 6.8, bromophenol blue, 5% β-mercaptoethanol). The concentration of the protein was normalized to load 10 μg of protein in 20 μl per lane.

3.5.3 Detailed Methods for SDS-PAGE and Membrane Transfer

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% resolving gel (30% Acrylamide, 1.5M Tris, pH 8.8) and a 5% stacking gel (30%

Acrylamide, 1M Tris at pH 6.8). 20 µg of protein was loaded per lane in a 12-well Bio-Rad. Gel was subjected to electrophoresis for 2 hours at 100 Volts (V). The gel was transferred to nitrocellulose blotting membranes (Bio-Rad Cat # 1620112) overnight at 30 V in the cold room. The transfer buffer contained 25 mM Tris, 190 mM glycine, 20% methanol. Total protein loaded and transfer was verified using Ponceau S Stain (P7170 Sigma).

3.5.4 Detailed Methods for Antibody Staining

Membranes were washed three times (3x) for 10 minutes using Tween-Tris buffered saline (TTBS – 0.1% Tween-20, 50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl (Sigma)). Membranes were blocked with 10% skim milk in TTBS for one hour at room temperature on a shaker. Membranes were washed 3x for 5 minutes with TTBS. Membranes were incubated with primary antibody at 4°C overnight. Membranes were washed 3x for 5 minutes with TTBS and incubated with appropriate secondary antibody (Horseradish Peroxidase (HRP)-Goat of anti-rabbit or anti-mouse depending on primary) at 1:2000 dilution for one hour at room temperature. Membrane blots were washed 4x for 10 minutes using TTBS. Membranes were immersed into enhanced chemiluminescence solution (GE Healthcare, Amersham ECL Prime Western Blotting Detection Reagents, Cat# RPN2232) for a minute. Bands imaging was performed using the Bio-Rad ChemiDoc Imager at the Charbonneau Centre. Band density was analyzed using ImageStudioLite (Licor).

3.6 Immunocytochemistry

Immunostaining was performed either for both 2D (on top of adherent and confluent cell monolayer) and 3D (intact aggregates) conditions. The samples were fixed using 4% paraformaldehyde (PFA) for 10 minutes at room temperature. The samples were then washed 3x

using PBS for 5 minutes. The samples were incubated in 0.5% Triton X-100 detergent for 5 minutes followed by three washes with PBS. Primary antibodies were then added to samples using PBT (0.1% TritonX-100 in PBS) and 1% BSA solution. The samples were incubated in the primary antibody solution overnight at 4°C. The following day, they were washed three times in PBS and then blocked in 1% BSA for 10 minutes at room temperature. The samples were incubated in the secondary antibody solution (secondary antibody and 1% BSA in PBS) for one hour at room temperature, then washed 3x with PBS. The sample nuclei were stained using DAPI (4′,6-diamidino-2-phenylindole) stain. Samples were imaged using an Olympus IX83 Microscope at 20X magnification using MicroManager software [99].

CHAPTER FOUR: RESULTS

4.1 Various Cell Lines Grown in 2D and 3D Culture

In this section, the ability of various cells to form aggregates is explored. Figure 4-1A shows that the shape of the microwell is an upside-down pyramid [100]. The single-cell suspension can be placed on top of the microwell to allow cells to cluster together. Overtime, these cells can form an aggregate. Figure 4-1B shows that the contact points of the aggregate to microwell do not depend on aggregate size. In addition, the contact points are very minimal and the Pluronic coating further ensures that the cells do not attach to the plate [100]. Majority of the cells are experiencing cell-to-cell and cell-to-matrix connections. The stiffness experienced by the cells are dependent on the cells and ECM surrounding them. Figure 4-1C shows the morphology of different cell types in 2D and 3D conditions. The first column of Figure 4-1C is 2D adherent culture, and it shows that different cell lines have distinctive morphology after being grown for 48 hours (2 days). Fibroblast cells seem to display the most spread out phenotypes followed by ARPE-19 and HES-2. In contrast, HT29 cancer line seems to show the most compact phenotype and seem to cluster close together in colonies. The second column shows 3D aggregates at Day 0 where the cells can be seen after being dissociated into single cell and centrifuged. This allows the cells to cluster together to promote aggregation. The different cell types seem to show a similar number of cells per well indicating that 100 cells/well were correctly seeded. At Day 0, It is difficult to tell cellular size differences, but it can be assumed that the cells retract when they are dissociated into single cell. The third column shows Day 2 where aggregate formation can be seen by the four cell lines indicated by the clear spherical boundary layer. The mesenchymal stem cells seem to show the smallest aggregate size. Moreover, ARPE-

19 and fibroblast cells seem to have a smaller aggregate size when compared to cancer and stem cell lines. In addition, the cancer cell line (HT29) shows rugged edges for its aggregate and some darker cell morphology. The stem cell line (HES-2) shows single cells in the well that did not incorporate into the aggregate which are presumed to be dead cells due to single-cell dissociation.

In efforts to normalize between culture conditions, cell counting was performed. However, this was found to be difficult as aggregates were difficult to dissociate into single cell presumably due to forming stronger connections with other cells and accumulation of ECM. In addition, DNA assay using Quant-iT™ PicoGreen™ dsDNA Assay Kit was performed. Although this was able to give a better evaluation on the number of cells, there was a lot of variation found between cell samples. This could be due to the fact that DNA quantification was performed on a subsection of the sample and the subsection could vary a lot between samples due to the clumping issue listed earlier. Due to interest of time, normalization using cell numbers was not performed but, in the future, this should be taken in account.

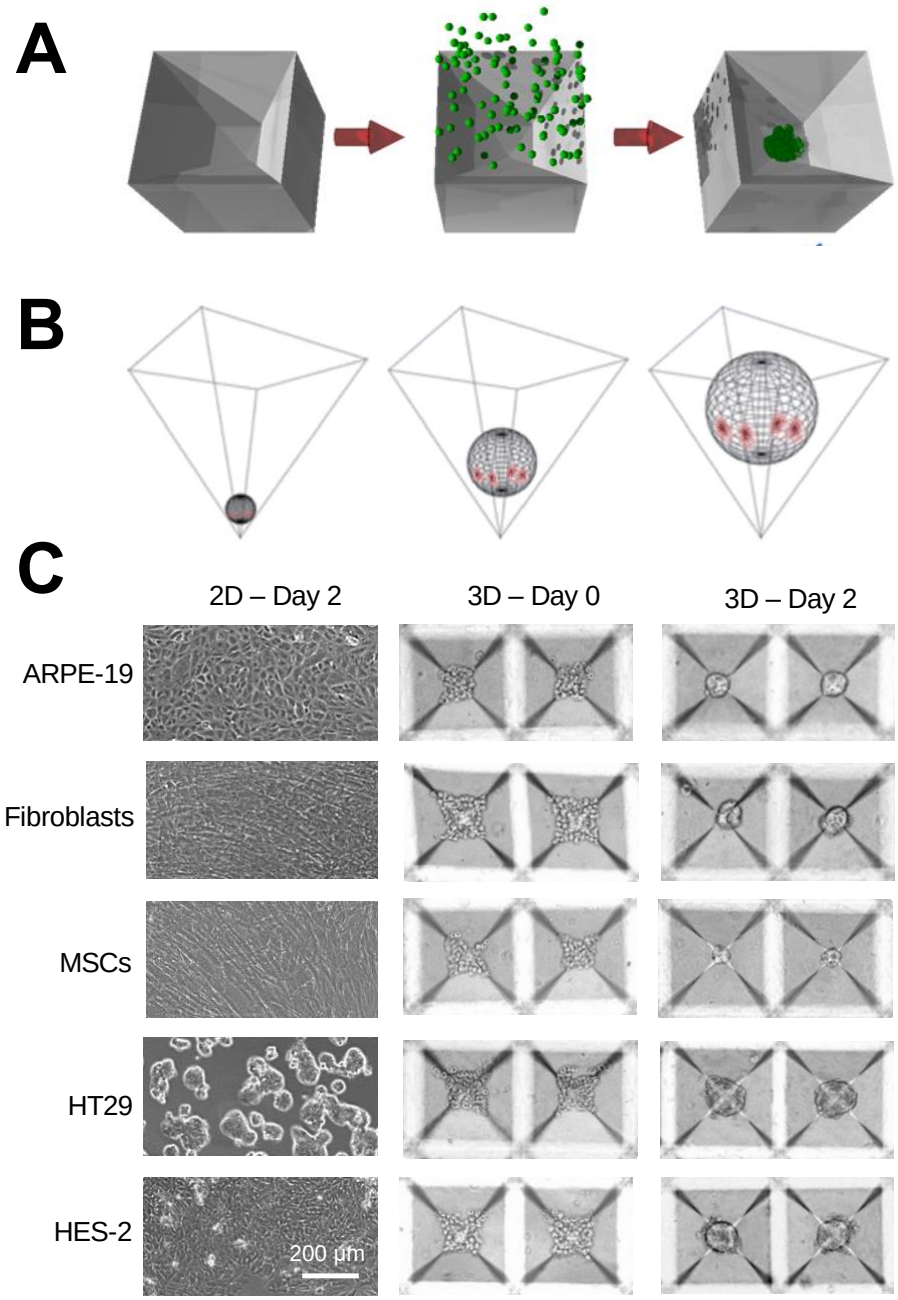


Figure 4-1. Cell Morphology in 2D vs 3D conditions. A. Aggregates are formed by centrifugation of suspension of single cells into microwells. The microwells are a square pyramidal shape and have a size of 400 µm. B. The geometric relationship between aggregate and microwell does not depend on aggregate size. The red highlights show contact points. C. Comparison of morphology of various cell lines in 2D and 3D adherent culture. Note that HES-2 was grown on top of a layer of Matrigel™. Aggregates are formed by centrifuging 100 cells/microwell (Day 0) and are usually formed in 24-48 hours depending on cell type (Day 2).

4.2 RT-qPCR

Author's Note: All of the experiments and analysis was performed by the author, Saba Aslam, using Microsoft Excel, R Studio, and GraphPad Prism 8. Results from 3.3 were included.

RT-qPCR was performed on the cell lines listed in Table 3. The total RNA amounts were normalized prior to performing RT into cDNA. Based on the stability analysis, all the results were normalized to the geometric mean of *EIF2B1*, *YWHAZ*, and *POLR2A*. The subsequent experiments were performed on the remaining cell lines on a smaller panel of genes: *ACTB*, *TUBB, ACTR2, FN1, KRT18, LMNA*, and *VIM*. This resulted in a full panel of ten genes.

Table 3. Human Cell Lines.

Human Cell Line Category	Cell Line Name	Cell Line Information
Common Cell Lines	ARPE-19	Adult Retinal Pigment Epithelial cell line-19
	ES-RPE	Embryonic stem cell derived retinal pigment epithelium
	Fibroblast	Foreskin fibroblast cells
	MSCs	Mesenchymal stem cells (adipose derived)
	HEK293	Human embryonic kidney 293 cells
Cancer Cells Lines	MCF7	Mammary gland epithelium (breast cancer) / Michigan Cancer Foundation-7
	Ishikawa	Endometrial cancer
	HeLa	Cervical cancer (Henrietta Lacks)
	HT-29	Colon cancer
Pluripotent Stem Cells	HES-2	Embryonic stem cells
	H9	Embryonic stem cells

Figure 4-2 and Figure 4-3 shows the variation between C_T values of common cell lines and cancer cell lines, respectively. The panels are separated by cell line and different genes are shown within each panel. Raw C_T values are graphed in order to compare base gene expression levels to observe which cell types express which genes. As such, no normalization was performed on this data. The C_T values are reversed on y-axis as low C_T values indicate high expression of the gene and high C_T values indicate low expression of the gene. Commonly, C_T cut-off value of above 35 is indicative of little to no expression [101]. In Figure 4-2, ARPE-19 and fibroblast cells of have C_T values within the 20 to 35 range but the C_T values for MSCs are in the high ranges of 30 – 40. This could be due to lower amounts of cDNA that was input into the experiment as all of the genes observed including vimentin show low expression. ARPE-19 and fibroblast cells show similar results with most of the genes showing upregulation in 2D compared to 3D apart from keratin and tubulin (*KRT18* and *TUBB*). Moreover, MSCs show similar upregulation of most genes observed in 2D compared to 3D including tubulin. In Figure 4-3, cancer cell lines values can be seen. Overall, it can be seen that all of the cancer cell lines highly express *ACTB* and *KRT18.* HT29 cells do not seem to have differences in expression levels in any gene between 2D and 3D. HeLa cells seem to show an upregulation of *ACTB* and *FN1* in 2D but show a downregulation of *VIM* in 2D. Although the results have a large range, so it is hard to determine significant changes. Ishikawa cells seem to show an upregulation of *ACTB*, *FN1,* and *VIM* in 2D with the rest not showing much changes. MCF7 results seem to be quite irregular with *ACTB, ACTR2*, and *VIM* showing some upregulation in 2D. However, *KRT18* and *TUBB* seem to be downregulated in 3D compared to 2D. When comparing across Figure 4-2 and Figure 4-3, it can be seen that cancer cells seem to show high expression levels with C_T in low 20 ranges. The variability within samples

and across cell lines will be further examined in the discussion. Please note that stem cell lines and stem cell derived RPE line were excluded due to limited amounts of RNA available. The ES-RPE cell line is grown for eight weeks in culture to ensure maturation of the RPE cells. The stem cell data for hPSCs was found to be quite variable and thus was checked with additional pluripotency genes such as *OCT4* and *GATA4*.

