AMERICAN UNIVERSITY OF BEIRUT

ENHANCING MIR-183-5P ABUNDANCE IN RODENT MAMMARY EPITHELIAL CELLS: EFFECT ON DIFFERENTIATION

by
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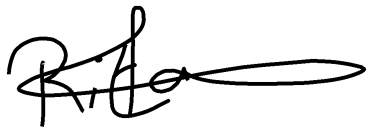

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ABSTRACT
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Title: Enhancing miR-183-5p Abundance in Rodent Mammary Epithelial Cells: Effect on Differentiation

Breast cancer is the most common type of cancer occurring in women worldwide. Many cellular mediators contribute to breast cancer initiation and progression, among those are microRNAs. microRNAs (miRNAs) are a class of single stranded RNA molecules that control important cell functions including differentiation, cell growth, apoptosis, and migration through post- transcriptional gene silencing. Panels of miRNAs are dysregulated in multiple cancer types including breast cancer. In breast cancer, miR-183-5p is among the overexpressed miRNAs in both ductal carcinomas in situ (DCIS) and lobular neoplasia subtypes. Previous reports correlated miR-183-5p overexpression to loss of epithelial cell polarity and enhanced migration and proliferation of breast epithelial cells of ductular origin. Despite being identified as overexpressed in lobular carcinoma, the mechanisms by which miR-183-5p mediate lobular cancer initiation remain uninvestigated. Knowing that differentiation and cancer initiation are fundamentally opposite processes, and that the common cellular factors contribute to both processes, we aimed to determine the effect of miR-183-5p on tumor initiation in a model that specifically recapitulates mammary epithelial differentiation in vitro. To investigate the role of miR-183-5p on loss of differentiation and tumor initiation in lobular mouse mammary epithelial SCp2 cells, we sought to trigger miR-183-5p abundance in SCp2 cells and monitor its effect on cell-cell/ cell-ECM-mediated differentiation through the expression of the differentiation marker β-casein.

Virally infecting SCp2 epithelial and SCg6 myoepithelia-like cells with miR-183-5p expression vector under the control of CMV promoter resulted in low transduction efficiency and diminished expression of miR-183-5p. Two alternative approaches were therefore, adopted to trigger miR-183-5p abundance in SCp2 cells. The first was treating SCp2 cells with conditioned media derived from miR-183-5p-infected S1 epithelial cells. The results suggested that miR-183-5p is elevated in SCp2 cells treated with conditioned media deriving from S1-miR-183-5p infected cells. However, inhibitors-possibly sodium selenite- within the conditioned media resulted in the abolishment of β-casein expression independent from miR-183-5p. An alternative solution was treating SCp2 with exosome extracts isolated from the conditioned media.

The exosomal extracts deriving from miR-183-5p-S1 conditioned media had the highest miR-183-5p levels compared to controls. Moreover, a higher level of miR-183-5p was detected in SCp2 cells after treatment with the aforementioned exosome extracts.

Further experiments are needed to assess the effect of exosome treatment on differentiation and β-casein expression. The third approach aimed to increase miR-183-5p in SCp2 by treating the cells with the potential carcinogen Glyphosate. Preliminary results suggest that Glyphosate treatment (10^{-11}M) increases in miR-183-5p expression in SCp2 cells. On the other hand, β-casein expression was diminished in SCp2 after being treated with 10^{-11} M glyphosates.

In conclusion, we suggest that human CMV promoter efficiency is reduced in mouse derived SCp2 cells. Moreover, miR-183-5p could be released into S1-culture conditioned media and transferred to target SCp2 cells through exosomes. Finally,

Glyphosate treatment induced the overexpression of miR-183-5p and culminated with the downregulation of β-casein expression and the loss of normal mammary epithelial differentiation in SCp2 cells despite being cultured in differentiation permissive conditions. Overall, to increase miR-183-5p in SCp2 and SCg6 cells we suggest virally infecting SCp2 and SCg6 with a viral vector suitable for mouse mammary epithelial cells. Alternatively, exosomes could also be purified from pre-tumorigenic Cx43 knockout cells expressing high miR-183-5p levels. We also propose that glyphosate treatment could be downregulating β-Casein expression and imposing other tumor-initiating, or cytotoxic effect on SCp2.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 1

ABSTRACT 2

ILLUSTRATIONS 7

TABLES 11

ABBREVIATIONS 12

INTRODUCTION 14

Literature Review 18

A. Mammary gland development and differentiation: 18

B. An Overview of Breast Cancer: 22

C. The Role of microRNAs in Differentiation and Cancer: 24

D. miR-183 in Breast Cancer: 27

E. Exosomes-Mediated Intercellular Communication: 29

F. Glyphosates and Breast Cancer: 32

MATERIALS AND METHODS 35

A. Cell Culture 35

B. Drug selection killing curve 36

C. Lentiviral Infection 36

D. Quantifying cell viability after Puromycin treatment 37

E. Total RNA isolation and quality control 37

F. miRNA expression by quantitative real time-polymerase chain reaction (RT-qPCR) 37

G. Reverse transcription qualitative Real time PCR (qRT-PCR) for β-Casein 38

H. Proliferation Assay 39

I. Scratch Assay 39

J. Isolation of exosome extracts from conditioned media 40

RESULTS 41

A. SCp2 and SCg6 cells express low levels of miR-183-5p upon viral transduction using a vector carrying a human-derived CMV promoter 41

B. SCp2 treated with culture conditioned media previously harvested from miR-183-5p infected S1 cells express high levels of miR-183-5p 47

C. Treatment with S1 conditioned media does not induce increase in proliferation in SCp2 cells 48

D. Treatment with S1 culture conditioned media does not impose any difference in cell migration on SCp2 cells 50

E. Additives of conditioned media inhibit the expression of β-Casein by SCp2 cells in EHS drip conditions 51

F. miR-183-5p is upregulated in exosome-containing extracts of conditioned media collected from S1-miR-183-5p infected cells 55

G. SCp2 show higher expression of miR-183-5p after treatment with exosome extracts from S1-miR-183-5p conditioned media 57

H. Triggering miR-183-5p overexpression by treating SCp2 cells with glyphosate (potential carcinogen) 58

I. miR-183-5p has 188 experimentally validated target genes in humans, and 17 experimentally identified targets in mice 60

DISCUSSION 63

CONCLUSION 71

REFERENCES OR BIBLIOGRAPHY 73

ILLUSTRATIONS

Figure

1. Mechanisms dictating β-Casein expression. SCp2 express β-Casein in response to prolactin signaling in combination with either an exogenously provided basement membrane, or when co-cultured with SCg6 in a GJIC dependent manner. 21

2. 1.5 μg/mL Puromycin concentration was the lowest concentration inducing significant cell death. Drug selection killing curves for SCp2 and SCg6 cells were carried out using varying concentrations of the antibiotic Puromycin, observed under a phase contrast microscope at 10X magnification. SCp2 and SCg6 cells were treated with Puromycin at concentrations 0.75 μg/mL, 1 μg/mL, 1.5 μg/mL, 2 μg/mL, 3 μg/mL. All concentrations showed some level of cytotoxicity after three days of selection, but the lowest concentration that showed significant cell death was 1.5 μg/mL. Thus, to avoid inducing cytotoxicity while ensuring successful drug selection post infection, the concentration 1.5 μg/mL was chosen to carry out the drug selection step. 42

3. Infected SCp2 and SCg6 cells showed low GFP fluorescence despite continuous resistance against Puromycin treatment. Infected- SCp2 and SCg6 cells were monitored at day 1, day 4, and day 6 post selection, and observed under a fluorescent microscope at 10X magnification. Compared to the total number of cells present in each culture plate, the proportion of miR-183-5p and miR-control infected SCp2 and SCg6 cells showing green fluorescence was low. This is reflective of the amount of GFP being expressed by the infected cells. However, the number of SCp2 and SCg6 cells kept increasing from day 1 to day 6 despite continuous Puromycin treatment, which shows that the cells were successfully infected and were expressing the Puromycin resistance gene..... 43

4. Infected SCp2 and SCg6 cells show minimal cell death compared to their uninfected counterparts after Puromycin treatment.A drug selection killing curve was established with higher Puromycin concentrations (2 μg/mL, 4 μg/mL,6 μg/mL) and monitored under a phase contrast microscope at 10X magnification. A high level of cell death was observed in uninfected SCp2 and SCg6 cells upon treatment with at least 2 μg/mL Puromycin concentration. On the other hand, infected SCp2 and SCg6 cells showed significant increase in cell number even after being treated with 6 μg/mL Puromycin while still showing low green GFP fluorescence (not shown). 45

5. Quantification of the number of cells surviving after treatment with Puromycin at 6 μg/mL concentration.The number of surviving uninfected SCp2 cells dropped to zero after the first day of Puromycin treatment. Infected SCp2 cells remained viable and proliferated until the cells became confluent and a plateau was reached. Similarly, the number of viable uninfected SCg6 cells kept decreasing to reach zero viability at day 3 post Puromycin treatment. The number of infected SCg6 cells kept increasing until full confluence was reached. 45

6. Infected cells express low levels of miR-183-5p as shown by RT-qPCR after infection.Fold change in normalized miR-183-5p expression in infected SCp2 (P2) and SCg6 (G6) cells. The graphs were plotted in excel and bars represent technical duplicate analysis of ± SEM. 46

7. SCp2 (P2) treated with miR-183-5p-S1 conditioned media show elevated miR-183-5p expression compared to controls.Fold change in miR-183-5p expression was assessed between SCp2 in growth medium (GM), and SCp2 treated with culture conditioned medium (CCM) collected from S1-uninfected cells, S1-miR-183-5p infected cells, and S1-miR-control infected cells. miR-183-5p expression was highest in SCp2 treated with CCM from S1-miR-183-5p infected cells compared to those treated with S1-CCM and S1-miR-control-CCM, and to those in GM. Bars represent technical duplicate analysis of ± SEM. 48