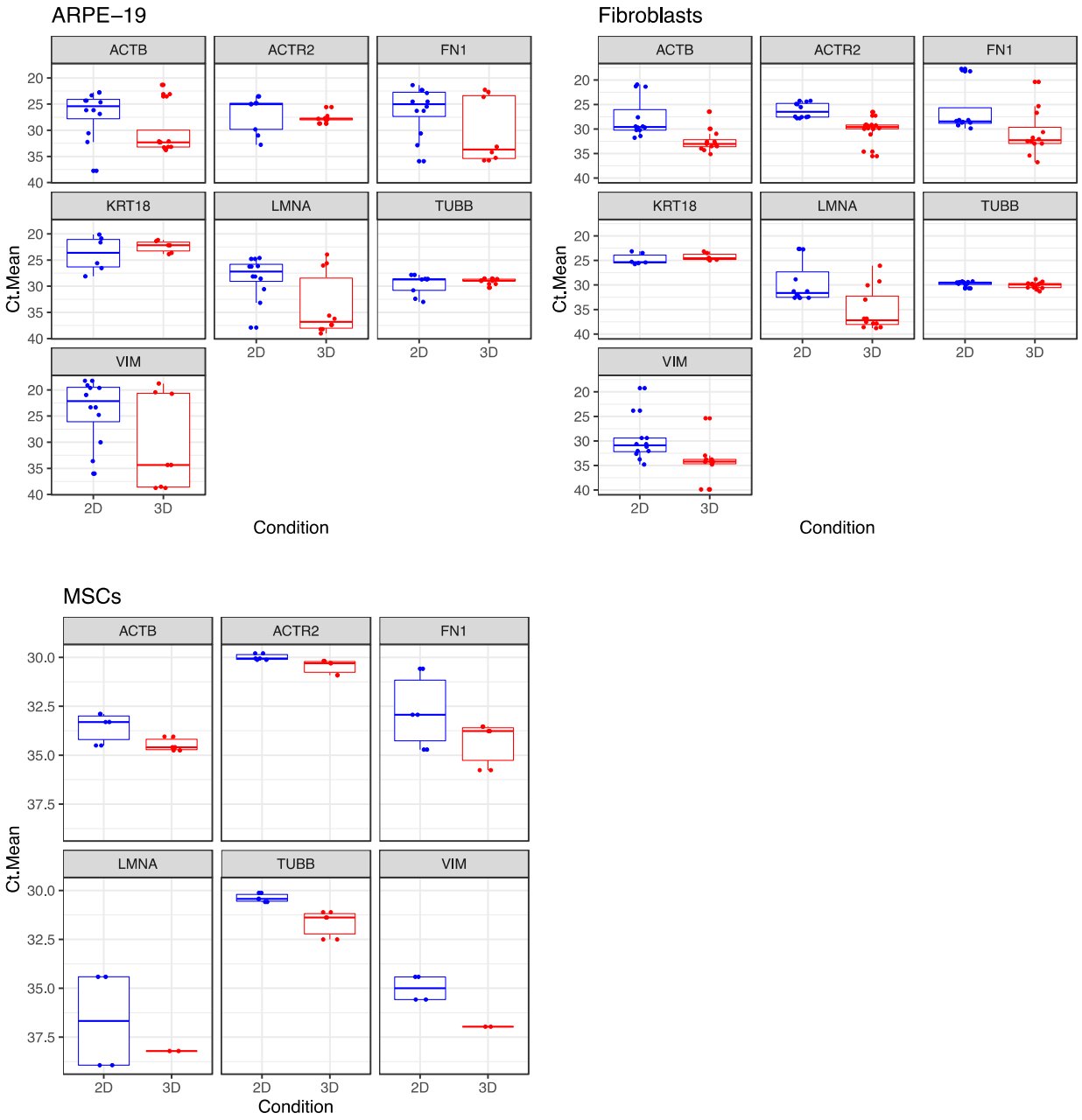


Figure 4-2. Boxplot of C_T Values for Common Cell Lines. Each Panel indicates a different cell line and each plot within the panel shows different genes. The y-axis is reversed as the low C_T values show highly expressed genes and high C_T values show low expression of the gene. The 2D culture is shown in blue and 3D is shown in red. This boxplot was generated using R Studio.

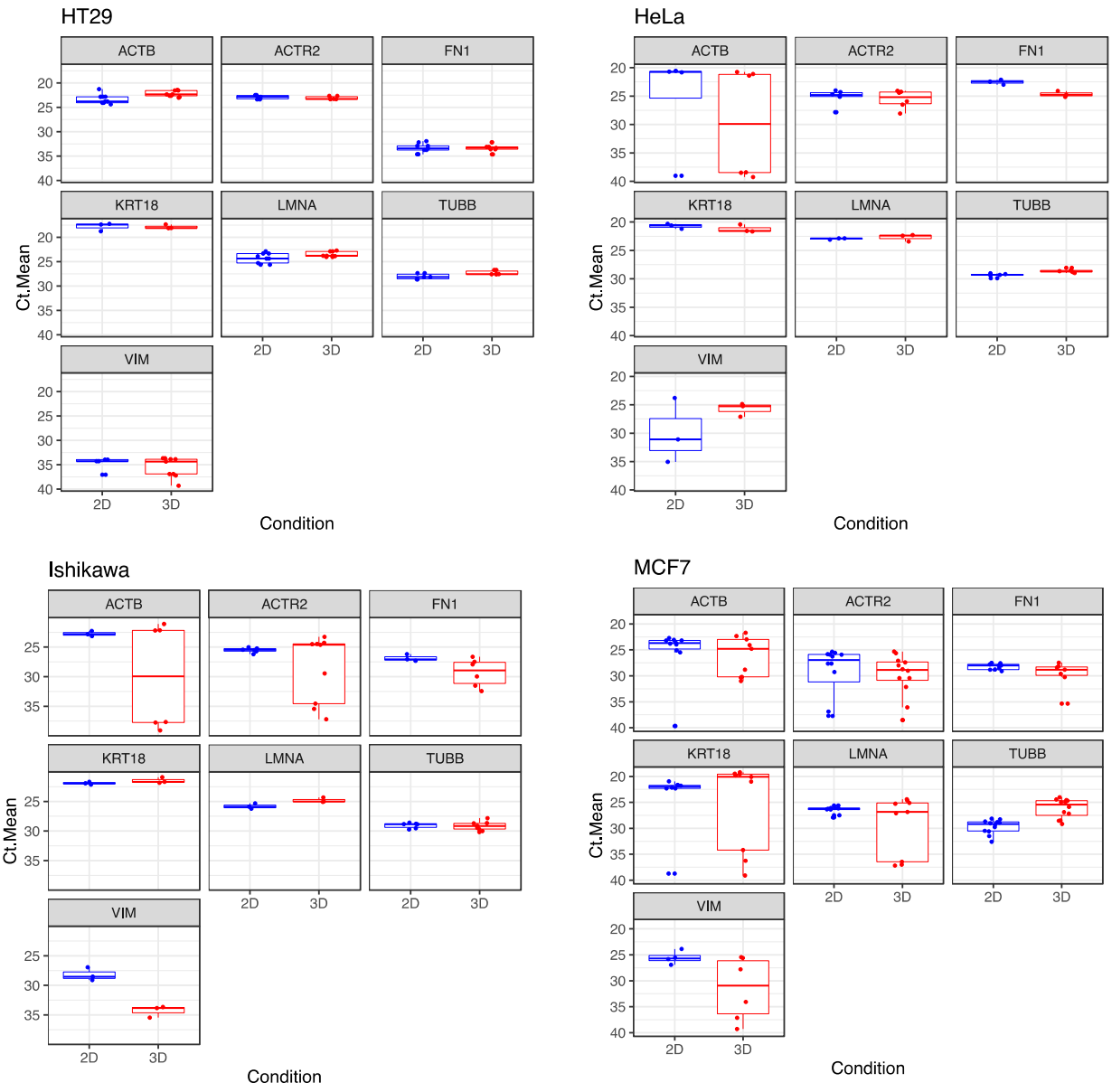


Figure 4-3. Boxplot of CT Values for Common Cell Lines. Each Panel indicates a different cell line and each plot within the panel shows different genes. The y-axis is reversed as the low CT values show highly expressed genes and high CT values show low expression. The 2D culture is shown in blue and 3D is shown in red. This boxplot was generated using R Studio.

In particular, β-actin expression was further analyzed. Figure 4-4 shows β-actin expression in the various cell lines normalized to the three reference genes (*EIF2B1*, *YWHAZ*, and *POLR2A)*. Statistical significance can be determined using various tests to calculate a p-value and $p<0.05$ is considered significant. Student t-test is based on the mean values between two groups of data under the assumption that two groups of data are independent and normally distributed. For a more stringent analysis, Mann-Whitney U test can be used. Mann-Whitney U is a non-parametric test which means that data is not assumed to be normally distributed. After performing statistical analysis with Mann-Whitney U test, β-actin was found to be significantly upregulated in 2D culture for various cell lines including ARPE-19, ES-RPE, Fibroblasts, and HES-2. β-actin also showed the trend of being upregulated in 2D in HeLa and H9 cells. Due to low n-values for HeLa cells, statistical significance ($p<0.05$) was not found with Mann-Whitney test. Unpaired t-test is not the preferred test for this data but performing the t-test provisionally on the HeLa cells result led to a very significant (**) p-value of 0.0056 for upregulation of *ACTB* expression in 2D. This analysis indicates statistical significance and will need to be repeated with more replicates for Mann-Whitney test. This shows the trend that common cell lines (RPE-related cell lines, fibroblasts) with the exception of HEK293 seem to show the trend of 2D cells having more β-actin expression. The cancer cell lines do not show the β-actin upregulation in 2D, apart from HeLa. The stem cells (HES-2 and H9) had a lot of variation in their C_T values which can be explained due to culture conditions and potential differentiation. It can be hypothesized that with better culture conditions and more n-values, H9s will also show similar result to HES-2. Thus, PSCs also seem to follow the same trend. MCF7 seem to also show the trend in 2D, however their 3D values seem to be extremely variable to hypothesize one way or another.

Overall, it can be said that cancer cell lines do not uphold the trend, and this could be explained by how cancer cell lines have deregulated β-actin expression in general. Some cell lines only had n of 3 and adding more n values will allow better statistical tests to be performed.

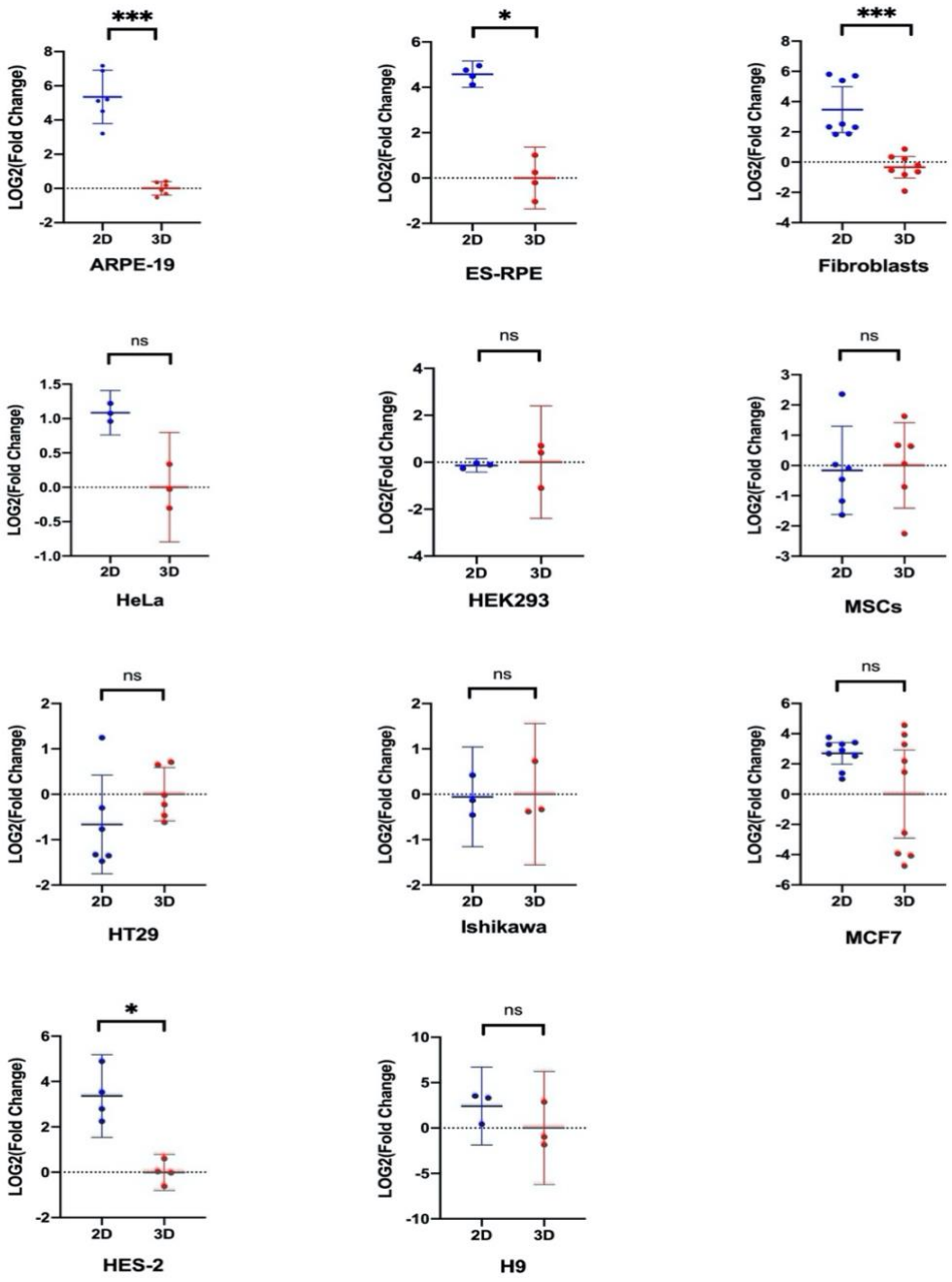


*Figure 4-4. β-actin Expression in 2D vs 3D Conditions Across Cell Lines. The 2D condition is shown in blue and the 3D is shown in red. The average log2(fold change) is graphed with 95% confidence interval. Fold change is representative of 2^(-ΔΔCT). The statistics were calculated using the Mann-Whitney test. *** denotes extremely significant (p value 0.0001-0.001), ** denotes very significant (p value 0.001-0.01), and * denotes significant (p value 0.01 to 0.05), ns denotes not significant (p value >0.05). The n values ranged from 3 to 9. The graphs were created using Prism 8 GraphPad. Please note that the y-axes are of different scales.*

4.3 Western Blot

Author's Note: Samples were collected and prepared by Jarin Thundathil and Bilal Hafeez under the supervision of Derek Toms. The western blot was performed by the author, Saba Aslam, with the help of Tarryn Bourhill. The analysis was performed by the author.

As the actin isoforms are >93% identical in their amino acid sequence, most antibodies against actin are able to recognize all of the actin isoforms. Most of the differences in amino acid sequences between the isoforms can be found in the N-terminal region [36], [37]. As such, most research papers use generic actin antibodies as controls.

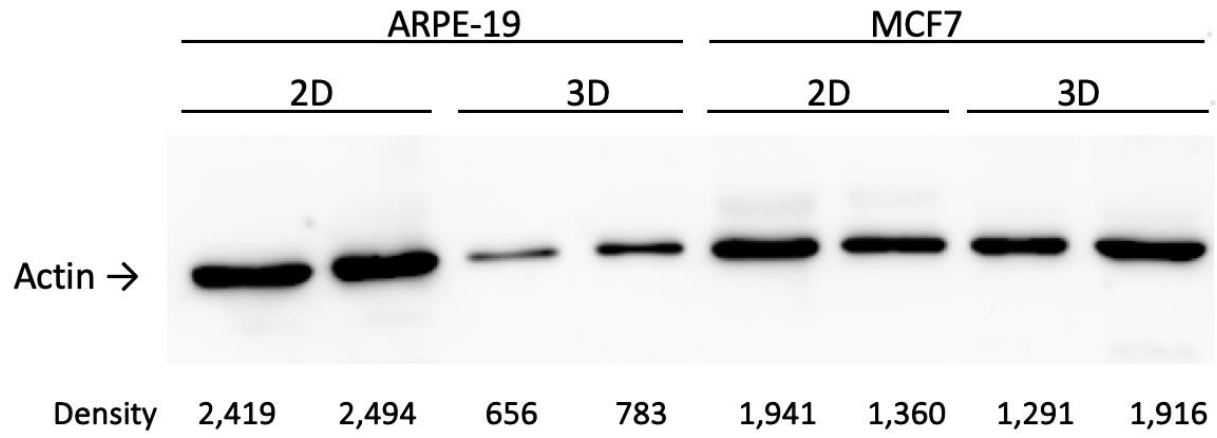


Figure 4-5. Western Blot Results of Actin in 2D and 3D conditions. The control for Western Blot was total protein content (Ponceau S staining). The densitometry values are listed below and were measured using ImageStudioLite from Licor.

Figure 4-5 shows the protein level expression of actin proteins in ARPE-19 and MCF7. The western blot was normalized by performing protein quantification and loading the same amount of protein through all samples. Furthermore, Ponceau S staining was performed (results not shown). The actin expression shows the trend of being highly upregulated in 2D in ARPE-19 similar to the RT-qPCR results. In MCF7, not much of a difference can be seen which also

corresponds to the RT-qPCR results. Although, proper error and statistics could not be calculated due to n of 2 for each sample.