8. No significant difference in SCp2 proliferation was detected upon treatment with S1 culture conditioned media.SCp2 cells were first starved then kept in growth media or starvation media, or treated with either fresh H14 (S1 growth media), culture conditioned media (CCM) collected from S1 uninfected cells (S1 CCM), or from miR-183/miR-control infected S1 cells (S1-miR-183 CCM and S1 miR-control CCM respectively). After 24 and 72 hours, SCp2 cells showed a slight increase in cell number upon treatment with growth media compared to starvation conditions. The increase in SCp2 cell number after 48 and 72 hours of CCM-treatment was not significant in all treatment conditions as compared to starvation conditions. The experiment was repeated three times using different batches of cells. One-way ANOVA was used for statistical analysis of the difference between the aforementioned treatments. Bars represent triplicate analysis of ± SEM. 49

9. **A)** Treatment with S1 conditioned media has no significant effect on SCp2 cell migration.SCp2 cells show no enhanced migration after treatment with conditioned media collected from S1-uninfected, S1-infected with miR-183-5p, S1 infected with miR-control as compared with controls of SCp2 not treated with conditioned media as assessed by wound healing assay. **B)** Cell migration was measured as the percentage of cells covering the wound area after 24 and 48 hours post wounding. The experiment was repeated three times. One-way ANOVA in Graph pad prism v.8.4 was used for statistical testing of the differences between the conditions. Bars represent triplicate analysis of ± SEM. 51

10. Conditioned media treatment disrupts β-Casein expression in SCp2 despite being induced to differentiate by EHS drip. 52

11. Sodium Selenite treatment leads to down regulation of β-Casein expression in SCp2 on drip.β-estradiol, Apo-transferrin, and Sodium selenite are cell culture media additives supplemented in S1 media for optimal cell growth, and therefore are present in the conditioned media used to treat SCp2. It was suspected that one of those additives could be responsible for blocking β-Casein expression by SCp2 cells as previously observed. To determine which of those additives is influencing β-Casein expression, SCp2 in differentiation media was supplemented with each additive alone on drip and the fold change in β-Casein

expression was quantified by RT-qPCR. Although not significantly shown, SCp2 supplemented with either one of the additives show reduced β-casein expression compared to SCp2 in differentiation media on Drip without additives supplementation (DM drip), however, sodium selenite treatement showed the most decrease in β-Casein expression as compared to the non-treated control. Statistical analysis was done using one-way ANOVA with multiple comparisons via GraphPad prism v8.4 software. This experiment was repeated twice and error bars were plotted to represent duplicate analysis of ± SEM. 54

12. miR-183-5p level is elevated in exosome extracts collected from conditioned media collected from miR-183-5p-infected-S1 cells.Exosome extracts were obtained by differential ultracentrifugation carried out on conditioned media of infected and uninfected S1 cells. RT-qPCR was done to assess the levels of miR-183-5p in those extracts. The results revealed an elevated level of miR-183-5p in exosome extracts from S1-miR-183-5p conditioned media but not in those deriving from miR-control and uninfected S1 conditioned media. The graph was plotted via GraphPad prism v8.4 software. This preliminary experiment was done once and error bars were plotted to represent duplicate analysis of ± SEM. 56

13. miR-183-5p levels are highest in SCp2 treated with miR-183-5p derived exosome extracts compared to exosomes from uninfected S1, and miR-control-infected S1 conditioned media.SCp2 treated with miR-183-5p derived exosomes show an approximate 200-fold increase in miR-183-5p compared to those treated with exosome extracts from uninfected S1, or miR-control-infected conditioned media. The graph was plotted via GraphPad prism v8.4 software. This preliminary experiment was done once and error bars were plotted to represent duplicate analysis of ± SEM. 58

14. **A)** Treatment of SCp2 with 10-11 M glyphosate triggers elevated miR-183-5p expression, and **B)** hinders β-Casein expression in SCp2 induced to differentiate by EHS drip.SCp2 cells were treated with glyphosate for 21 days before being cultured with EHS matrix for four days to induce their differentiation. miR-183-5p expression increased under differentiation permissive conditions when the cells were treated with glyphosate 10^{-11}M showing an approximate 2-fold increase in expression compared to untreated controls. β-Casein expression in SCp2 on drip was diminished upon treatment with glyphosate as compared to the untreated positive control. As a negative control for drip in both experiments, SCp2 cells were seeded on plastic and supplied by non-differentiation media (NDM) lacking prolactin (not shown). The graphs were plotted via GraphPad prism v8.4 software. This preliminary experiment was done once and error bars were plotted to represent duplicate analysis of the SEM. 60

15. **A)** 188 experimentally validated targets of miR-183-5p were identified in various tissue in humans.**B)** 17 targets interact with miR-183-5p in breast cancer cell lines MDA-MB-231.**C)** In mouse, miR-183-5p interacts with 9 targets in different tissue. **D)** According to the bioinformatics tool TragetScan, β-1 integrin

-a mjor contributor to β-Casein expression- is a predicted target for miR-183-5p. 62

TABLES

Table

1. Real time qPCR primers with their relatives forward and reverse sequences. ... 39

ABBREVIATIONS

miRNA: microRNA
SC: Stem cell
EMT: Epithelial to mesenchymal transition
ILC: Invasive lobular carcinoma
LCIS: Lobular carcinoma in situ
GJIC: Gap junction intercellular communication
JAK2: Janus Kinase 2
STAT5a: Signal transducer activator of transcription 5a
Connexin 43: Cx43
ECM: Extracellular matrix
FEA: Flat epithelial atypia
DCIS: Ductal carcinoma in situ
TME: Tumor microenvironment
Chi3L1: Chitinase-3-like-1
pri-miRNA: primary microRNA
pre-miRNA: precursor miRNA
Ago-2: Argonaute 2
RISC: RNA induced silencing complex
P-body: Processing body
miR-183: microRNA-183
NSCLC: Non-small cell lung canrcinoma
MAP1: Metastasis associated protein 1
ILV: Intraluminal vesicles
ESCRT: Endosomal sorting complex
vSNARES: vesicle SNARES
NET: Neutrophil extracellular traps
hUCMSC: Human umbilical cord mesenchymal stem cells
CAFs: Cancer associated fibroblasts
PBMCs: Peripheral blood mononuclear cells
TET3: Ten-eleven translocation 3
DMEM/F12: Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 Ham
FBS: Fetal bovine serum
NRT: No reverse transcription control
NTC: No template control
qRT-PCR: Reverse transcription qualitative real time polymerase chain reaction
GFP: Green fluorescent protein
CMV: cytomegalovirus
HCMV: Human cytomegalovirus
P2: SCp2
G6: SCg6
CCM: Culture conditioned media
GM: Growth media
DM drip: Differentiation media on drip
NDM: Non-differentiation media
ITGB1: Integrin β-1

ES: Embryonic stem cells
MSC: Mesenchymal stem cells
CRC: Colorectal cancer

CHAPTER I

INTRODUCTION

Over the past decade, microRNAs (miRNAs) have emerged as important regulators that orchestrate various cellular functions including differentiation, proliferation, cell death, and migration. Due to their crucial regulatory functions, miRNAs are widely expressed in tissues during early development of organs and even after their differentiation. Importantly, miRNAs have been shown to be implicated in the normal development and differentiation of the mammary gland. For instance, specific patterns of miRNA expression were characterized during varying stages of differentiation of mammary stem cell-like (SC) cells, HC11 cells. Twenty-one miRNAs were found to be regulated during the differentiation of HC11 Stem cell like cells from SC-like stage to pre-differentiation stage and then to a fully differentiated state. Among the miRNAs identified are miR-200a, miR-200b, which are involved in the regulation of epithelial to mesenchymal transition (EMT) [1]. In addition, the overexpression of miR-30b in the mammary gland of transgenic mice lead to the development of acini structures with abnormally small lumens during lactation, and even though all milk proteins were being produced, the number and structure of lipid droplets produced was altered. The transgenic mice also had a delay in involution of the mammary gland post weaning which suggested an important role for miR-30b in the various stages of the developing gland [2]. Not only do miRNAs contribute to normal cellular processes, but many studies have reported altered patterns of their expression in pathological conditions, suggesting an additional contribution of miRNAs to the development of disease. A recent study identified a panel of miRNAs that are exclusively dysregulated in breast cancer patients belonging to the Lebanese population. Among the dysregulated

miRNAs is miR-183 which was found to be upregulated in invasive ductal carcinoma samples obtained from both Lebanese and US patients [3]. miR-183 is a member of the microRNA-183-96-182 cluster located at the position 7q31-34 locus of the human chromosome. It has a tissue specific pattern of distribution and is found to be dysregulated in many types of cancers [4]. The overexpression of miR-183 was found to promote proliferation, invasion, and survival in breast cancer, as well as in other cancers such as pancreatic cancer, gastric cancer, and hepatocellular carcinoma [5-8].