4.4 Immunocytochemistry

Author's Note: All of the experiments, imaging, and analysis was performed by the author, Saba Aslam, using Fiji ImageJ.

RT-qPCR and Western blots are both tissue disruptive techniques as they require cell lysis. Immunocytochemistry was used as a non-disruptive technique. Immunocytochemistry was performed to give insight into the quantity and localization of actin proteins. Figure 4-6 shows the differences in actin proteins within different cell types and across 2D (A panel) and 3D conditions (B panel). In Figure 4-6A, clear differences between cell sizes and their corresponding actin proteins can be seen. Fibroblast cells seem to be the most spread out with the more elongated cell shape. In contrast, HT-29 cancer cell line seems to have the smallest size. Actin seems to run across the length of the cell for all cell types. Ideally, the 3D aggregate images could be taken on a confocal microscope to see better differences. Please note, aggregates of ARPE-19 and HT-29 were also stained but have not been imaged yet. It would have been useful to perform semi-quantification of fluorescence of actin proteins between 2D and 3D conditions for each cell line. This could have then been compared with the Western Blot results. However, the antibody for actin did not seem to be very specific. As such, quantification was found difficult to perform.

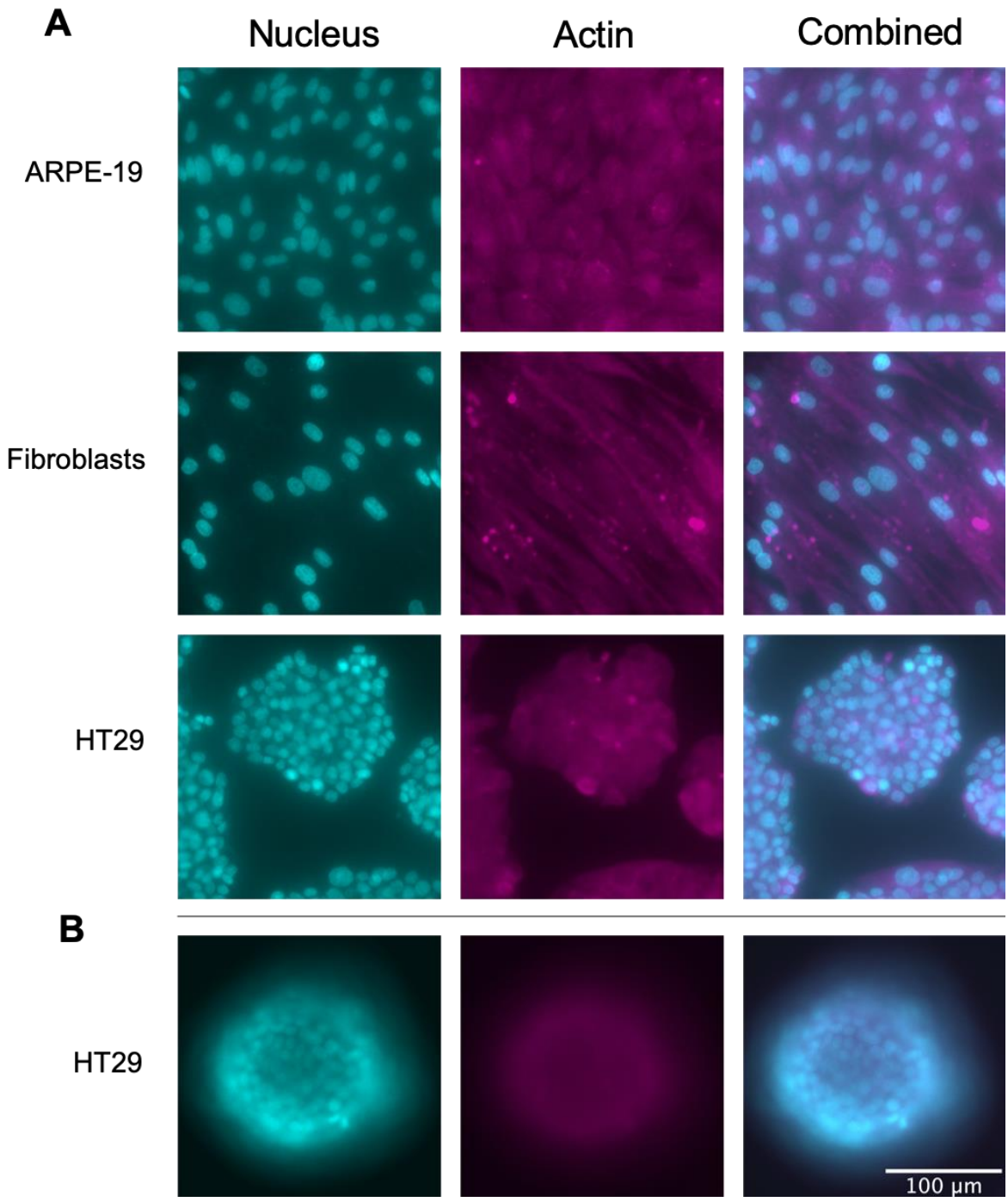


Figure 4-6. Immunohistochemistry for β-actin levels in 2D vs 3D cell culture of various cell lines. A: 2D cell culture. B: 3D cell culture. A primary antibody to actin was used. This figure shows the localization of actin and the differences between cell sizes.

4.5 Microarrays

Due to lack of resources and time, being able to perform stability analysis on each individual experiment may not be feasible. Instead, publicly available data from similar experimental conditions can be utilized to check the validity of reference genes of interest. This section will discuss how publicly available data from GEO was used to study the hypothesis of downregulation of cytoskeleton genes in 3D. The full methodology can be found in Figure 3-3. Then, studies that explored cell culture methods in both 2D and 3D contexts were found. In particular, two main research articles will be highlighted.

4.5.1 Study 1 – Fibroblasts vs Glioblastoma Cell Line (2D, 3D)

Author's Note: The experiments were performed by Jack et al. [102] *who provided publicly available microarray data. The analysis was performed by the author, Saba Aslam, using GEO2R, RStudio, and Ungrin lab ReceptoR Software.*

The first study looked at two cell types, primary human foreskin fibroblasts (HFF-2) and glioblastoma cell line (T98G), in both monolayer (2D) and spheroid (3D) conditions [102]. In the paper, he HFF-2 were grown in DMEM with 15% FBS, and T98G were grown in EMEM with 10% FBS and 1% non-essential amino acids. Both cell types were grown in 5% CO_2 humidified air at 37°C. They grew the monolayers in adherent T-185 flasks to 60% confluency and then suspended the cells on a flask coated with 1% (w/v) agarose mix in a 2:1 media/water ratio. The suspended cells were allowed to grow for four days to form spontaneous aggregates. Total RNA was extracted, synthesized into cDNA, which was then hybridized onto the Affymetrix U133 Plus 2.0 Array. Their microarray protocols, equipment, and data were fully MIAME compliant and were deposited at GEO with Series: GSE4217-4218; Samples: GSM96262-96285. [102]

This study was performed to study the effect of metabolic arrest on utilizing an autocrine response in aggregates of fibroblast and cancer cells. This data was then processed through Ungrin lab ReceptoR software to discover which cytoskeleton-related genes were differentially expressed (DE) between the 2D and 3D conditions. ReceptoR can then create boxplots or heatmap of the DE genes. The heatmap for cytoskeleton genes is shown in Figure 4-7 for fibroblast cells, and Figure 4-8 for glioblastoma cells. The ten genes that were studied in previous RT-qPCR experiments are pointed out on the right side. As seen by the orange colour, a larger number of cytoskeleton-related genes are significantly upregulated in 2D vs 3D for both cell types. Moreover, there is a higher number of upregulated cytoskeleton-related genes in 2D for fibroblasts compared to glioblastoma cells.

In addition, GEO2R, a tool created by NCBI, was also used to analyze these samples. It allows assigning samples to certain conditions and analysis of a single GEO dataset at a time. It calculates a list of the top 250 statistically significant results that are differentially expressed between the samples selected. Through GEO2R, *ACTB* was found to be significantly differentially expressed in fibroblast cells but not in the cancer cells. This indicates that GEO2R has more stringent statistical significance calculations compared to ReceptoR. This is because ReceptoR was designed to predict a novel active signaling pathway so including more genes would be beneficial [96]. The GEO2R results further validate the RT-qPCR findings, with fibroblast cells showing a significant change in cytoskeleton genes but not cancer cells.

For future analysis, GEO2R could be used for more stringent analysis. In addition, actin cytoskeleton organization (GO:0030036) is a group underneath the cytoskeleton organization (GO:0007010). Moreover, GO:0005200 is the gene list for structural constituent of cytoskeleton.

Performing the analysis using a more specific list of actin/structural cytoskeleton genes could give a better understanding of the actin and related proteins which is more relevant to the objective of this thesis.

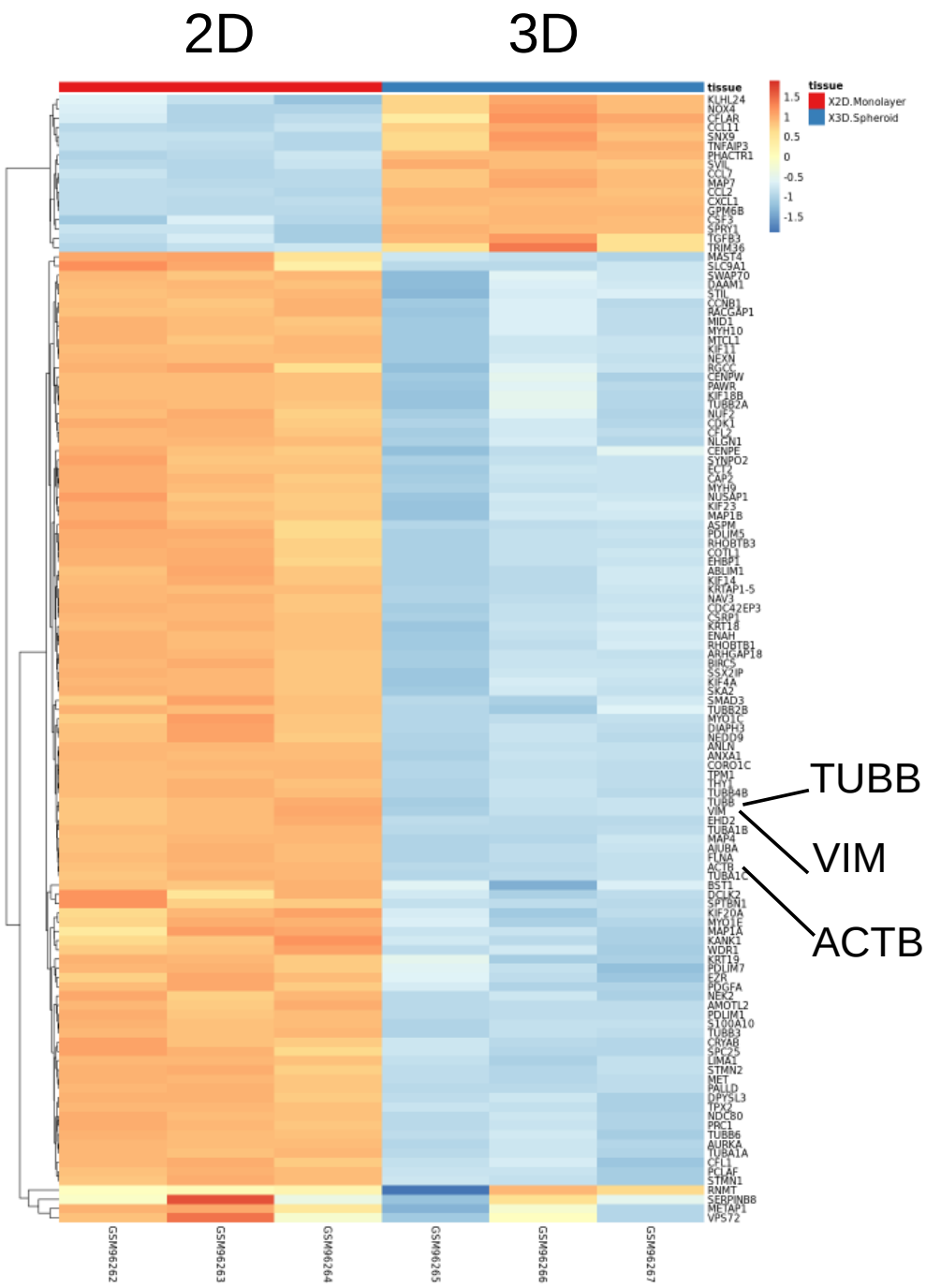


Figure 4-7. Heatmap of cytoskeletal gene expression in Fibroblast cells. Significantly differentially expressed genes between the two conditions (2D and 3D) are plotted on the heatmap. High expression is shown in red and low expression is shown in blue. This graph was generated in ReceptoR.

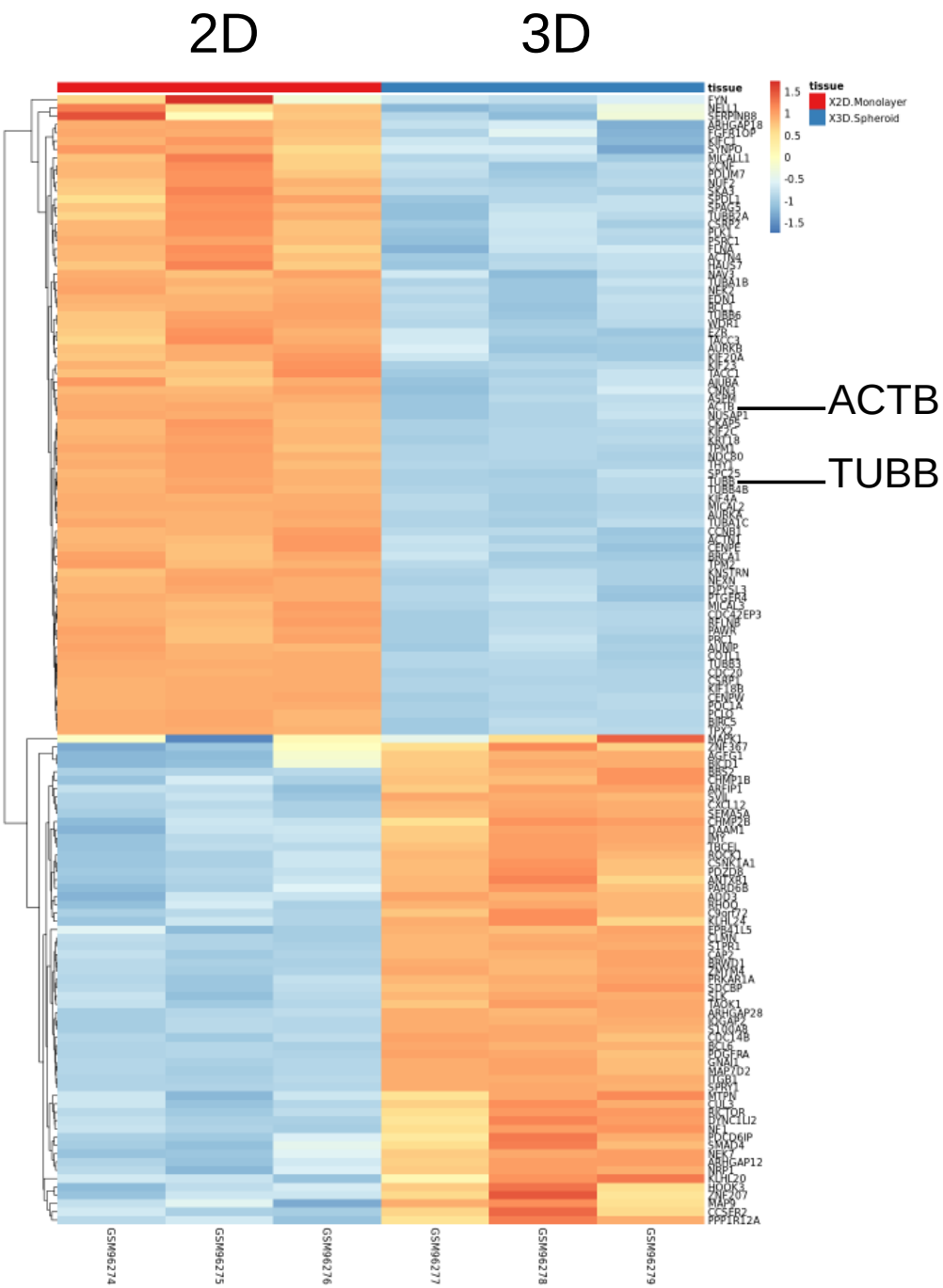


Figure 4-8. Heatmap of cytoskeletal gene expression for glioblastoma cells. Significantly differentially expressed genes between the two conditions (2D and 3D) are plotted on the heatmap. High expression is shown in red and low expression is shown in blue. This graph was generated in ReceptoR.