Differentiation and neoplastic phenotypes are fundamentally opposite processes. miRNAs are among the mediators that regulate both processes [9, 10]. The interrelatedness between differentiation and cancer makes studying cellular differentiation in the mammary gland important for better understanding both normal functions and malignant transformation [11]. Previous studies at our lab have revealed a link between miR-183 over-expression, breast cancer initiation, and the disruption of polarity in ductal epithelial cells as indicated by disrupted localization of polarity markers (Naser Al Deen et al. 2021; *manuscript in preparation*). Moreover, one study showed that in tissue samples obtained from invasive lobular carcinoma (ILC) and lobular carcinoma in situ (LCIS), miR-183 was among the overexpressed miRNAs along with miR-182 and miR-375 identified in the epithelium. These findings suggested that miR-183 was associated with the development of lobular neoplasia. Although the latter study established a link between the levels of miR-375 overexpression and loss of appropriate tissue organization - which is accounted as a loss of differentiation - in MCF-10A model of mammary morphogenesis, the potential effect of miR-183 on lobular epithelial differentiation and the induction of lobular neoplasia remains uninvestigated [12]. Therefore, we aimed to investigate the effect of miR-183 on

differentiation within a model that specifically recapitulates mammary epithelial differentiation in vitro. The model used consists of SCp2 and SCg6 cells of lobular mammary epithelial and myoepithelia-like origins respectively. SCp2 cells provide suitable models to study mammary epithelial differentiation since they exclusively express β-casein as a differentiation marker under differentiation permissive conditions achieved by supplying lactogenic hormones and appropriate basement membrane components. Three approaches were adopted to increase the level of miR-183-5p in SCp2 cells for better understanding its role in mammary epithelial differentiation. The first was through stable infection of SCp2 and SCg6 with a lentivirus carrying a miR-183-5p gene. The aim of this approach was to determine the effect of miR-183-5p overexpression on gap-junction-intercellular communication (GJIC) and cell-ECM interactions-mediated-lobular tumor initiation. As previously mentioned, SCp2 cells produce β-Casein as a differentiation marker upon stimulation with prolactin, and when provided with proper integrinβ-1-mediated-ECM interactions [13], or alternatively, when SCp2 cells are co-cultures with SCg6 cells in a GJIC dependent mechanism [14, 15]. Thus, monitoring the expression of the differentiation marker β-Casein upon miR-183-5p overexpression might provide mechanistic insight into the association of miR-183-5p with possible tumor-initiation induced by altered cell-ECM or cell-cell interactions. The second approach was done as an alternative to viral infection. SCp2 cells were treated with conditioned media and exosome-containing extracts derived from S1 breast epithelial cells overexpressing miR-183-5p by viral infection. This approach was based on previous reports showing that exosome-derived miRNAs from various tissue types (myoblasts, keratinocytes, tumor adipocytes) affect the differentiation of target tissue of different origins (osteoblasts, osteoclasts, stromal

adipocytes) [16-18]. However, data obtained from treating SCp2 with conditioned media was not conclusive since conditioned media components-in specific Sodium Selenite- showed an inhibitory effect on β-Casein expression. Therefore, treating cells with purified exosome preparations isolated from conditioned media could present a more suitable approach for exogenously providing miR-183-5p to SCp2 cells. In fact, studies have shown that breast cancer associated miRNAs can be released into exosomes and transferred to other cells through blood sera and body fluids in vivo, or via cell culture media of breast cancer cell lines that are induced to overexpress miRNAs in vitro [19-21]. The third approach was to treat SCp2 cells with the herbicide and potential carcinogen Glyphosate. Glyphosate induces global DNA hypo-methylation which is one way by which miRNA biogenesis and gene expression is regulated [22]. Duforestel et al, 2019 showed that glyphosate treatment in combination with high miR-182 expression levels triggers breast cancer initiation in mice and primary breast cancer cells [23]. We proposed that by applying the aforementioned approaches, we could be able to impede β-casein expression by SCp2 cells under differentiation permissive conditions and trigger a breast cancer-like phenotype in SCp2 cells.

CHAPTER II

LITERATURE REVIEW

A. Mammary gland development and differentiation:

The mature mammary gland consists of a series of branching milk ducts and milk producing alveoli. The ducts and alveoli are lined by a layer of luminal epithelial cells surrounded by myoepithelia-like cells on the basal side. The gland is embedded within fatty tissue that extend throughout the breast. Mammary gland development starts during embryogenesis, stops after birth, and then resumes following puberty. During embryogenesis the nipple and a rudimentary ductal tree are formed from epidermal cells and elongating ductal epithelial cells that branch into the underlying fat pad. At puberty, branching morphogenesis is initiated, and is tightly regulated by multiple factors including hormones, soluble factors, cell to cell interactions, and cell-extracellular matrix interactions. During pregnancy, the increase in estrogen and progesterone levels enhances the branching and growth of the mammary ducts. At this stage, prolactin and other lactogenic hormones induce differentiation and maturation of the alveolar epithelium in preparation for milk production. Luminal epithelial cells of the alveoli produce and secrete milk upon differentiation. Myoepithelia-like cells then contract in response to stimulation by oxytocin and suckling to squeeze the milk out of the lobular lumen into the ductal lumen during lactation. When lactation is ceased, the gland regresses and the mature luminal epithelial cells undergo apoptosis [24].

Multiple factors contribute to mammary gland development and differentiation. Hormonal signals tightly regulate mammary gland morphogenesis. At puberty estrogen, growth hormone, and insulin-like growth factor-1 induce branching morphogenesis and result in the formation of a branching ductal tree within the fat pad. During pregnancy

prolactin and progesterone are required for alveolar differentiation and milk production [25]. Normally, prolactin binds to its receptor on the plasma membrane of mammary epithelial cells and activates Janus Kinase 2 (JAK2). The activation of JAK2 results in the phosphorylation of the prolactin receptor leading to the recruitment of signal transducer activator of transcription 5a (STAT5a). STAT5a is then phosphorylated by JAK2 promoting its dimerization and translocation to the nucleus. Phosphorylated STAT5a dimer causes transcriptional activation of milk proteins including β-Casein. Extracellular matrix (ECM) component laminin activates β1-integrin mediated signaling required for mammary epithelial cell polarization and β-Casein expression. Laminin-1/ β1-integrin signaling leads to the activation of Rac1 which inhibits the activation of Shp2 tyrosine phosphatase. Shp2 tyrosine phosphatase de-phosphorylates STAT5a and prevents its translocation to the nucleus thereby inhibiting the transcriptional activation of β-Casein [26, 27]. The contribution of the basement membrane to the differentiation of mammary epithelia is only partial. Gap junction mediated cell communication is a major contributor to the epithelial cell differentiation in vitro. Gap junction protein isoforms Connexin43 (Cx43) and Cx26, Cx30, and Cx32 are expressed in both rodent and human mammary gland tissue, while Cx46 is exclusively expressed in humans [28, 29]. Cell lines that could replicate both the hormone dependent regulation, and the cell-cell interactions-dependent gene expression in mammary epithelial cells were established to facilitate studying epithelial differentiation in vitro, an example is CID-9 mammary epithelial cell line cultures and their derivatives, SCp2 and SCg6 cell strains. CID-9 cell lines originate from COMMA-1D cell lines derived from primary mammary epithelial cells isolated from mid-pregnant mice. The CID-9 cell cultures are suitable models for studying mammary epithelial cell differentiation in vitro because they are

capable of producing β-Casein under differentiation permissive conditions. CID-9 can recapitulate the hormone dependent changes of gene expression of mammary epithelial cells by differentiating only in the presence of hormonal signals. The role of the extracellular matrix (ECM) in mammary epithelial differentiation of CID-9 was also evident when β-Casein was exclusively produced upon supplying the CID-9 cultures with EHS matrix (a laminin rich ECM) [30]. Later on, the involvement of the gap junction mediated communication in the differentiation of ECM-supplemented CID-9 was revealed. It was shown that not only is the β-Casein expression enhanced, but the function of the gap junctions is promoted in EHS supplemented cultures. This was first attributed to the fact that in the presence of an exogenously provided basement membrane, connexin proteins adopt a membranous localization as compared to their cytoplasmic accumulation when cultured on plastic. Using cAMP to enhance gap junctional communication, and 18αGA to block it, it was shown that gap junction communication was sufficient to drive β-Casein expression even in the absence of an ECM [13]. Importantly, CID-9 cells contain a heterogeneous population of cells including luminal epithelial cells, myoepithelia-like cells, and fibroblasts. The gap junction mediated heterocellular communication between luminal and myoepithelia-like cells is responsible for inducing differentiation. This finding was demonstrated using co-cultures of two cell strains purified from CID-9, the SCp2 luminal epithelial cells, and the myoepithelia-like cells, SCg6. It was shown that the heterocellular communication between SCp2 and SCg6 is sufficient to promote mammary epithelial differentiation in culture without the need for a basement membrane. It was proposed that the assembly of connexin proteins on the membrane, and their interaction with α-catenin, β-catenin, and ZO-2 under co-culture conditions sequester β-catenin to the

membrane and prevent it from translocating to the nucleus, this in turn reduces β-catenin/TCF transcription and might partially contribute to the expression of β-casein by mammary epithelial cells SCp2 [14]. It is postulated that the mechanism by which GJIC mediates β-Casein expression involves signaling through transcription factor Oct-1 rather than STAT5a. Oct-1 was found to activate the transcription of β-Casein in response to prolactin by binding to promoter regions distinct from those of STAT5 [15, 31]. Furthermore, in the presence of the gap junction inducer cAMP and the absence of adherent substrata, CID-9 cells were capable of expressing β-Casein despite the minimal expression of phosphorylated STAT5. It is worth noting that under the latter conditions three isoforms of Oct-1 were exclusively and simultaneously expressed; 28 KDa, 40 KDa, and 75 KDa isoforms. These findings had suggested that the GJIC-mediated β-Casein expression is independent from STAT5 signaling, but might be mediated through a mechanism involving Oct-1 [15] (Figure 1).

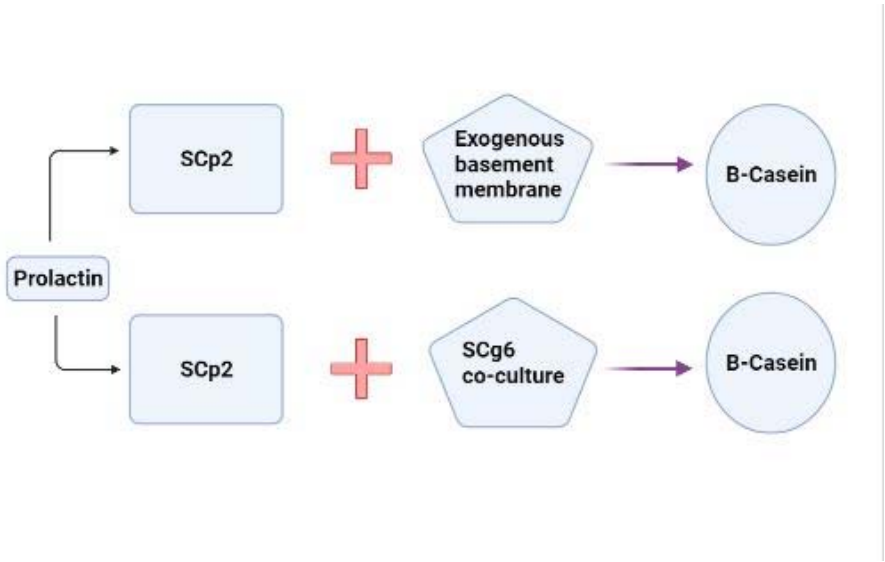


Figure 1 Mechanisms dictating β-Casein expression. SCp2 express β-Casein in response to prolactin signaling in combination with either an exogenously provided basement membrane, or when co-cultured with SCg6 in a GJIC dependent manner.

B. An Overview of Breast Cancer:

Breast cancer is most common type of cancer in women worldwide. Breast cancer is reported mostly in postmenopausal women aged between 44 and 49 years [32]. Breast cancer occurs after breast ductal or lobular epithelial cells undergo hyper-proliferation and develop benign tumors. Ductal carcinomas account for 40-70% of the diagnosed cases, and are believed to progress first by the transformation of normal epithelium into flat epithelial atypia (FEA), followed by atypical ductal hyperplasia, which then progresses to ductal carcinoma in situ (DCIS), and then finally culminate in invasive ductal carcinoma and malignant BC. On the other hand, breast cancers of lobular origin start when normal lobular epithelial cells develop atypical lobular hyperplasia which then evolves into lobular carcinoma in situ (LCIS). LCIS may further progress into a more aggressive form referred to as invasive lobular carcinoma (ILC) [33]. Breast sarcomas are another rare form of breast neoplasms (less than 1%) arising from heterogeneous neoplasms of the mesenchymal tissue associated with the breast [34]. In our study, we focus only on the more frequently occurring breast carcinomas. Breast carcinomas were classified into five molecular subtypes according to the surface receptors they display. Luminal A breast cancer cells are ER-positive, HER2-negative, Ki-67-low, and PR-high. The Luminal B HER2 negative subtype is ER-positive, HER2-negative and either Ki-67 high or PR low. Luminal B-HER2 positive are ER-positive, HER2-high, Ki-67, PR positive. In HER2 positive subtype HER2 is overexpressed, ER and PR are not expressed, and finally, triple negative breast cancer cells are ER-negative, HER2-negative, and PR-negative [35].

Multiple factors are thought to contribute to the progression of mild breast tumors into more invasive and aggressive forms. Those factors include hormones, age,

genetic mutations and faults in the DNA repair system correlated to family history, chemical and environmental carcinogens, diet, and lifestyle of women [36-38]. The tumor microenvironment (TME)-comprising the basement membrane and extracellular matrix (ECM), in addition to non-cancerous cell types such as endothelial cells, immune cells, adipocytes, fibroblasts, and infiltrating inflammatory cells- plays a key role in the progression of breast cancer. The stromal compartment may release chemokines, cytokines, growth factors, and ECM proteins that can influence epithelial cells and therefore, affect major characteristics of the tumor including its degree of proliferation and invasion [39]. Triple negative and ER negative breast cancer subtypes are characterized by mediating immune suppression with the aid of immune regulatory cells such as Tregs, MDSC, Th_2, Th_{17}, M2 macrophages, $HLADR^-$ T cells, and $T\gamma\delta$ cells. On the other hand, ER^+ breast tumors have higher NK cells, CTL, Th,, and Tfh cells which are associated with anti-tumor activity [40].The role of TME in carcinogenesis is highlighted in a study by Maffini et at, which showed that treating mammary epithelial cells with the carcinogen (NMU) was not sufficient to induce neoplastic transformation. However, transplanting the same cells into a stroma that is exposed to NMU triggered transformation and cancer initiation in the epithelial cells [41]. It has been shown that components of the TME interact by autocrine or paracrine means with the cancer cells to promote tumor growth. For example, cancer associated fibroblasts CAFs -which are also components of the stromal compartment- demonstrated a crucial role in promoting breast tumor growth and immune-suppression. CAFs release a glycoprotein chitinase-3-like-1 (Chi3L1) which induces tumor cell invasion and growth by promoting the activation of MAPK and PI3K signaling pathways in breast tumors. It also facilitates tumor infiltration and disrupts T cell recruitment and differentiation leading to immune-

suppression thus further favoring tumor growth and invasion [42]. The interaction of the tumor with its microenvironment has demonstrated importance in terms of cancer drug resistance as well. It is well known that cancer cells can display resistance to drugs by changing their interaction with the surrounding stroma [43]. Therefore, breast cancer is complex heterogeneous disease that is influenced by a range of diverse factors.

C. The Role of microRNAs in Differentiation and Cancer:

miRNAs are important post transcriptional regulators contributing to a wide range of normal cellular functions and pathological conditions, including breast cancer. miRNAs are reported as either oncogenes (onco-miRs) or tumor suppressors of breast cancer [44-47]. Due to their dysregulated patterns of expression in breast cancer [48, 49], recent studies have highlighted the importance of miRNAs expression patterns as early stage diagnostic and prognostic biomarkers [50]. miRNA biogenesis occurs through a multistep process involving initial transcription by RNA polymerase II. Briefly, a large primary miRNA (pri-miRNA) is first transcribed and cleaved in the nucleus into a shorter hairpin double stranded RNA called precursor miRNA (pre-miRNA) by an enzyme called Drosha. Pre-miRNA is exported by Exportin-5 to the cytoplasm where it is further cleaved into an RNA duplex by the endonuclease DICER and its associated co-factor TRBP. Argonaute 2 (AGO-2) protein then cleaves one strand of the miRNA duplex and the remaining mature strand is incorporated into an RNA induced silencing complex (RISC). The mature miRNA strand has a seed sequence located on its 5' end. The seed sequence enables the miRNA to bind with either perfect of imperfect complementarity to the 3' UTR of its target mRNA, thereby inhibiting its expression at the post transcriptional level [51]. In the cytoplasm, miRNA-

mediated post transcriptional silencing occurs in the Processing body (P-body). The P-body is a site rich in enzymes specialized in mRNA turnover. miRNAs were also found to exert regulatory cellular functions within multiple organelles such as the Golgi apparatus, the mitochondria, and the endoplasmic reticulum. Several studies have indicated the localization of miRNAs and the miRISC components within the nucleus, which suggests that the miRISC complex can assemble and mediate RNA silencing within the nucleus. Nuclear miRNA may regulate the expression of other non-coding RNAs. They may also interact with pri-miRNAs to regulate their biogenesis [52].

Dysregulated expression of miRNAs has been reported in many cancer types [53, 54], including breast cancer [3, 55, 56]. On the other hand, 35 miRNAs were found to be exclusively dysregulated in renal cell carcinoma-derived patient samples compared to controls. The robust pattern of miRNA dysregulation makes it a reliable means for molecular-based diagnosis of renal cell carcinoma [57]. Similarly, a set of 50 to 576 differentially expressed miRNAs were identified in acute lymphoblastic leukemia patients with relapse and multidrug resistance. The same abnormally expressed miRNAs were associated with pathways leading to leukemia stem cell differentiation and self-renewal [58]. Moreover, one study identified 153 miRNAs that were dysregulated between the gastic cancer cell line GC9811-P and its higly variant GC9811-P which is associated with peritoneal metastasis[59]. In prostate cancer, 51 miRNAs were found to be differentially expressed between in vivo samples from benign prostate hyperplasia, and prostate carcinoma, of which 14 were upregulated and 37 downregulated [60]. In another study, signature miRNAs were used to differentiate between blood samples derived from triple negative breast cancer patients before and after being subjected to non-adjuvant chemotherapy. The study identified 321 miRNAs

that were differentially expressed upon chemotherapy treatment. These findings, highlight the role of miRNAs as both cancer predictive biomarkers, and prognostic biomarkers for anti-cancer therapy [61]. The change in miRNA expression in cancer is influenced by multiple factors acting on the transcriptional and post transcriptional levels. On the transcriptional level, intragenic miRNA expression can be regulated by the transcription factors that regulate the host gene. Intergenic miRNAs genes have their own promoters, and thus are regulated by the transcription factors that directly interact with their promoter regions. Epigenetic modifications such as promoter hyper-methylation, and mutations in the miRNA genes also affect miRNA expression [62]. For example, promoter hyper-methylation of miR-132 reduces its expression and leads to poor prognosis in colorectal cancer [63]. Also, a germline mutation in the miR-161-1 gene leads to an alteration in the 3' flanking region of the pri-miR-161. This causes a defect at the Drosha processing step that ultimately results in severe downregulation of miR-16 expression observed in chronic lymphocytic leukemia patient-derived cells [64] . Post transcriptional regulation may be presented as changes in the activity of miRNA biogenesis enzymes. This could be attributed to somatic and germline mutations in the genes encoding miRNA biogenesis enzymes such as Dicer and Drosha, or to epigenetic modifications on the promoters of those enzymes. Studies have shown that some chemical compounds being either endogenous (hormones, chemokines), or exogenous (xenobiotics) may also interfere with the processing and stability of miRNAs, thereby, regulating their expression at the post transcriptional level [62]. For instance, Malliot et al showed that a wide set of miRNAs are repressed upon treatment with estrogen, and that the re-expression of those repressed miRNAs reduced estrogen-dependent cell growth of breast cancer cell lines [65].

miRNAs circulate in body fluids including blood plasma, serum, urine, and breast milk [66]. Circulating miRNAs are usually bound to lipoproteins like HDL, or are enclosed in micro-vesicles such as exosomes [67]. So far, high throughput technology has facilitated the identification of panels of dysregulated miRNAs in both tissues and breast cancer patient sera. Compared to normal tissues, many miRNAs have been found to be dysregulated in breast cancer. One cohort study by Godfrey AC et al, revealed 21 dysregulated miRNAs in the blood serum of breast cancer patients compared to disease-free controls [68]. In another study, 51 dysregulated miRNAs were identified with the potential of regulating 719 tumor promoting mRNAs in breast cancer. The mRNAs were either involved in increasing proliferation or limiting migration and invasion [3]. These findings and others, suggest a potential role for miRNAs in the diagnosis, prognosis, and therapeutic assessment of diseases such as breast cancer.