4.5.2 Study 2 – Dermal Papilla Cells (intact, 2D, 3D)

Author's Note: The experiments were performed by Higgins et al. [103] *who provided publicly available microarray data. The analysis was performed by the author, Saba Aslam, using GEO2R, RStudio, and Ungrin lab ReceptoR Software.*

The second study looked at primary human dermal papilla cells that were obtained from three male donors during hair transplantation surgery [103]. They looked at these cells in three different conditions: primary, 2D, and 3D. This study showed that when dermal papilla cells go from their primary environment (3D) to adherent culture (2D), they quickly change their transcriptional signature and can no longer induce de novo hair (hair neogenesis). Although they did not look specifically at cytoskeleton genes, they demonstrated that growing these cells in 3D spheroid culture can partially restore their transcriptional signature, along with their capability to perform hair neogenesis in the human skin.

In the paper, the primary cells were dissected directly from the adult scalp hair follicles. These cells were cultured and then grown in 2D adherent culture and samples were taken at passage 0, 1, 3, and 5. For 3D cultures, they resuspended 300 cells per μl in a hanging drop method to form spheroids of 3,000 cells in 10 μl. The mRNA was extracted and then two-cycle 3'IVT (*in vitro* transcription) was performed to generate biotin-labeled cRNA. This cRNA was hybridized onto the Affymetrix U133 Plus 2.0 Array. The data was deposited in the GEO database with accession number for Series: GSE44765. [103]

This data was then processed through Ungrin lab ReceptoR software to produce the following heatmap of differentially expressed genes in Figure 4-9. The actin related and tubulin genes are pointed out on the right. A larger number of differentially expressed cytoskeletal-

related genes were shown to be significantly upregulated in 2D compared to both 3D and primary conditions. Importantly, the 3D and primary conditions clustered together. This signifies that 3D conditions are more representative of native conditions in the body compared to 2D conditions.

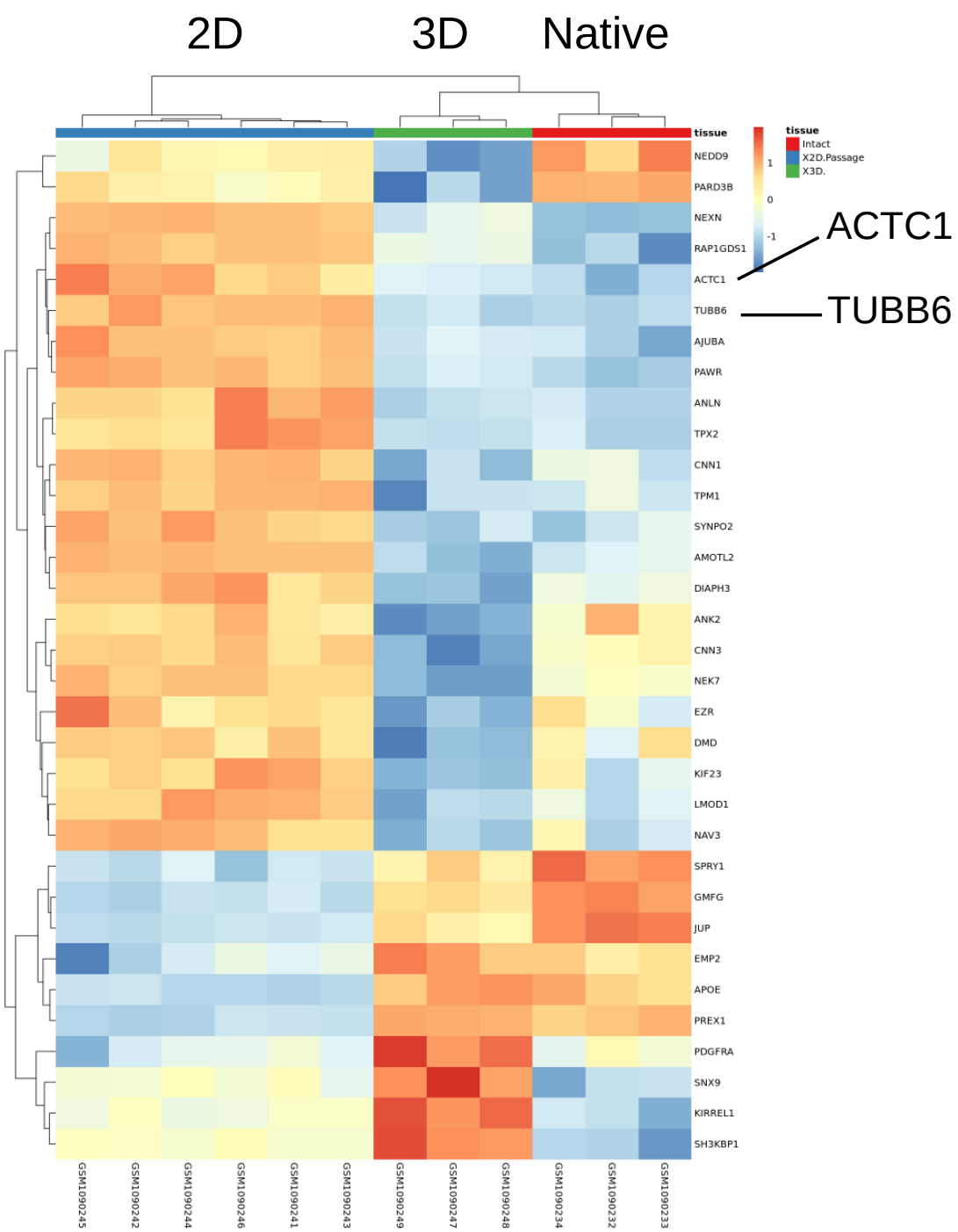


Figure 4-9. Heatmap of cytoskeletal gene expression in dermal papilla. Significantly differentially expressed genes between the two conditions (2D and 3D) are plotted along with genes expressed in the native condition (intact). High expression is shown in red and low expression is shown in blue. This graph was generated in ReceptoR.

CHAPTER FIVE: DISCUSSION

5.1 Cell Culture

Various cell types have the ability to form aggregates using the microwell system. This has also been previously shown by the Ungrin lab and others using the AggreWell system [8], [98], [100], [104]. In this thesis, the cell lines were divided into three categories: common cell lines, cancer cell lines, and stem cell lines. The cell lines were arbitrarily placed in these categories to allow for comparison across different groups. The same number of cells (120k) were seeded onto one well of 24 WP for both the 2D (tissue culture adherent plate) and 3D (microwell plate). This allowed cells to form an aggregate of 100 cells each.

In addition, efforts were made to minimize other differences between the two different conditions. This included keeping the passage number and quality of the cells consistent across conditions. Cells in both conditions used the same type and amount of media (1 mL). Furthermore, both the 2D and 3D conditions were grown for 48 hours (2 days). This was found to be the shortest amount of time needed for aggregate formation for most cell types and also allowed cells in 2D conditions to reach ~85% confluency. Figure 4-1 shows the ability of the selected cell lines to form aggregates using the microwell system. The microwell system allows for cells to cluster together which promotes aggregation over the time period of few hours to days depending on the cell type. Due to the clustering aspect, the microwell system can be a better tool for aggregate formation, particularly for the cell types that do not form spontaneous aggregates when placed in single-cell suspension in media on a non-adherent culture plate.

Although efforts were made to minimize other differences across the two culture conditions, it was difficult to control the conditions experienced at the cellular level for each cell.

For example, in 3D aggregates, there is a gradient experienced by the cells where the cells on the periphery have more contact with the media, nutrients, oxygen, and other factors in comparison to the cells found in the core of the aggregate. Whereas, the cells in 2D conditions more or less experience similar conditions at the cellular level. As such, small sized aggregates were used to minimize the extent of the gradient. Aggregates of a 100 cell/aggregate were found to be the smallest size that allowed for aggregate formation in the cell types utilized. In addition, there are various differences that exist based on the cell type. For instance, Table S2 shows that different cell types experience varying amounts of oxygen in their immediate microenvironment due to differences in their metabolic rates. These differences were important to note but did not seem to have a correlation with β-actin expression results.

Figure 4-1C shows the morphology of various cell types in both 2D and 3D conditions. Fibroblasts seem to show the most spread-out phenotype in 2D conditions and cancer cell show the least spread. For 3D conditions, similar numbers of cells were seeded in each well. It was found that aggregates can be formed from various types of cells in a size-controlled manner. The mesenchymal stem cells show the smallest sized aggregates. The cancer cell line and stem cell line showed a larger aggregate size compared to the ARPE-19 and fibroblast cell lines. Mainly, this could be due to the proliferation and doubling time of cancer cells and stem cells being higher than those of other cell types.

The aggregate size differences could also be attributed to many factors that depend on the cell type such as: the size of the cell, amount of ECM secreted, and incorporation of cells into the aggregate. Moreover, if the cell type proliferates more in a 2D environment compared to a 3D environment. In addition, cell lines such as hPSCs (human pluripotent stem cells) do not

typically prefer single cell suspension, although HES-2 is less susceptible to single cell culture. Due to this, sometimes not all the cells get incorporated in the aggregate and typically are found to be dead cells using live-dead staining. As such, even though the same number of cells are seeded for both 2D and 3D conditions, this may not be the case after some time (day 2) and post-aggregation. In addition, some of the aggregates are lost during media changes which is not applicable to 2D adherent culture. Efforts are made to ensure that this number remains small. Due to all these factors, typically the 3D culture conditions have a smaller number of cells compared to 2D cell culture. In addition, the 3D aggregates are known to secrete their own extracellular matrix (ECM). Attempts were made to normalize the number of cells prior to lysing for further analysis. These attempts were unfruitful due to difficulties in dissociation of 3D aggregates into single cells due to their ECM and possibly stronger cell-to-cell and cell-to-matrix connections. As such, the amount of RNA was normalized for gene expression studies and the total protein was normalized for protein expression studies.

5.2 Gene Expression

The gene expression of cytoskeleton related proteins or ECM proteins and other common housekeepers was analyzed using RT-qPCR across many different types of cell lines in 2D or 3D conditions. The gene expression was also observed by performing analysis on publicly available microarray data. Two main studies were selected as they fit the parameters of 2D vs 3D conditions and array type that can be analyzed through ReceptoR. The first study looked at fibroblasts and glioblastoma cancer cell line, and the second study looked at dermal papilla cells. Finally, the gene expression analysis from bulk RNA-sequencing of naïve H9 cells was also included.

5.2.1 RT-qPCR

For RT-qPCR, first stability analysis was performed to determine which reference gene and how many reference genes needed to be used. This was important because this thesis questions the role of β-actin as a reference gene so stable alternative reference genes had to be verified. From the stability analysis it was found that the geometric mean of three reference genes: *EIF2B1*, *YWHAZ*, and *POLR2A* would be sufficient as a reference (Figure 3-2).

Following this, RT-qPCR was performed.

Figure 4-2 and Figure 4-3 showed the variation in raw C_T values for various genes and cell lines in 2D and 3D conditions. Overall, common cell lines showed the trend of upregulation of cytoskeletal and ECM related genes whereas cancer cell lines showed irregular expression.

The variability in the data within one gene could be due to number of cells in each sample, technical differences within various individuals performing the experiments, differences in cell quality for specific batches, and differences in the cells themselves. It is important to note that the expression levels across different cell types could vary due to the amount of cDNA input in the experiment. For instance, although the same number of cells were seeded in 2D and 3D conditions, different cell types have different proliferation rates. The doubling time of cancer cells and stem cells is typically higher than other types of cell lines. As such, this could explain high expression values (low C_T values) seen for cancer cell lines. It is also possible that cancer cell lines show the trend of upregulation in 2D, but it is not detected as the limit of qPCR is being reached. This can be looked into by using a diluted cDNA sample in the future.

Cancer cell results could also be due to de-regulation processes that occur when a cell becomes cancerous. It is key to highlight that established cell lines, in particular cancer cell lines,

can come with their own set of problems. Over the years of their use, cell lines can have a drastically different genome and transcriptome compared to their origin. For example, a study of HeLa cells found that they had abnormally high number of chromosomes and extensive genomic rearrangements indicative of catastrophic chromosome shattering [105]. In addition, HEK293 cells were created by transfection of human embryonic kidney cells [106]. Similarly, they show many abnormalities such as properties of immature neurons and chromosomal abnormalities [107], [108]. Moreover, since 2D cell culture was the most common cell culture technique, these cells could have evolved to become more accustomed to adherent culture. As such, these cell lines may not be truly representative of cancer conditions found in the human body. Future experiments using primary cancer cell lines need to be performed.

Stem cell results could be variable due to differentiation. Although stem cells are attempted grown in conditions to promote their pluripotency, and have the potential to undergo differentiation [109]. As such, stem cell lines more difficult to culture compared to other cells lines. This could happen due to many reasons such as lack of nutrients/media, oxygen fluctuations, Matrigel variability, and other growth conditions [109]. As they were found relatively recently, the exact conditions to promote their optimal growth are not yet known [109].

The proteins involved with intermediate filaments (vimentin, keratin, lamin A) were variable across different cell types as expected. Since different cell types selectively express different kind of proteins involved in intermediate filaments. However, tubulin which is involved in microtubules does not seem to show much of a change in most of the cell types between 2D and 3D. It is possible that the larger types of fibres are less susceptible to culture conditions

within the experimental timeframe, whereas intermediate and smaller fibres are more susceptible. These result correlate well with the mouse embryonic stem cell study showcased earlier where they observed that cytoskeletal proteins (actin α 1&2, keratin 8, lamin, vimentin) showed an upregulation in 2D culture, whereas tubulin did not.