D. miR-183 in Breast Cancer:

MicroRNA-183 (miR-183) is a member of the microRNA-183-96-182 cluster family. miR-96 was the first member of the cluster to be discovered [69]. miR-183 and miR-182 were later identified using bioinformatics tools [70]. The three microRNAs were grouped in a cluster due to their sequence homology and chromosomal location at the 7q31 locus in humans, and the 6qA3 locus in mice. The miR-183 cluster is highly conserved among various organisms such as Zebra Fish, Drosophila Melanogaster, and mice. Differences in the seed sequence between the three members of the cluster result in them having different RNA targets [71]. The expression of miR-183 cluster occurs in a tissue specific manner and is exceptionally high in sensory organs including the eyes,

nose, and inner ears. In fact, studies have shown the expression of the members of the miR-183 cluster plays a crucial role in the development of the retina and the cochlea of the inner ear, and their absence in early stages of development leads to hearing loss [72].

miR-183-5p is dysregulated in many cancer types and can act as either an onco-miRNA or a tumor suppressor depending on the context. For example, in non-small cell lung carcinoma (NSCLC), the overexpression of miR-183-5p initiates carcinogenesis by targeting PTEN, suppressing P53 and activating signaling pathway. This was concluded after proliferation and migration of NSCLC cells increased upon stably infecting the cells with miR-183-5p [73]. Similarly, in gastric cancer AGS cells, miR-183-5p overexpression promoted proliferation and migration of cells by targeting TPM1, and inhibited apoptosis by suppressing Bcl-2/P53 signaling pathway [74]. Additionally, miR-183-5p promotes proliferation in hepatocellular carcinoma HCC cells by targeting FOXO1, FOXN3, DYRK2, AKAP12, and IRS1 [75, 76]. In breast cancer, miR-183-5p overexpression leads to the downregulation of PDCD4, which increases cell proliferation and decreases cell death in MCF7 and MDA-MB-231 breast cancer cell lines [77]. On the other hand, some studies have reported that miR-183 can act as a tumor suppressor in multiple cancer tissue. In one study, Lowery et al showed that induced miR-183-5p expression in MDA-MB-231 breast cancer cells suppresses migration by downregulating the expression of VIL2 encoding for ezrin protein. Ezrin is a membrane cytoskeleton cross linker that controls cell adhesion and motility by controlling the actin cytoskeleton [78]. Similarly, ectopic expression of miR-183 suppressed metastasis by inhibiting Metastasis-associated 1 protein (MTA1) and preventing epithelial to mesenchymal transition in nasopharyngeal carcinoma spheroids

[79]. miR-183 also regulates migration in cervical cancer HeLa cells by targeting ITGB1 that normally promotes cell migration and adhesion [80]. These findings show that miR-183-5p has a dual role in cancer through regulating the expression of a wide range of genes involved in both cancer initiation and suppression.

E. Exosomes-Mediated Intercellular Communication:

Exosomes are extracellular nano-vesicles- 40 to 100 nm in diameter - released by many cells within the body. Exosomes are released either constitutively or in response to physical or chemical stress such as shear stress, oxidative stress, and hypoxia [81]. It was suggested that exosomes function in the removal of cell waste, and both maintaining cellular fitness [82] and cell-cell communication in normal and pathological conditions [83, 84]. Exosomes are formed from the internalized endosomal compartments. Early endosomes form intraluminal vesicles (ILV) that carry cargo targeted for degradation or extracellular release. The ILVs form multi-vesicular bodies MVBs as the early endosome matures into a late endosome. Endosomal sorting complexes (ESCRT) facilitate exosome loading and transport by recognizing ubiquitylated proteins marked for lysosomal degradation. Some MVBs fuse with the lysosome releasing cargo into the acidic environment of the lysosome, resulting in their degradation. Cargo can also be loaded into exosomes in a ubiquitin independent manner by direct and indirect binding to exosome associated proteins such as ALIX [85, 86]. Not all MVBs fuse with the lysosome, rather a large portion of MVBs fuse with the plasma membrane and release ILVs, referred to as exosomes at this stage, outside of the cell. The fusion of ILV with the plasma membrane and exosome release is mediated by SNARE proteins. Vesicle SNARES (vSNAREs) on the surface of MVBs recognize

target SNARE on the plasma membrane which initiates the fusion and extracellular release of exosomes outside of the cell. Rab family protein members are also involved in regulating mechanisms of exosome release [86, 87]. On the other hand, tetraspanins such as CD9, CD63, CD81, and CD82 regulate exosome fusion, migration, and adhesion to target cells [85]. The released exosomes circulate until they reach the target tissue where they are internalized, and their contents released into the cytoplasm. Several pathways could be implicated in exosome internalization. Those include fusion of the exosome with the plasma membrane [88], micropinocytosis [89], phagocytosis [90], Clathrin-mediated endocytosis [91], Caveolin-dependent and lipid raft-mediated endocytosis [92, 93].

Some cytosolic proteins are expressed by all exosomes regardless of the cell type they originate from. Those include tubulin, actin, TSG101, Alix, heat shock proteins HSP, Rab family proteins, tetraspanins, and MHC class I [94]. Additionally, exosomes can transport virtually every type of protein, RNA, breakdown products of signaling pathways, viruses, lipids, therapeutic drugs and miRNAs to target cells [87]. This highlights the role of exosomes as mediators of cell-cell communication. For example, exosome derived miRNAs from varying tissue types such as myoblasts, keratinocytes, and tumor adipocytes can influence the differentiation of target tissue from different origins (osteoblasts, osteoclasts, stromal adipocytes respectively) [14-16]. Moreover, oncogenic proteins, mRNAs, and miRNAs can travel to distant sites through exosomes and initiate pro-tumorigenic environments for cancer metastasis [95]. In one example, mutated KRAS protein was transported in exosomes and its uptake by target colon cancer tissue lead to the deterioration of colorectal cancer in mouse models by increasing IL-8 production, neutrophil recruitment and formation of neutrophil

extracellular traps (NETs) [96]. Recently, tumor associated miRNAs were identified in circulating exosomes. Precursor miRNAs (pre-miRNA) in association with miRNA processing complexes were identified in breast cancer derived exosomes where they were processed into mature miRNAs [97, 98]. Double stranded miRNAs are sorted into exosomes by various modes. Those include miRISC-related pathway, nSMAse2-dependent pathway, miRNA motif and sumoylated hnRNPs-dependent pathway, and miRNA sequence dependent pathway [97].

Several studies highlighted the role of exosome-derived miRNA in cancer. Tumor cells may get rid of tumor suppressor miRNAs by releasing them outside of the cells in exosomes-for instance. Tumor suppressing miRNA let-7 is concentrated in exosomes obtained from highly metastatic gastric cancer cells compared to those originating from less metastatic gastric cancer cells [99]. Additionally, tumor suppressing miRNAs could reduce cancer progression by exogenously being delivered to tumor cells through exosomes from distant normal tissue. Yuan et al showed that delivering miR-148b-3p to MDA_MB-231 breast cancer cells through exosomes originating from human umbilical cord mesenchymal stem cells (HUCMSCs) inhibited proliferation, invasion, and migration, and promoted apoptosis in the breast cancer cells [100]. Neighboring tissue could also affect tumor growth by exosome-mediated delivery of miRNAs. For example, cancer associated fibroblasts (CAFs) reduce tumor growth and metastasis of gastric cancer cells by releasing miR-139 into exosomes that target MMP11 in the tumor microenvironment [101]. On the other hand, exosomal miRNAs may promote oncogenic effects in target tissue. miR-3613-3p in exosomes released from CAFs promote breast cancer cell survival and metastasis. This was due to SOCS suppression by miR-3613 in BT474 and MCF7 breast cancer cells [102].

Similarly, miR-223 levels were significantly increased in breast cancer cell lines MDA-MB-231 and HER2+ SKBR3 after being treated with miR-223 rich-exosomes derived from IL-4 activated macrophages. Notably, this increase in miR-223 promoted significant cell invasion in the exosome treated cells [103, 104].

F. Glyphosates and Breast Cancer:

Glyphosate (*N*-(phosphonomethyl) glycine) is a broad spectrum herbicide widely used in agriculture for weed control. Microorganisms in the soil degrade glyphosate into AMPA. Glyphosate has a broad spectrum of activity since it targets an enzyme -involved in the synthesis of aromatic compounds in microorganisms and plants- called 5-enolpyruvylshikimate-3-phosphate synthase [105]. Glyphosate residues were reported in water, soil, crops and processed food, maternal and umbilical cord serum, breast milk samples, and human urine samples [106]. In mammals, glyphosate does not get completely degraded by gut microflora. Notably, the international agency of research considers glyphosate as a potential carcinogen [107]. Several studies suggested that glyphosate exposure negatively affects both female and male reproductive health in humans and mice models. Glyphosate-induced abnormalities include disrupted hypothalamic-pituitary axis, uterine and ovary abnormalities, testicular lesions, and pre- and post-implantation embryo losses [106]. Since glyphosate is suspected to be a carcinogen, one study aimed to link glyphosate metabolite AMPA levels in urine to breast cancer risk. A case control study of 250 postmenopausal women (124 breast cancer cases and 126 healthy controls) was therefore established. AMPA was detected in 90% of breast cancer cases and 84% in controls. This study provided a preliminary association between excreted glyphosate

metabolite AMPA levels and breast cancer [108]. Moreover, glyphosate treatment has been linked to alterations in estrogen receptor expression (ER) in both in-vivo and in-vitro models, and in a dose dependent manner. For example, developmental exposure to glyphosate induces epigenetic changes in ERα of F1 rats as shown in two studies conducted by Gomez at al [109] and Lorenz et al [110]. Those changes were accompanied by increased DNA methylation of the ERα promoter regions [106]. Sritana et al also showed that glyphosate treatment at concentrations 10^{-5} and 10^{-11} has the same effects as estradiol treatment on cholangiocarcinoma HuCCA cells. Both Glyphosate and estradiol induce cell proliferation by promoting the expression of proteins associated with proliferation, including ERα, VEGFR2, pERK, and PCNA. Additionally, an increase in the S phase of the cell cycle and cyclin family protein levels was also observed after glyphosate treatment. Treating HuCCA cells with estrogen receptor and MEK antagonists U0126 and 4-hydroxytamoxifen respectively reversed the effect of both treatments, suggesting that glyphosate induces cell growth in an ER/MEK/ERK1/2-dependent signaling pathway [111]. On the other hand, De Almeida et al showed that treating MCF-7 and MDA-MD-231 breast cancer cells with moderate concentrations of glyphosate did not show any significant effect on cell viability, but rather triggered considerable DNA damage [112]. The effect of glyphosate on DNA methylation was first reported in a study by Kwiatkowska et which showed that exposing peripheral blood mononuclear cells (PBMCs) to high concentrations of glyphosates leads to the formation of DNA lesions, decreases the percentage of global 5mC, and increases methylation of p53 promoter [113]. It was recently shown that even low concentrations of glyphosate and AMPA can trigger DNA damage. For example, Santovito et al showed that exposing human leukocytes to low glyphosate

concentrations (0.025-0.500 μg/ml) increases the frequency of micronuclei and chromosomal aberrations in glyphosate treated cells [114]. Further support was provided in a study by Duforestel et al showing that glyphosate treatment at low concentrations induces global DNA hypo-methylation and ten-eleven translocation 3 (TET3) activity in non-tumorigenic MCF10A cells. It was also shown that glyphosate exposure in combination with increased miR-182-5p expression induced breast cancer initiation in mice. Thus, the latter study suggested that glyphosate-induced DNA hypomethylation in combination with another risk factor, miR-182-5p in this case, affects TET3 pathway and triggers breast tumor initiation [23]. Therefore, glyphosates at both high and low concentrations can trigger global genomic aberrations, which when affecting tumor-associated genes, can lead to tumor initiation.

CHAPTER III

MATERIALS AND METHODS

A. Cell Culture

Cell culture experiments were performed using low passage number (20–35) of the SCp2 mouse mammary epithelial cell line and the mouse myoepithelia-like SCg6 mammary cells (Kindly provided by P.Y. Desprez, Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, CA). All cells were grown in Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 Ham (DMEM/F12, Sigma, St. Louis, MO) with 5% fetal bovine serum (FBS, Gibco, Paisley, UK), insulin (5 μg/ml, Sigma), and 1% penicillin/streptomycin (Gibco) in a humidified incubator (95% air 5% CO2) at 37 °C (Forma Scientific Inc., Ohio, USA). The above-described medium referred to as growth medium was changed every other day until SCg6 cells formed a confluent monolayer. SCp2 cells were transferred as they reached 80% confluence. To trigger differentiation, SCp2 cells were plated at $5x10^5$ cells/ml in 60 mm dishes in growth medium. The next day, growth medium was replaced by either differentiation medium or S1-derived conditioned medium mixed with 1.5% volume/volume of Matrigel (BD Biosciences, 354234) and then dripped over the cells. Differentiation medium consisted of DMEM/F12 media supplemented with insulin (5 μg/ml; Sigma), hydrocortisone (1 μg/ml; Sigma), ovine prolactin (3 μg/ml; Sigma) and 1% penicillin/Streptomycin (Gibco). Infected and uninfected S1 cells -non-tumorigenic human mammary HMT-3522 epithelial cells- were cultured ($0.75x10^6$ cells/ml) on plastic in T-75 cm^2 flasks in chemically defined serum free H14 medium [115], at 95% air 5% CO2 in a humidified incubator. H14 was changed every 2 days, and EGF was

omitted from the culture medium at day 6 post seeding. H14 conditioned media was collected from infected and uninfected S1 cells at days 11-14 in culture. For drip experiments, conditioned medium was supplemented with 1.5 IU of prolactin to compensate for the difference in prolactin concentration between H14 and differentiation media.

B. Drug selection killing curve

The drug selection killing curves of SCp2 and SCg6 were carried out according to the protocol supplied by abm. Briefly, SCp2 and SCg6 cells were seeded in 6 well plates at $2x10^5$ cells per well. Puromycin was diluted in growth medium to varying concentrations 0.75-3µg/ml for initial killing curve establishment, and 2-6µg/ml for the repeated killing curves done on infected SCp2 and SCg6 cells. Puromyicn containing growth medium was fed to the cells 24 hours after seeding. The cells were monitored daily and images were taken using a phase contrast microscope at 10X magnification.

C. Lentiviral Infection

Lentiviral infection was carried out according to the protocol supplied by abm. Briefly, SCp2 and SCg6 cells were plated in 10 mm cell culture dishes at $3x10^5$ cells/ml in growth medium. The next day, Polybrene (8µg/ml) was prepared from a working stock of 2 mg/ml, mixed with freshly prepared growth medium and then added onto the cells. Previously packaged viruses with miR-183-5p containing vectors were added (1.2 ml per plate) onto the cells. The cells were then incubated for 6 hours, then the infection media was removed and replaced with freshly prepared growth media. Infection was

repeated two additional times, and Puromycin selection (1.5μg/ml) in growth medium was started 48 hours after the last infection.

D. Quantifying cell viability after Puromycin treatment

Infected and uninfected SCp2 cells were seeded in growth media in 12 well plates at $1x10^5$ cells/ml. The next day, the media was replaced with growth media containing 6 μg/ml Puromycin. The cells were counted and monitored daily.

E. Total RNA isolation and quality control

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) for total RNA isolation from animal tissue according to the manufacturer's instructions. Purity and concentration of RNA samples were examined spectrophotometrically by absorbance measurements at 260, 280 and 230 nm using the NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). OD260/OD280 ratios between 1.8 and 2.1 were considered acceptable.

F. miRNA expression by quantitative real time-polymerase chain reaction (RT-qPCR)

Reverse transcription of 10 nanograms of the total RNA was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions and as previously described by Nassar et al.[3]. Briefly, small nuclear RNA RNU6B, miR-182-5p primers and probes were purchased as part of the TaqMan microRNA Assays Kit (Applied Biosystems, USA) with validated efficiency. cDNA synthesis was carried out for miR-183-5p in each

reaction with the endogenous control, RNU6B. RT-qPCR was performed using BioRad CFX96 Real Time System, C1000 Thermal Cycler (Germany). Reactions using 10 µl of SYBR Green JumpStart Taq ReadyMix (SIGMA S4438), 0.4 µl of the corresponding microRNA primer set from Hairpin-it^{TM} miRNA and U6 snRNA normalization RT-PCR quantification kit (GenePharma), 5.6 µl of DEPC treated water, and 4 µl of cDNA were performed in duplicates for each miRNA probe. cDNA Synthesis and RT-qPCR were repeated twice for each sample and each plate included no reverse transcription control (NRT), no template control (NTC). The cycling conditions were 94 °C for 3 min and 40 cycles of 94 °C for 15 s and an annealing temperature of 55 °C for 25 s, and 12°C for 25 s. The relative expression of miRNA was determined using the ΔCt equation.

G. Reverse transcription qualitative Real time PCR (qRT-PCR) for β-Casein

Total RNA (1 µg) was reversed transcribed to cDNA using Quantitect Reverse Transcription kit (Qiagen,Valencia,CA) according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green JumpStart Taq ReadyMix (SIGMA S4438) in a CFX96system (Bio-Rad Laboratories, Hercules,CA). Products were amplified as per the table.1 below. The incubation conditions consisted of 3 min at 95ºC followed by 40 cycles of 15s at 95ºC and 1 min at 60ºC. To quantify changes in gene expression, the ΔΔCt method was used to calculate the relative-fold changes normalized to GAPDH.

Table 1 Real time qPCR primers with their relatives forward and reverse sequences.

Gene	***Forward Primer***	***Reverse Primer***
β-Casein	GTGGCCCTTGCTCTTGCAAG	AGTCTGAGGAAAAGCCTGAAC
GAPDH	AAGGTGAAGGTCGGAGTCAAC	GGGGTCATTGATGGCAACAATA

H. Proliferation Assay

For each replicate, SCp2 cells were seeded in three 6 well plates at $5x10^4$ cells per well in growth medium. The next day, growth medium was removed and the cells were washed twice with serum free medium. Growth medium lacking fetal bovine serum (FBS) was added onto the cells to promote starvation conditions. The cells were kept in starvation conditions for 5 days, after which the cells were spiked with either complete growth medium, or S1 derived conditioned media from infected and non-infected cells. Cells left in starvation media were used as a negative control. The cells were then counted after 24 hours, 48 hours, and 72 hours. The fold change in cell number was compared to cells kept in starvation conditions, and the data was analyzed by multiple comparison two-way ANOVA.

I. Scratch Assay

For each replicate, SCp2 cells were seeded in two six well plates in growth medium at $2x10^5$ cells/ml. When the cells became fully confluent, growth medium was removed and the cells were washed twice with 1 ml PBS (1x). Serum free growth media

or conditioned media was added to the corresponding wells. Wounds were made using a sterile 200 µl pipette tip. Images across the entire wound were taken directly after wounding (t=0), 24 hours, and 72 hours post wounding. The size of the scratched area was measured using the commercially available imageJ software, and the percent coverage of the scratched area was calculated as follows: % coverage= (Δdistance/time)x100.

J. Isolation of exosome extracts from conditioned media

Exosomes were prepared from culture supernatants of S1 cells by differential centrifugations. Briefly, S1 cell lines were maintained in serum free H14 medium and conditioned media was collected at day 14 in culture. EGF was omitted from the medium starting day 6 in culture. The cells are 70–80 % confluent upon collection of conditioned media. After collection, the culture supernatants were sequentially centrifuged at 300*g* for 10 min, 2000*g* for 20 min and 10,000*g* for 30 min at 4 °C to pellet cells, dead cells and cell debris, respectively. The supernatants were then filtered using a 0.22 µm filter and centrifuged at 100,000*g* for 70 min at 4 °C to pellet the exosomes using the T865 rotor in a Sorvall WX Ultra Series Floor Model Centrifuge (Thermo Scientific,USA). The exosome pellet was resuspended in 1 ml PBS and stored at -80 ºC.