In particular, β-actin data was normalized using the geometric mean of three control genes from stability analysis. From the RT-qPCR, it was found that majority of common cell lines showed a significant upregulation of β-actin gene in 2D when compared to 3D conditions (Figure 4-4). This included ARPE-19, ES-RPE, fibroblasts. However, HEK293 cells and MSCs did not show a similar trend. In addition, HeLa cells showed a strong trend of the same upregulation of β-actin in 2D conditions. The results for HeLa were statistically significant through t-test but not through Mann-Whitney. None of the cancer cell lines (except HeLa) showed a similar trend of β-actin upregulation in 2D culture. This can be explained due to the fact that β-actin is very de-regulated in cancer cell lines. The pluripotent stem cell lines also showed the trend of β-actin upregulation in 2D culture with HES-2 showing significant results, and H9 showing the trend non-significantly.

5.2.2 Microarrays

When publicly available microarray data was analyzed to cytoskeleton related genes, similar results were seen. In the first study analyzed through ReceptoR, fibroblasts showed majority of cytoskeleton related genes to be upregulated in 2D culture, whereas cancer cells showed a similar trend to a lesser extent (Figure 4-7, Figure 4-8). In addition, when the same study was analyzed through GEO2R, ACTB was found to be significantly differentially expressed in fibroblast cells and not in the cancer cells. This further reflects the RT-qPCR results showing that the common cell lines show the trend and not cancer cell lines.

In the second study, primary dermal papilla cells were observed in 2D, 3D, and their primary conditions (Figure 4-9). It was found that cytoskeleton genes were upregulated in 2D conditions. The 3D and primary conditions were similar in expression to each other. This illustrates that the 3D culture is more representative of the native conditions in the human body. The results agree with the paper's findings which demonstrated that 2D culture can quickly perturb the primary phenotype of dermal papilla cells [103]. This leads to abrupt changes in the cell's transcriptomic signature and function. They show that this happens very rapidly, as quickly as the first passage, which indicates that these changes are due to the microenvironment conditions. They go on further to illustrate that 3D culture can help restore native dermal papilla transcriptomic signature, morphology, and function. The structure of the primary dermal papillae consists of a spheroid of a single cell type with a complex extracellular matrix. As such, creating spheroids of these cells closely mimic the primary dermal papillae morphology. Additionally, these 3D spheroids can induce hair neogenesis on human skin which has major clinical applications. Cytoskeletal proteins are involved in a variety of functions including structure, cell signaling, cell division, and cell behaviour. Thus, it is not surprising that cytoskeleton genes could be playing an important role behind the functional differences seen in the 2D and 3D conditions. This further emphasizes the importance of using physiologically relevant culture systems, particularly in the cases of clinical research.

5.2.3 RNA Sequencing

Microarrays were quite popular a decade ago but as next generation sequencing (NGS) became more feasible, microarrays are being replaced by these sequencing technologies [14]. In comparison to microarrays, RNA sequencing (RNA-seq) can observe differential expressions at a

much broader dynamic range [14]. This allows the examination of DNA variations (SNPs, insertions, deletions), discovery of new genes, or alternative splice variations within just one dataset [14], [110]. However, RNA-seq is found to be more expensive and is more difficult to plan the experiment and analyze the data. Most publicly available RNA-seq data requires alignment before meta analyses can be run [111].

Two labs that are part of the Reproductive and Regenerative Medicine (RRM) Group at University of Calgary, Dr. Derrick Rancourt's and Dr. Michael Kallos's, are currently collaborating on a project focusing on the naïve pluripotency of human pluripotent stem cells [112]. Most common stem cell lines are considered to be in the "primed" state [113]. Recently, it has been discovered that hPSCs can be induced to enter a "naïve" state [113]. This naïve state represents an earlier stage in embryonic development (pre-implantation of embryo) compared to primed (post-implantation) [114]. Currently, a lot of research is being performed to determine how to culture naïve PSCs optimally *in vitro* [114].

In their project, Leili et al. observed the effects of three different culture conditions on pluripotency of naïve H9 hPSCs [112]. The three conditions included 2D adherent culture, 3D suspension culture, and 3D bioreactor culture. Their analysis involved performing Bulk RNA Sequencing to discover pathways that show significant changes. They found that the pathway involved in actin cytoskeleton showed significant results when comparing across 2D adherent culture and 3D suspension culture. It was found that actin cytoskeleton genes were downregulated in 3D culture when using 2D culture as the control group. This was done by analyzing RNA-seq data compared to IPA databases. This agrees with the results observed earlier through RT-qPCR and microarray data. From the RT-qPCR results, HES-2 were found to be

statistically significant and H9s were not. Based on this naïve H9 data, it is presumed that primed H9 cells would also be significant with further analysis and addition of samples. To conclude, it is hypothesized that majority of human PSC lines would show similar findings.

5.2.4 Summary of Gene Expression Findings

Overall, the gene expression results were found to be consistent across the various techniques (RT-qPCR, microarrays, RNA-seq) and across various types of 3D culture (microwells, spontaneous, hanging drop, etc.). They were also found to be significant in a variety of different cell types including common cell lines and stem cell lines. The cell types also included primary fibroblast cells, along with commercial cell lines. The RT-qPCR results for this data was reproducible and shown be similar across experiments that were prepared and performed by different people. In addition, the ability of this data to be shown through data collected by other groups such as publicly available microarray data and RNA-seq collected by another lab indicates the strength in reproducibility of this data. Furthermore, microarray data and RNA-seq data are reduce confirmation bias as they are performed on the full transcriptomics of the cell instead of being able to choose specific genes/primers in RT-qPCR. In addition, they were useful for studying gene expression changes as they do not require normalization to other genes.

This research fills a gap in the field because the role of cytoskeleton in different microenvironments has not been explored in human cell types. Even in mice studies, there is a lack of literature surrounding this topic. In Relevant Studies, a study which explored differentiation patterns of mouse embryonic stem cells (mESCs) grown in 2D or 3D was introduced. In a closer look into their results, they noted that both actin α 1 and 2 genes (*ACTA1, ACTA2*) were upregulated in 2D culture [91]. Moreover, they observed changes in *KRT8*, *LMNA*,

and *VIM* but not tubulin-α1b gene (*TUBA1B*). These results in mice correlate with the RT-qPCR results observed in human cell lines in this thesis. In most cell types, the intermediate filament genes show changes in gene expression but not microtubule genes such as tubulin (Figure 4-2 and Figure 4-3).

5.2.5 Main Takeaway from Gene Expression Studies

Overall, the main takeaway from this data is that β-actin is not a stable reference gene when comparing across different culture conditions, especially in the common cell lines and hPSCs. It is possible that β-actin could be used as a reference gene in some cancer cell lines. Since cancer cell lines are very de-regulated when it comes to β-actin expression, it would be suggested not to use β-actin when comparing across different types of cancer cell lines.

This further highlights the stable reference genes should be picked based upon the specific experimental conditions. It is not enough to rely on past literature for a reference gene, unless the literature explicitly takes in account the culture conditions and other experimental conditions/factors that will be specific to the experiment being performed. Publicly available microarray and RNA-seq databases are a useful resource that could be used to pick reference genes especially if the cell type and cell conditions match those used in previous research. ReceptoR is an easy-to-use tool as it allows users to search across multiple array data and automatically normalizes the data. Currently, many different free and straightforward methods exist to analyze reference genes in Microsoft Excel including GeNorm, BestKeeper, and Norm-Finder [17], [60], [64]. The drawback of performing these initial reference gene finding experiments could include time constraints and could be difficult in cases of limited RNA sample. However, if there is enough RNA available, the benefits of performing an RT-qPCR run to determine stable reference genes could outweigh the disadvantages. The wrong reference gene could lead to skewed results that future experiments could be based upon. Thus, it is important to pay attention to the reference genes and fluctuations in their C_T values and when possible, use more than one reference gene. In cases of limited amount of RNA, previous databases could be

utilized to search for a reference gene/protein based on the experiment. There is a rapid advancement in next-generation sequencing technologies, along with continuous improvement of better prediction methods [115]. With the improvement of technologies and addition of more data, machine learning approaches will become more accurate, allowing databases to become an important research tool [115].

5.3 Protein Expression

The protein expression of actin in the western blot correlates with the gene expression results. Figure 4-5 shows that ARPE-19 cell line upregulates actin proteins in 2D and cancer cell line, MCF7, does not. The immunocytochemistry results show the differences in actin expression across the different four cell lines (Figure 4-6). The morphology of the cell lines is quite different from one another and as the actin fibres seem to span the length of the cell, it could give insight into the cell type specific differences in expression of actin proteins in 2D vs 3D conditions. The current protein results could be improved by adding n values to perform statistical tests and studying the variation of other cytoskeletal proteins to see if they correlate with RT-qPCR results. Although more work needs to be performed, so far, the current data correlates with the RT-qPCR results and it can be theorized that similar results will be seen at the protein level for the rest of the cell types.

This is important because just as β-actin is used as a reference gene in gene expression studies, β-actin is used as a reference protein in western blot and other protein expression studies. As such, it may also indicate that β-actin is not a stable reference gene for protein studies relating to different culture conditions. This further demonstrates the point that reference genes/proteins should be picked based on the specific experimental conditions and

not based on previous research. Although there are protein databases and tools to perform analysis on previous data to determine a reference protein, they are not easily accessible. Since the protein result seem to correlate with the gene expression studies, it is possible that the tools used for picking a reference gene could be extrapolated towards a reference protein. This would need to be tested based on the specific proteins and the experiments being performed.

5.4 Mechanism

It is possible that the morphology of the cells in 2D conditions (Figure 4-1 and Figure 4-6) gives insight into their gene and protein expression results. In particular, if cells that spread out in 2D show drastic upregulation of β-actin in 2D. In contrast, cells with a cluster morphology in 2D do not show the upregulation of β-actin in 2D as they have less morphological changes going from 2D to 3D culture. This would be interesting to observe with additional experiments. The main hypothesis for the upregulation of cytoskeletal markers, particularly β-actin, in 2D culture is due to the stiffness of the substrate. As previously mentioned, the stiffness of the 2D adherent surfaces is unphysiological and in order of gigapascals [86]. Moreover, this supra-physiological stiffness led to formation of actin stress fibres in the cells. The stiffness experience in 3D culture is not due to the culture plates but rather due to cell-to-cell or cell-to-matrix connections. As illustrated in Figure 4-1B, the aggregates in the microwell have minimal contact with the surface and majority of their contact is with their surrounding cells. Moreover, 3D cell culture allows cells to secrete their own ECM and recreate their native environment. These cells experience the dimensionality and stiffness they would experience in the human body. This was illustrated in dermal papillae study where expression of cytoskeleton in 3D culture conditions was closely aligned to primary cells. Moreover, it is possible that some of the cell types are undergoing

mesenchymal-to-epithelial transition or some other sort of differentiation when they are relocated from 2D to 3D conditions.

CHAPTER SIX: FUTURE STUDIES AND CONCLUSION

6.1 Future Studies

6.1.1 Cell Culture

In future directions, additional human cell lines and primary human cell types could be added for a more comprehensive study. In addition, different species could be studied in order to see if they follow the same trend as humans. It can be hypothesized that these results can be seen across different mammalian species and possibly avian (bird) species due to the conservation of all six isoforms of actin and their 93% identical amino acid sequence across the different isoforms and species [37]. In fact, multiple isoactins have been found in non-vertebrates as well. For example, *Drosophila melanogaster*, the common fruit fly has both non-muscle and muscle specific actin isoforms [37]. The simplicity of *Drosophila* along with its fully sequenced genetics could make this organism a valuable model system for addressing the role of actin in an isoform specific fashion and changes in actins due to the microenvironment.

The mouse embryonic stem cells study seems to indicate that similar pattern would be seen in mice [91]. In addition, some colleagues in the Veterinary Medicine Department at University of Calgary who use the microwell system were looking at fibrosis in equine endometrial cells in 2D, 3D, and primary cells [116]. They observed similar results of overexpression of α-actin gene (*ACTA2*) in 2D adherent culture through RT-qPCR [116]. This further indicates that these results can be shown across different mammals. This also indicates that other actin isoforms will show a similar pattern in the cell types they are most commonly expressed in. As a quick check, microarray data analysis from mouse studies and other common

species used in research could be used to determine if this hypothesis holds true across species and across different actin isoforms.

In addition, different cell culture methods could be explored in detail. These could include diverse surfaces for 2D culture on different stiffnesses, plates made of various plastics, and coated with hydrogels. The various methods of 3D culture could also be expanded to include other methods such as bioreactor, embedding into hydrogels, etc. Further experiments could include measuring the size, shape, and stiffness of the cell in relation to the cell microenvironment in 2D, 3D, and different substrate stiffnesses. It would be hypothesized that the cells would become stiffer to reflect the substrate stiffness as discussed in literature review section. Based upon the current literature, it can also be hypothesized that β-actin is not a stable reference when comparing across different substrate stiffnesses, with stiffer substrates showing higher β-actin expression.

Note that the thickness of the matrix can be important as the cells can feel the rigid surface beyond the substrate. For example, mesenchymal stem cells grown on ~1 kPa gel were able to respond to the underlying rigid surface at about 10-20 μm thickness [117]. This thickness could vary depending on the cell type, stiffness of the substrate, etc. and should be taken in account. In addition, being able to measure the stiffness experienced by a cell in an aggregate would be very useful. Traction force microscopy has been used to measure tractional forces in 2D gels, but the need for a 3D equivalent was mentioned in [3]. There have been advancements in the 3D atomic force microscopy (AFM) that could be used in future studies [118]. In addition, AFM has been used to directly measure and visualize the cytoskeletal elements to give information about the surface density and amount of fibres [119].

Time course experiments could be performed to see how the cytoskeletal/actin markers change overtime in both 2D vs 3D culture. It is hypothesized that both cell conditions will upregulate their actin protein expression overtime as seen by the mouse ESC study. This is due to the higher amount of proliferation of the cells in 2D and the cells in 3D secreting more extracellular matrix (ECM) and actin proteins for stronger connections [91]. Previous time-course experiments have been shown that changes in ECM are very rapid, similar to the speed of cytoskeletal changes [120]. Being able to observe these changes at a shorter time scales would be very critical as it was shown that stress fibres in bone osteosarcoma cells retain their spreading memory for only ~50 minutes [120].

It would be important to perform more studies using primary cells and determine how cells in their native environment compare to 2D vs 3D culture. It would also be important to observe cells that are taken from the native environment directly to 3D culture without being first cultured in 2D conditions. It is hypothesized that even a short-term culture in 2D conditions can negatively alter the cells gene expression profile significantly. In the dermal papilla study, only 22% of the transcriptomic signature was restored by reverting from 2D to 3D conditions when compared to the primary cells [103]. In addition, it would be interesting to observe the changes and reversion of cytoskeletal and ECM when cells are cultured in 2D → 3D → 2D conditions. It would be hypothesized that the reverted 2D conditions would show similar results to the original 2D conditions, both being significantly different from the 3D conditions.

6.1.2 Gene Expression

In order to get absolute gene expression of *ACTB* gene, digital PCR can be performed through Translational Laboratories services at Tom Baker Cancer Centre. Digital PCR is

performed by taking a single sample and dividing into very small portions; so tiny that each partition contains either a single copy of target mRNA or none. Then, fluorescently labeled probes are used to measure the target in each partition similar to qPCR. Poisson statistical analysis is then used to determine the absolute quantification of target mRNA in the original sample. This is important as this measurement does not rely on a reference sample or standard. Thus, it can be used to give information to study the reference or standard samples themselves. [121]

Moreover, additional genes could be examined in addition to cytoskeletal ones. In order to see if this change is seen in other functionalities of the cell, bulk RNA sequencing across different cell types could be performed. In order to analyze if the cells are undergoing cell type transitions such as mesenchymal to epithelial transition, specific markers could be observed. Epithelial and mesenchymal cells can be distinguished from each other as they express different proteins. Epithelial cells express high levels of E-cadherin, whereas mesenchymal cells highly express fibronectin and vimentin [27]–[31]. Further microarray data could be further analyzed to determine other genes that change in relation to cytoskeletal genes. This could allow us to correlate cytoskeleton changes from RT-qPCR with other changes in the cell to give insight into the mechanism and pathways behind the cytoskeletal changes. Microarray data based on different substrate stiffnesses could allow being able to relate a specific stiffness to 3D culture based on the gene expression profile.

6.1.3 Protein Expression

It would be fascinating to see the cytoskeleton changes using live cell imaging from 2D to 3D. This could give information on their polymerization, assembly, and localization within the

cell. The localization could possibly give some indication into the function of the cytoskeleton at that timepoint. It would also be though-provoking to explore if the actin proteins upregulated in supra-physiologically stiff surfaces found in 2D culture are made up of actin stress fibers. This could help give insight into the negative drawbacks associated with common 2D culture techniques and further emphasize the use physiologically relevant culture systems.

6.1.4 Metabolomics

It would be interesting to observe the changes between 2D and 3D culture in their metabolomics profile and secreted factors. This could give further insight into the mechanism involved in the upregulation of actin. It could also shed insight into the differences in cell-to-cell signaling behaviour between different culture conditions.

6.2 Conclusion

This research provides important insight into the differences between 2D and 3D cultures and their impact on cytoskeletal genes/proteins. Overall, it has been found that actin genes are significantly upregulated in 2D culture compared to 3D culture across various cell types. This was shown by RT-qPCR, microarrays, and bulk RNA-Seq. In addition, β-actin was also shown to be upregulated in 2D culture using Western Blots. This research emphasizes the importance of selecting proper reference genes based on the experiment and highlights the instability of β-actin in different cell microenvironments. This could shed insight into previous wrong analyses where β-actin and other cytoskeleton markers were used for normalization between different culture conditions. For instance, this could explain discrepancies between RT-qPCR data and genomics/proteomics data within the same experiment. The impact of this research could be far-reaching as β-actin is used as a control in various research fields and in many different species across the animal kingdom.

Previous literature demonstrates that a stiff surface upregulates cytoskeletal proteins expression so the reason behind cytoskeletal upregulation in 2D culture could be due to the supraphysiological stiff surface. This could be impacting many downstream processes and key cell behaviour because cytoskeleton proteins are involved in many different functions of the cell. From a biological perspective, this research can be built upon to determine the mechanism behind these actin changes and their biological consequences. From a tissue engineering perspective, this would also indicate that the 3D culture techniques better represent the physiological environment inside the human body. This could improve bioengineering practices, possibly leading to more adaption of 3D culture in the future. Furthermore, less stiff 2D culture

substrates can be used. Research is constantly changing and adapting to new cell types, new techniques, methodologies, and culture conditions. As such, researchers should also change their approach to selecting controls and ensure that they take these new elements into consideration. Overall, this research shows that cytoskeletal proteins change significantly according to the cell microenvironment. This emphasizes accurate normalization practices and selecting stable references in various laboratory techniques, and the importance of implementing suitable culture and tissue engineering models.

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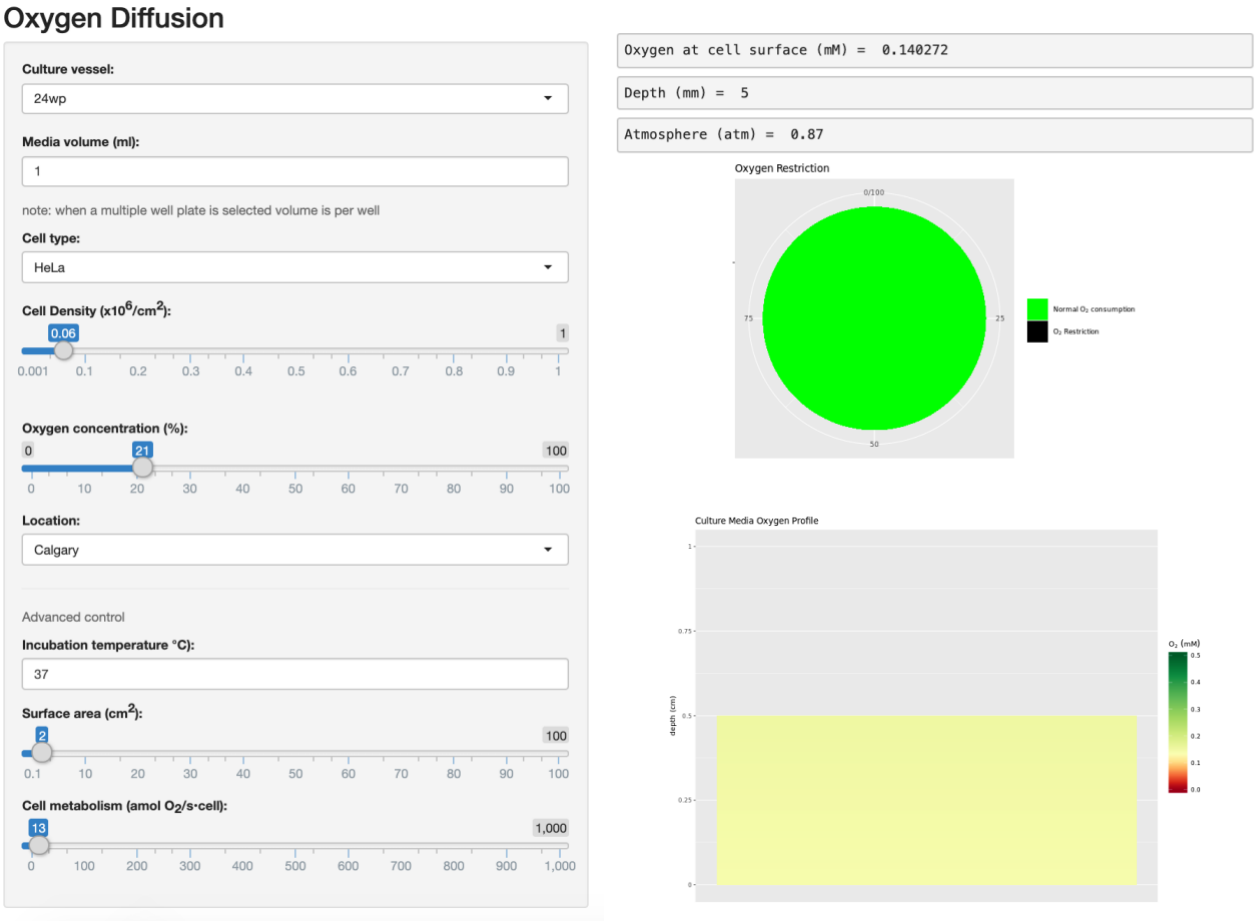
APPENDIX

Table S 1. Cell Line, Media, and Source Information

Cell Type Name	Cell Type Information	Cell Type Media	Source
ARPE-19	Adult Retinal Pigment Epithelial cell line-19	RPMI 1640 Media (ThermoFisher, Cat# 11879-020)	[122]
ES-RPE	Embryonic stem cell derived retinal pigment epithelium	RPMI 1640 Media (ThermoFisher, Cat# 11879-020)	Derived from hESCs using [123]–[125]
Fibroblast	Foreskin fibroblast cells	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	See 1 Below
MSCs	Mesenchymal stem cells (adipose derived)	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	See 2 Below
HeLa	Cervical cancer (Henrietta Lacks)	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	[126]
HEK293	Human embryonic kidney 293 cells	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	[106]
MCF7	Mammary gland epithelium (breast cancer) / Michigan Cancer Foundation-7	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	[127]
Ishikawa	Endometrial cancer	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	[128]
HT-29	Colon cancer	DMEM-F12/HEPES (Life Technologies, Cat#. 11330057) + 10% FB Essence (VWR Life Science Seradigm, Cat#. 10803-034)	[129]
HES-2	Embryonic stem cells	StemMACS iPS-Brew XF (Cat#. 130-104-368)	
H9	Embryonic stem cells	StemMACS iPS-Brew XF (Cat#. 130-104-368)	

1. Fibroblast Cells Source: human skin fibroblast cells were isolated by the Biernaskie Laboratory at the University of Calgary (Ethics file ID: AC14-0019) from a scalp skin sample taken from 58-year-old female patient (159S). The full details about the isolation of skin-derived precursors can be found in isolation protocol published by Biernaskie Lab [130]. A vial of cryopreserved passage 3 (P3) skin fibroblast cells was further expanded the cells in culture. Cells were used at a low passage number (P4 – P7) to ensure the quality and phenotype remains consistent.
2. MSC Source: human adipose-derived mesenchymal stem cells (MSC) (Donor 8) were ethically isolated from abdominal subcutaneous adipose tissue by the Sen laboratory at the University of Calgary (Ethics file ID: REB15-1005). The procedure can be found in [131]. A vial of cryopreserved MSC passage (P4) was obtained from Sen laboratory and expanded in culture.



S 1. Oxygen Consumption and Diffusion of HeLa cells in a 24-well adherent culture plate (2D). This result was generated using the link http://136.159.176.176:3838/o2/. The parameters of cell culture can be added on the left side of the page. This leads to the results on the right side to give calculation for oxygen at cell culture surface, and graphical representations of oxygen restriction and media oxygen profile. Conditions include Culture Vessel: 24 well plate (TC or microwell), Media volume/well: 1 mL, Cell Type: HeLa, Cell density: 120,000/2 cm^2 = 60,000 (input 0.06), Oxygen concentration: 21%, Location: Calgary, Incubation temperature: 37°C, Surface area: 2 cm^2, Cell metabolism (amol O2/s•cell): 13. Please note that cell metabolism will change depending on cell type. For this thesis, all other conditions remain the same across both 2D and 3D cell culture.

Table S 2. Oxygen at Cell Culture Calculated for Each Cell Line

Cell Line Name	Cell metabolism (amol O_2/s•cell)	Predicted oxygen at culture surface (mM)	β-actin expression in 2D Cell Culture in Comparison to 3D Cell Culture	Source for Cell Metabolic Rate
ARPE-19	42	0.108	Significantly upregulated	[132]
ES-RPE	42	0.108	Significantly upregulated	[132]
Fibroblast	18	0.133	Significantly upregulated	[133], [134]
MSCs	25	0.128	No significant results	[135]
HeLa	12.5	0.140	Possibly upregulated	[136]
HEK293	25	0.128	No significant results	Based on murine kidney duct cells in [137]
MCF7	32.5	0.120	No significant results	[138]
Ishikawa	45.3	0.108	No significant results	Based on prostate adenocarcinoma PC-3 in [139]
HT-29	27	0.125	No significant results	Based on similar cell line A549 in [140]
HES-2	40	0.112	Significantly upregulated	Based on murine ESCs in [141]
H9	40	0.112	Possibly upregulated	Based on murine ESCs in [141]

Table S 3. Primer Sequences for Genes

Gene	Forward Primer	Reverse Primer	Full Info
ACTA	CACGATGTACCCTGGGATCG	GCCGATCCACACCGAGTATT	P1
ACTB	AGAGCCTCGCCTTTGCCGATCC	CACATGCCGGAGCCGTTGTCG	P2
ACTR2	ATGTATCCTGGCCTGCCATC	CTAGAACTGCACCACCCAGG	P3
ACTR3	CTGTGGCACGGGGTATACAA	ACCCACTTTTGCTGACTCCT	P4
FN1	TTGCTCCTGCACATGCTTTG	CATGAAGCACTCAATTGGGCA	P5
KRT8	ATGTTTGCGGAATGAATGGGG	AGAGGTGGACACCTTGTAGGA	P6
KRT18	TGGAGAGCAAAATCCGGGAG	TGGCCAGCTCTGTCTCATAC	P7
LMNA	GACTCAGTAGCCAAGGAGCG	GCAGCTATCAGGTCACCCTC	P8
TUBB	ATCCAGAGCAGGGAAAGCTG	CTCAGGCCGTTGTTCTAGGG	P9
VIM	GGACCAGCTAACCAACGACA	AAGGTCAAGACGTGCCAGAG	P10
EIF2B1	AGGGTCTGAGGGCGAATCTC	CAGGGAGGCAAGACTGATGAA	
G6PD	AGGCCGTCACCAAGAACATTCA	CGATGATGCGGTTCCAGCCTAT	
POLR2A	GCGGAATGGAAGCACGTTAAT	CCCAGCACAAAACACTCCTC	
PPIA	GCCGAGGAAAACCGTGTACT	CTGCAAACAGCTCAAAGGAGAC	
TBP	CAACAGCCTGCCACCTTACGCTC	AGGCTGTGGGGTCAGTCCAGTG	
UBC	CTGGAAGATGGTCGTACCCTG	GGTCTTGCCAGTGAGTGTCT	
YWHAZ	TGATCCCCAATGCTTCACAAG	GCCAAGTAACGGTAGTAATCTCC	

List of Full Information for Cytoskeleton/ECM Related Primers: Page Number

Primer-BLAST» JOB ID:joRR3siexTbiCN8N0m37P6h26g2FZfEQhA

Primer-BLAST Results

[?]

Input PCR template

NM_001100.3 Homo sapiens actin, alpha 1, skeletal muscle (ACTA1), mRNA

Range

1 - 1509

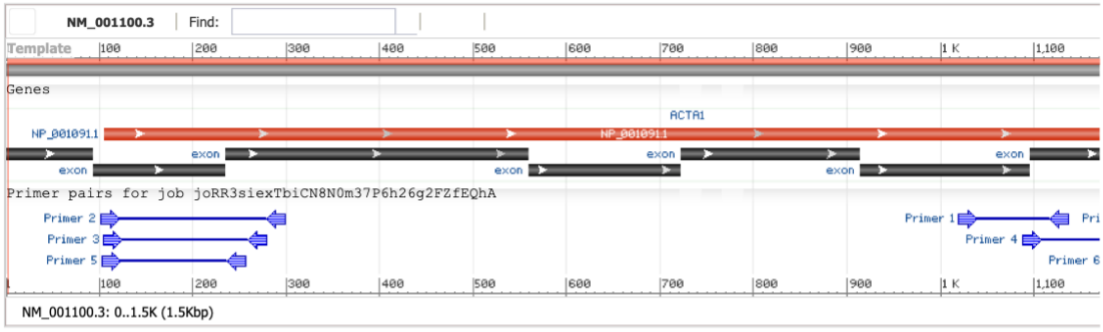
Specificity of primers

Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports

Search Summary

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

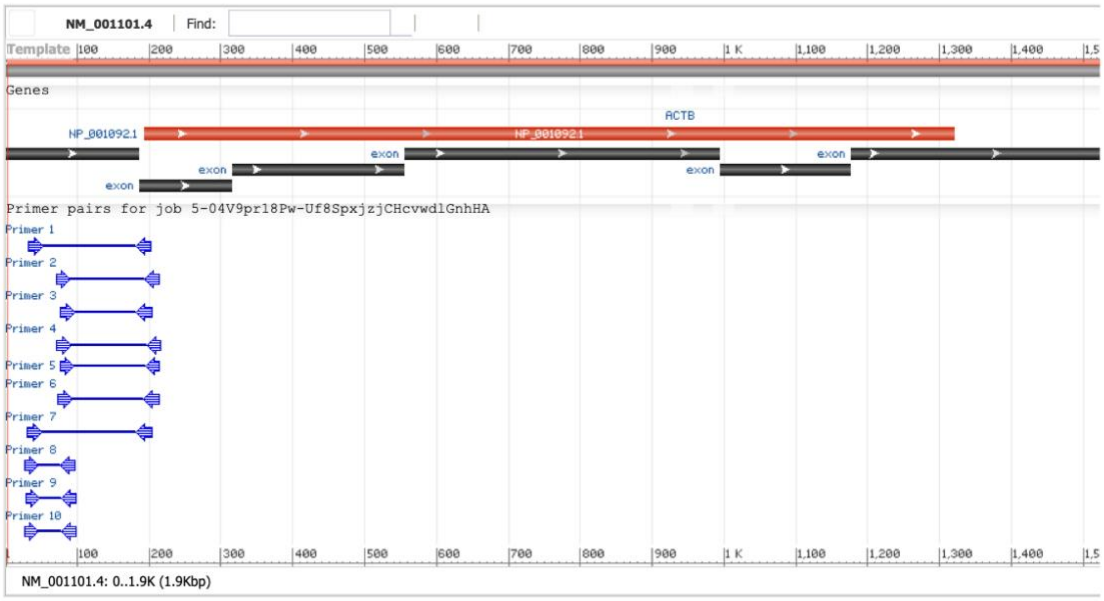
	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CACGATGTACCCTGGGATCG	Plus	20	1020	1039	59.97	60.00	5.00	2.00
Reverse primer	GCCGATCCACACCGAGTATT	Minus	20	1137	1118	59.90	55.00	4.00	2.00

P 1. ACTA Primer BLAST Information from NCBI

Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=5-04V9pr18Pw-Uf8SpxjzjCHcvwdlGnhHA more...