CHAPTER IV

RESULTS

A. SCp2 and SCg6 cells express low levels of miR-183-5p upon viral transduction using a vector carrying a human-derived CMV promoter

To induce constitutive overexpression of miR-183-5p in SCp2 and SCg6 cells, we first sought to stably infect SCp2 and SCg6 with a lenti-virus having an expression vector carrying miR-183-5p gene. The viral vectors that were used contained either a miR-183-5p gene or a miR-control gene (scrambled miRNA gene) and a green fluorescent protein (GFP) gene downstream of a human-derived cytomegalovirus (CMV) promoter, and a Puromycin resistance gene downstream an SV40 promoter. Prior to performing viral transduction, a drug selection killing curve was established for both SCp2 and SCg6 cells to determine the optimal concentration of the antibiotic Puromycin to be used for selecting against cells that were not successfully infected with the virus. SCp2 and SCg6 cells were treated with varying Puromycin concentrations ranging from 0.75 to 3 μg/mL. High cell viability and lowest cell death was observed at concentrations 0.75 μg/mL and 1 μg/mL. The concentration 1.5 μg/mL was the lowest concentration showing significantly low cell viability- and thus, high killing potential- in both cell types within three days post first selection. Similarly, the concentrations of 2 μg/mL and 3 μg/mL also show significant cell death (Figure 1). Therefore, to avoid cytotoxicity of infected cells and to ensure selection against uninfected cells, 1.5 μg/mL Puromycin concentration was chosen to carry out the drug selection step post infection. Both infected SCp2 and SCg6 cells showed low expression of GFP as observed by monitoring green fluorescence under the microscope after viral infection, and several

days post selection. This was despite successful resistance to Puromycin and the continuous increase in cell number observed between day one and day six following the start of Puromycin selection (Figure 2).

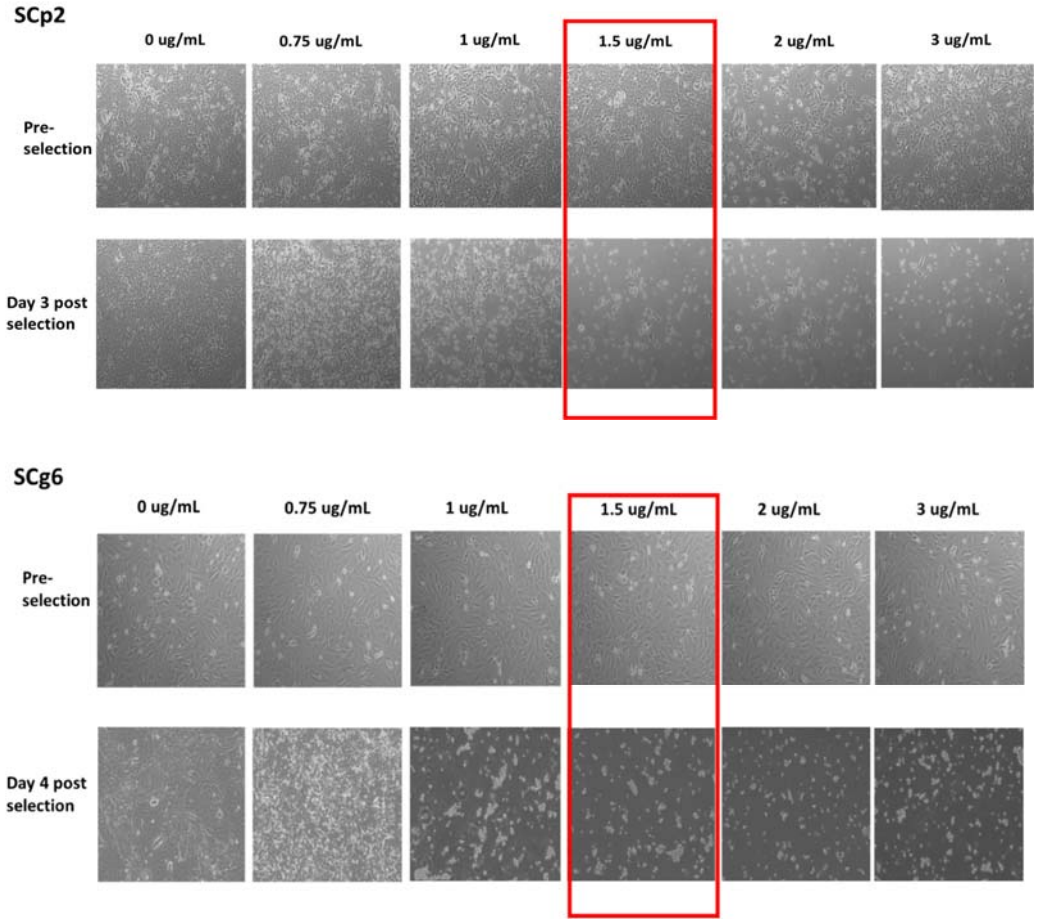


Figure 2 1.5 µg/mL Puromycin concentration was the lowest concentration inducing significant cell death. Drug selection killing curves for SCp2 and SCg6 cells were carried out using varying concentrations of the antibiotic Puromycin, observed under a phase contrast microscope at 10X magnification. SCp2 and SCg6 cells were treated with Puromycin at concentrations 0.75 µg/mL, 1 µg/mL, 1.5 µg/mL, 2 µg/mL, 3 µg/mL. All concentrations showed some level of cytotoxicity after three days of selection, but the lowest concentration that showed significant cell death was 1.5 µg/mL. Thus, to avoid inducing cytotoxicity while ensuring successful drug selection post infection, the concentration 1.5 µg/mL was chosen to carry out the drug selection step.

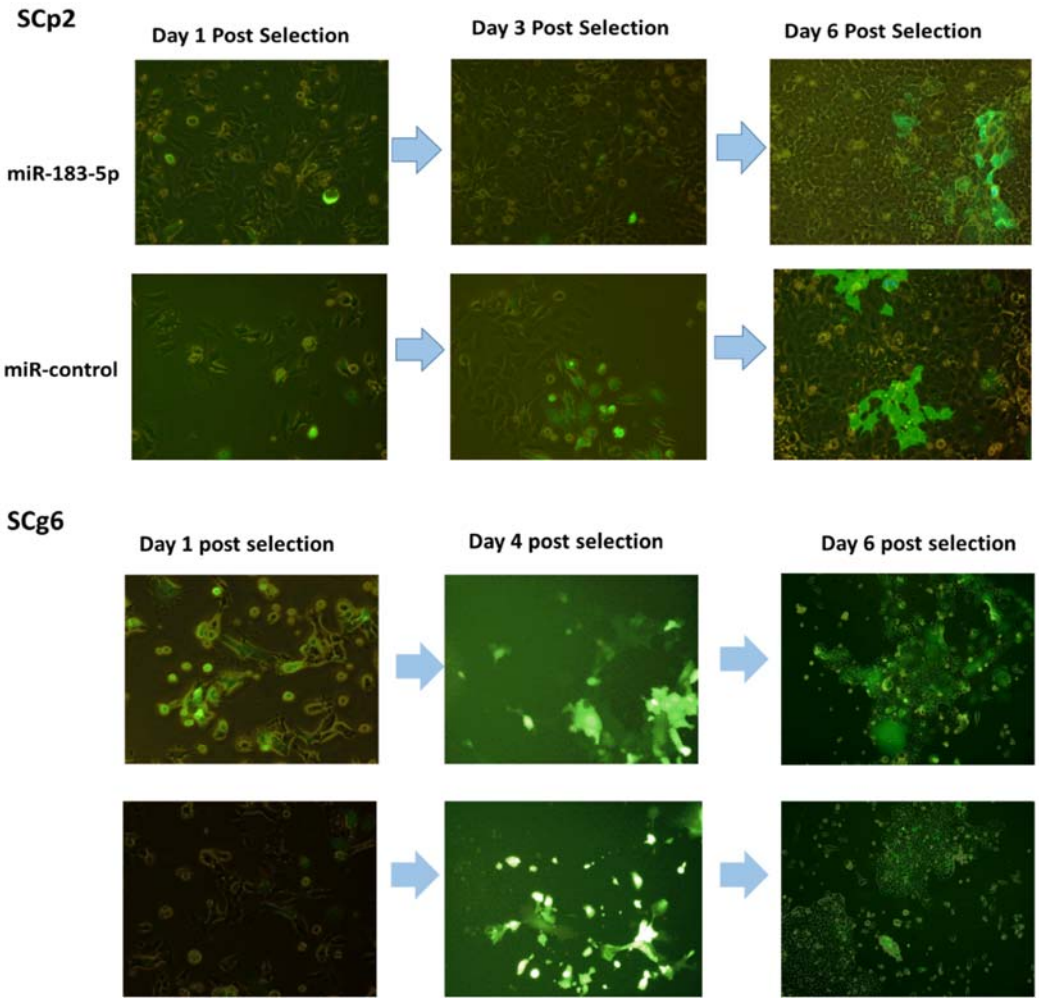
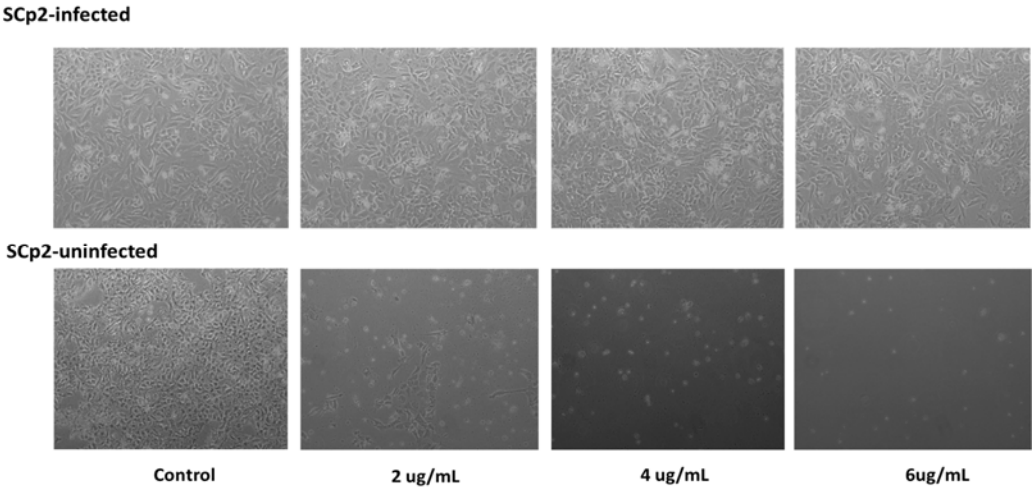