Input PCR template
NM_001101.4 Homo sapiens actin beta (ACTB), mRNA
Range
1 - 1940
Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CCGTTCCGAAAGTTGCCTTTT	Plus	21	32	52	59.93	47.62	5.00	2.00
Reverse primer	ATCATCCATGGTGAGCTGGC	Minus	20	201	182	60.18	55.00	8.00	2.00
Internal oligo		Plus							
Product length	170								
Product									

P 2. ACTB Primer BLAST Information from NCBI

Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=6OI3WNVr2MP_-Uj8RZxszj-HffwSlGbhEw more...

Input PCR template
NM_001005386.2 Homo sapiens ARP2 actin related protein 2 homolog (ACTR2), transcript variant 1, mRNA
Range
1 - 3944
Specificity of primers
primers may **not** be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers
Other reports
Search Summary

Graphical view of primer pairs

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATGTATCCTGGCCTGCCATC	Plus	20	1155	1174	59.60	55.00	6.00	2.00
Reverse primer	CTAGAACTGCACCACCCAGG	Minus	20	1314	1295	60.04	60.00	4.00	3.00
Internal oligo		Plus							
Product length	160								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									

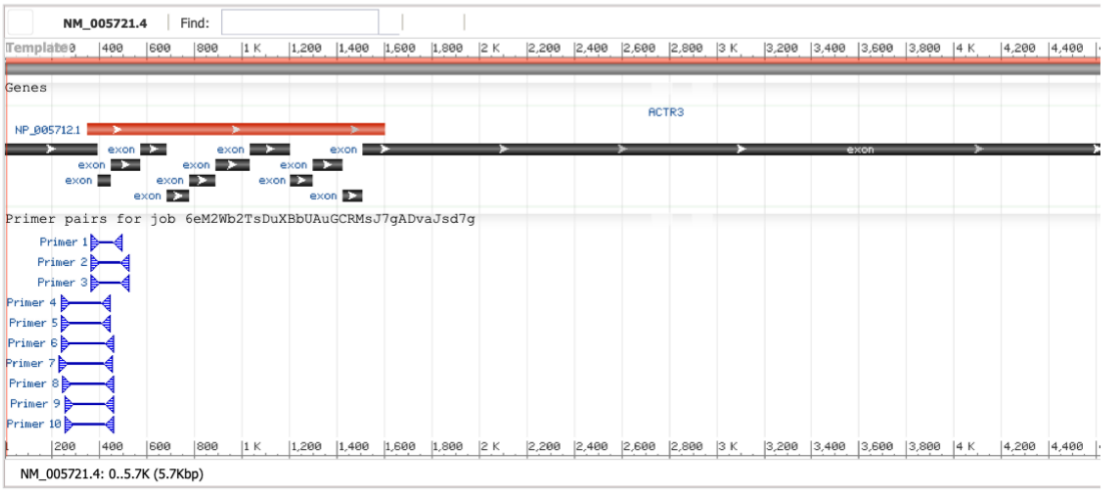
Total intron 3388 (between pos. 65265175 and 65268564 on NC_000002.12)

P 3. ACTR2 Primer BLAST Information from NCBI

Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=6eM2Wb2TsDuXBbUAuGCRMsJ7gADvaJsd7g more...

Input PCR template
NM_005721.4 Homo sapiens ARP3 actin related protein 3 homolog (ACTR3), transcript variant 1, mRNA
Range
1 - 5708
Specificity of primers
Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTGTGGCACGGGGTATACAA	Plus	20	379	398	59.75	55.00	6.00	2.00
Reverse primer	ACCCACTTTTGCTGACTCCT	Minus	20	478	459	59.15	50.00	3.00	0.00
Internal oligo		Plus							
Product length	100								
Product Tm									
Product Tm - min(OLIGO Tm)									
Exon junction									
Total intron size	3656 (between pos. 113913227 and 113916884 on NC_000002.12)								

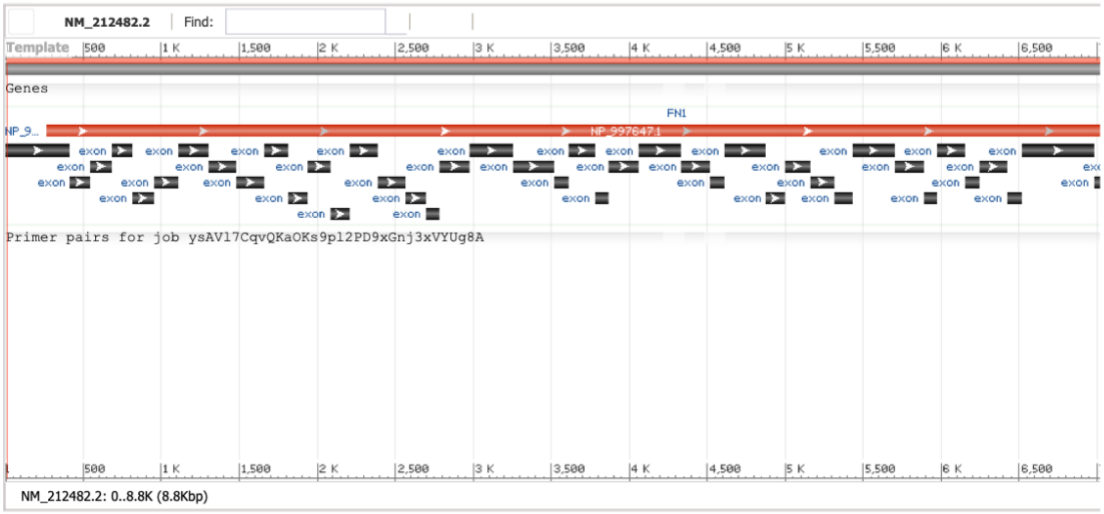
Products on intended target

P 4. ACTR3 Primer BLAST Information from NCBI

Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=ysAVl7CqvQKaOKs9pl2PD9xGnj3xVYUg8A more...

Input PCR template
NM_212482.2 Homo sapiens fibronectin 1 (FN1), transcript variant 1, mRNA
Range
1 - 8833
Specificity of primers
primers may **not** be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers
Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTGCTCCTGCACATGCTTTG	Plus	20	7487	7506	59.68	50.00	4.00	2.00
Reverse primer	CATGAAGCACTCAATTGGGCA	Minus	21	7658	7638	59.45	47.62	6.00	2.00
Internal oligo		Plus							
Product length	172								
Product Tm									
Product Tm - min(OLIGO Tm)									

P 5. FN1 Primer BLAST Information from NCBI

Primer-BLAST» JOB ID:mpBFxWshZolBs3C2fdZUhAfNRbYq3l6rKw

Primer-BLAST Results

[?]

Input PCR template

NM_001256282.1 Homo sapiens keratin 8 (KRT8), transcript variant 1, mRNA

Range

1 - 1807

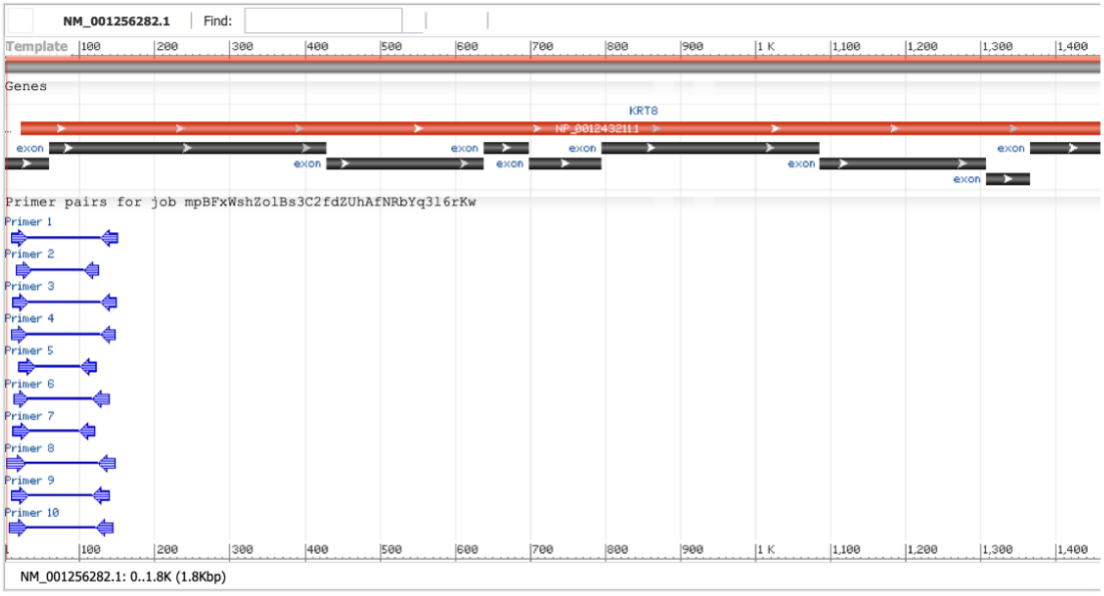
Specificity of primers

Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)

Other reports

Search Summary

Graphical view of primer pairs



Detailed primer reports

P 6. KRT8 Primer BLAST Information from NCBI

Primer-BLAST» JOB ID:9_0oRSQUKbwOgiyHIecItVv8GYd27wKadw

Primer-BLAST Results

[?]

Input PCR template

NM_000224.2 Homo sapiens keratin 18 (KRT18), transcript variant 1, mRNA

Range

1 - 1485

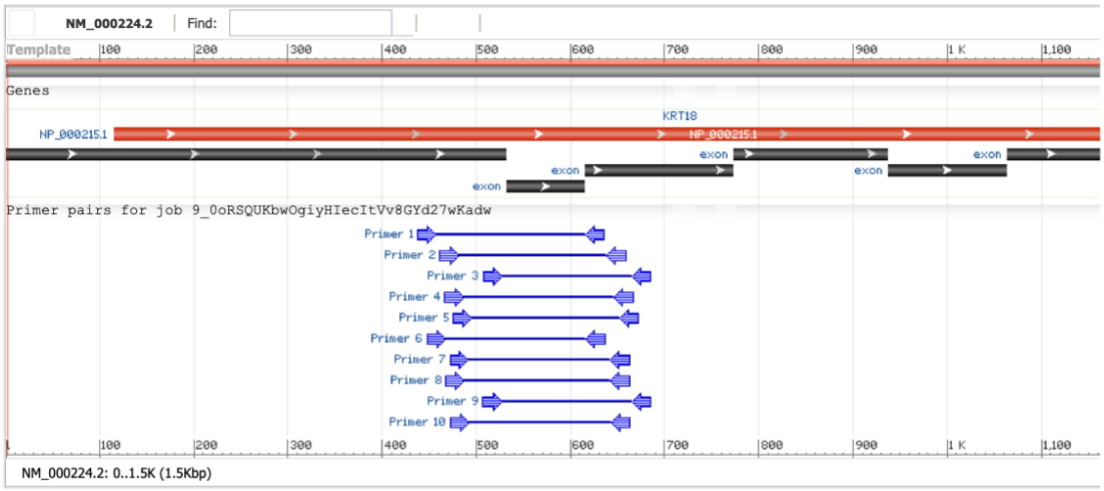
Specificity of primers

primers may **not** be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers

Other reports

Search Summary

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

P 7. KRT18 Primer BLAST Information from NCBI

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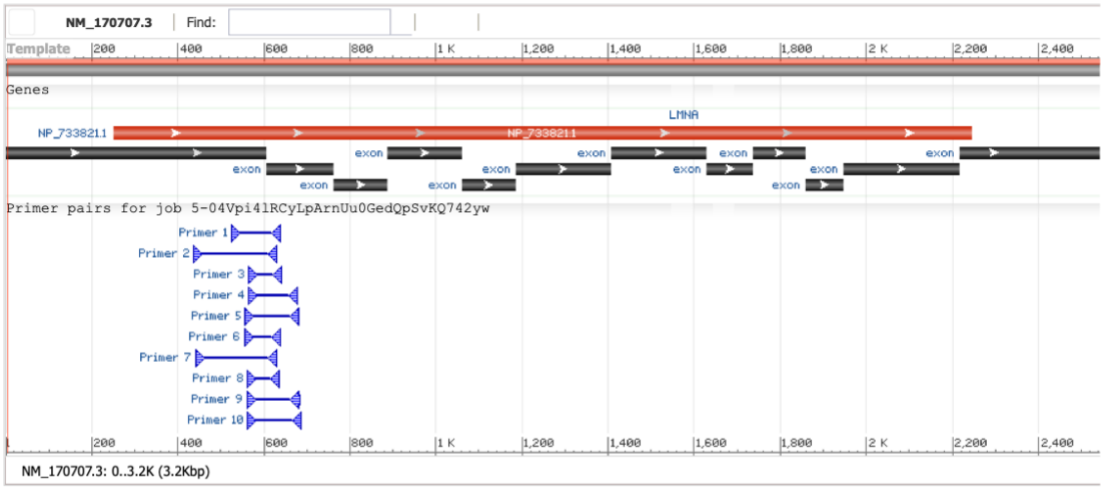
Primer-BLAST» JOB ID:5-04Vpi4lRCyLpArnUu0GedQpSvKQ742yw
Primer-BLAST Results
[?]

Input PCR template
NM_170707.3 Homo sapiens lamin A/C (LMNA), transcript variant 1, mRNA
Range
1 - 3239
Specificity of primers
primers may **not** be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers
Other reports
Search Summary

Graphical view of primer pairs



Detailed primer reports

Primer pair 1

P 8. LMNA Primer BLAST Information from NCBI

Primer-BLAST Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=m5FExuGq7ALLOPo9913eD41Gzz2gVdQgoQ more...

Input PCR template
NM_001293212.1 Homo sapiens tubulin beta class I (TUBB), transcript variant 1, mRNA
Range
1 - 2772
Specificity of primers
primers may **not** be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers
Other reports
Search Summary

Graphical view of primer pairs

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATCCAGAGCAGGGAAAGCTG	Plus	20	214	233	59.74	55.00	4.00	2.00
Reverse primer	CTCAGGCCGTTGTTCTAGGG	Minus	20	314	295	60.11	60.00	4.00	2.00
Internal oligo		Plus							
Product length	101								
Product Tm									
Product									

P 9. TUBB Primer BLAST Information from NCBI

Primer-BLAST» JOB ID:-PInSkboS0Bsett_1h__TawE7n-BF_VigA

Primer-BLAST Results

[?]

Input PCR template

NM_003380.4 Homo sapiens vimentin (VIM), mRNA

Range

1 - 2195

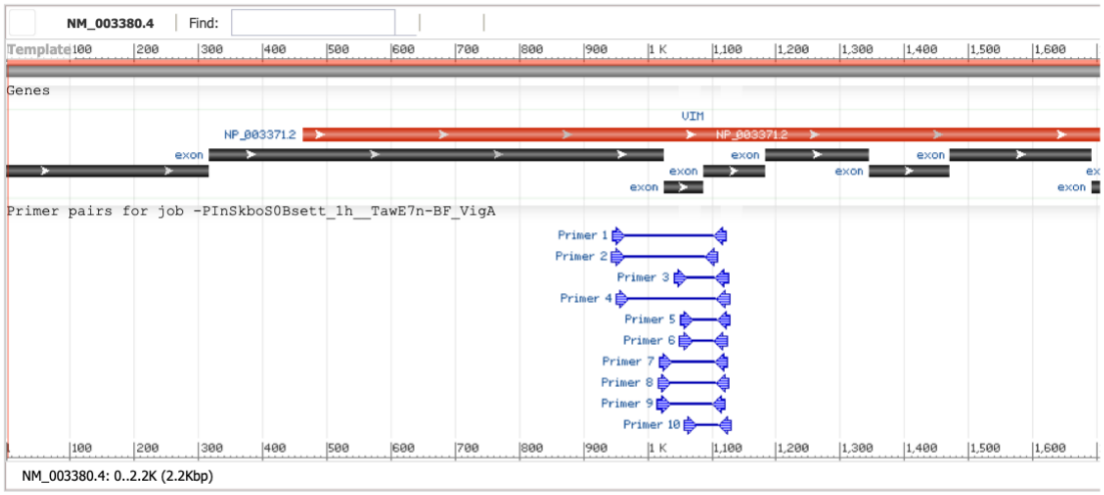
Specificity of primers

primers may **not** be specific to the input PCR template as targets were found in selected database:Refseq mRNA (Organism limited to Homo sapiens)...help on specific primers

Other reports

Search Summary

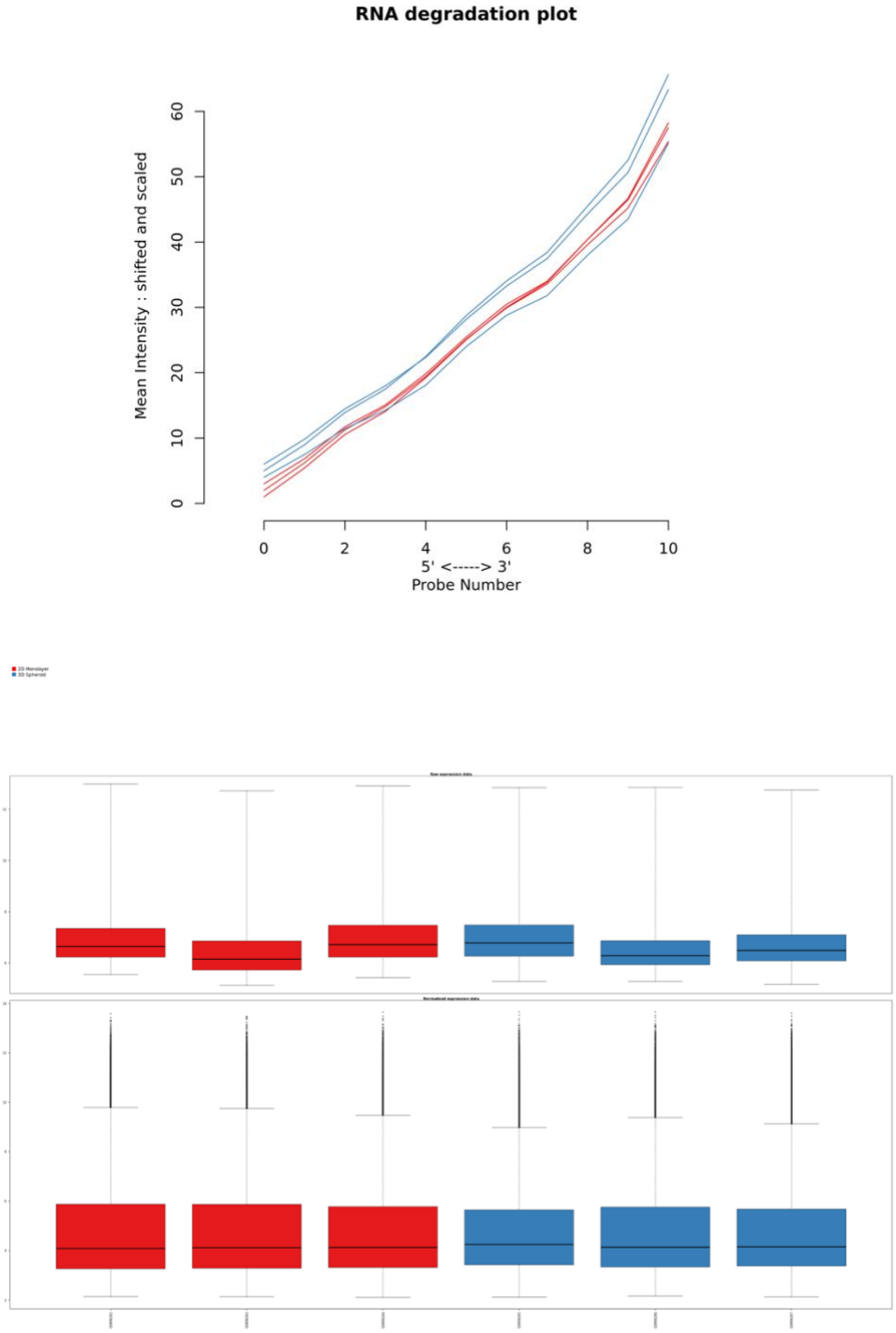
Graphical view of primer pairs



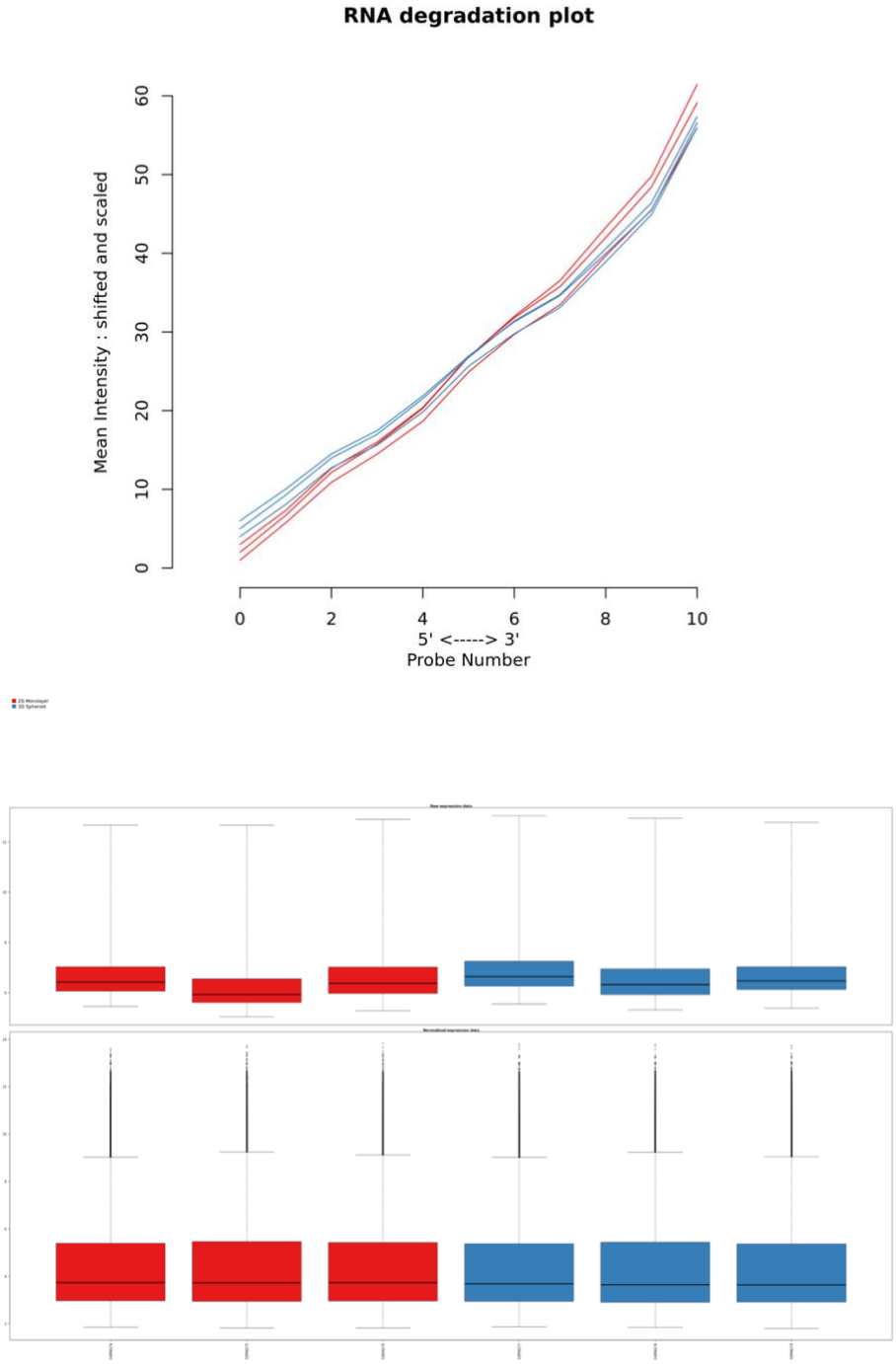
Detailed primer reports

Primer pair 1

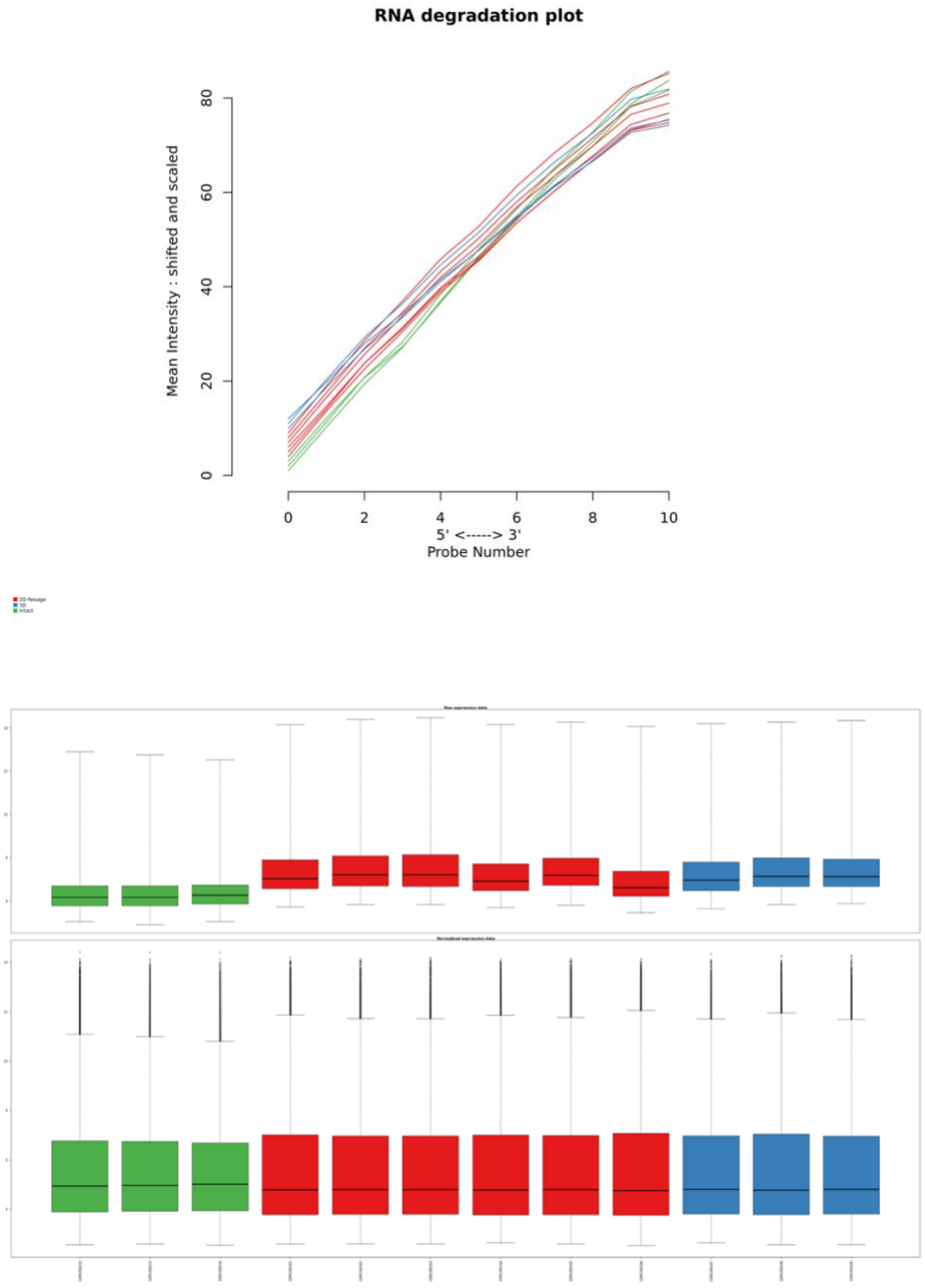
P 10. VIM Primer BLAST Information from NCBI



S 2. Quality Control of Fibroblast (HFF-2) Microarray Data. A. RNA Degradation Plot. B. Expression Normalization of array intensity. Top shows before and bottom shows after. The red are samples in 2D conditions and blue in 3D conditions. Both graphs show that the samples are consistent between the groups and biological replicates. These graphs were generated in ReceptoR.



S 3. Quality Control of Cancer Cell (T98G) Microarray Data. A. RNA Degradation Plot. B. Expression Normalization of array intensity. Top shows before and bottom shows after. The red are samples in 2D conditions and blue are samples in 3D conditions. Both graphs show that the samples are consistent between the groups and biological replicates. These graphs were generated in ReceptoR.



S 4. Quality Control of Dermal Papilla Microarray Data. A. RNA Degradation Plot. B. Expression Normalization of array intensity. Top shows before and bottom shows after. The green are primary samples, red are samples in 2D conditions, and blue are samples in 3D conditions. Both graphs show that the samples are consistent across the groups and biological replicates. The native samples seem to be less intensity in the above (before experiment) graph which could be due to lower amounts of RNA extracted. These graphs were generated in ReceptoR

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v 1.1

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