Figure 3 Infected SCp2 and SCg6 cells showed low GFP fluorescence despite continuous resistance against Puromycin treatment. Infected- SCp2 and SCg6 cells were monitored at day 1, day 4, and day 6 post selection, and observed under a fluorescent microscope at 10X magnification. Compared to the total number of cells present in each culture plate, the proportion of miR-183-5p and miR-control infected SCp2 and SCg6 cells showing green fluorescence was low. This is reflective of the amount of GFP being expressed by the infected cells. However, the number of SCp2 and SCg6 cells kept increasing from day 1 to day 6 despite continuous Puromycin treatment, which shows that the cells were successfully infected and were expressing the Puromycin resistance gene

To further validate the previous observation, a drug selection killing curve was concomitantly conducted on both infected and non-infected SCp2 and SCg6 cells using a higher range of Puromycin concentrations (2 μg/mL, 4 μg/mL, and 6 μg/mL). Cells

untreated with Puromycin were used as a control. Uninfected SCp2 and SCg6 cells showed significant cell death when treated with all three Puromycin concentrations. On the other hand, the infected cells showed minimal cell death, as expected in case the cells took up the viral vector and expressed the Puromycin resistance gene. Infected cells also proceeded to proliferate and increase in number (Figure 3). To quantify the rate of resistance to Puromycin, both infected and uninfected SCp2 and SCg6 cells were seeded in six well plates and treated with the highest tested Puromycin concentration 6 µg/mL, then counted for four days following treatment. Uninfected SCp2 and SCg6 cells showed a rapid drop in cell number, reaching zero viable cells after one day of Puromyicn treatment. In the meantime, infected SCp2 and SCg6 cells kept gradually increasing in number until reaching a plateau at day four (Figure 4).



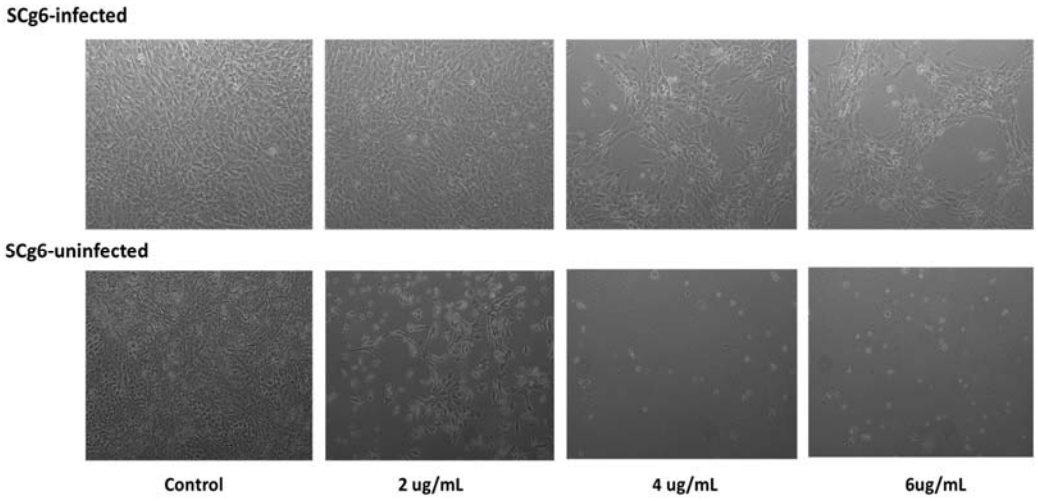


Figure 4 Infected SCp2 and SCg6 cells show minimal cell death compared to their uninfected counterparts after Puromycin treatment.A drug selection killing curve was established with higher Puromycin concentrations (2 µg/mL, 4 µg/mL,6 µg/mL) and monitored under a phase contrast microscope at 10X magnification. A high level of cell death was observed in uninfected SCp2 and SCg6 cells upon treatment with at least 2 µg/mL Puromycin concentration. On the other hand, infected SCp2 and SCg6 cells showed significant increase in cell number even after being treated with 6 µg/mL Puromycin while still showing low green GFP fluorescence (not shown).

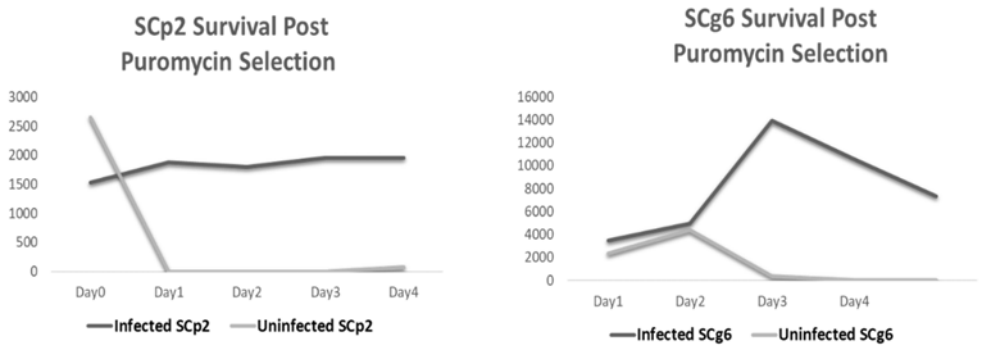


Figure 5 Quantification of the number of cells surviving after treatment with Puromycin at 6 µg/mL concentration.The number of surviving uninfected SCp2 cells dropped to zero after the first day of Puromycin treatment. Infected SCp2 cells remained viable and proliferated until the cells became confluent and a plateau was reached. Similarly, the number of viable uninfected SCg6 cells kept decreasing to reach zero viability at day 3 post Puromycin treatment. The number of infected SCg6 cells kept increasing until full confluence was reached.

To assess the miRNA expression efficiency after infection, the level of miR-183-5p expression was assessed in infected SCp2 and SCg6 cells using quantitative real-time PCR. The results showed that there was no significant expression of miR-183-5p in both infected SCp2 and SCg6 cells (Figure 5). This could possibly be attributed to low miR-183-5p promoter efficiency or the presence of transcriptional inhibitors acting on the promoter in SCp2 and SCg6 cells. Therefore, an alternative approach to viral transduction would be to exogenously supply miR-183-5p to SCp2 cells through conditioned media containing miR-183-5p, then determining the effect of miR-183 on differentiation and possible cancer initiation phenotypes.

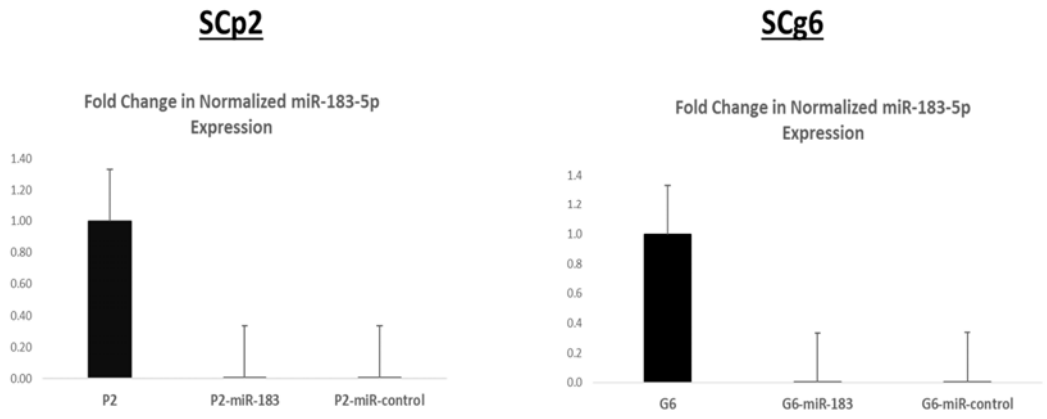


Figure 6 Infected cells express low levels of miR-183-5p as shown by RT-qPCR after infection.Fold change in normalized miR-183-5p expression in infected SCp2 (P2) and SCg6 (G6) cells. The graphs were plotted in excel and bars represent technical duplicate analysis of ± SEM.

B. SCp2 treated with culture conditioned media previously harvested from miR-183-5p infected S1 cells express high levels of miR-183-5p

One way for enhancing miR-183-5p abundance in SCp2 cells is by exogenously supplementing the cells with miR-183-5p and ensuring its uptake. Thus we aimed to treat SCp2 cells with conditioned medium collected from S1 cells previously infected and overexpressing miR-183-5p. We hypothesized that S1 cells overexpressing miR-183-5p could be releasing miR-183-5p into their medium. Therefore, by treating SCp2 with conditioned medium the latter might either directly take up the miRNA from the culture conditioned media or, other conditioned media components could induce miR-183 expression in SCp2 cells. To assess the validity of our hypothesis, SCp2 cells were first seeded in cell culture plates and then treated with culture conditioned medium (CCM) either harvested from S1-miR-183-5p infected cells, S1-uninfected cells, or miR-control-S1-infected cells. The levels of miR-183-5p expression in SCp2 in growth medium was also examined. miR-183-5p expression was highest in SCp2 cells treated with CCM collected from S1 infected with miR-183-5p compared to all other conditions and upon normalization to uninfected-S1 CCM (Figure 6). Therefore, the results were consistent with our hypothesis.

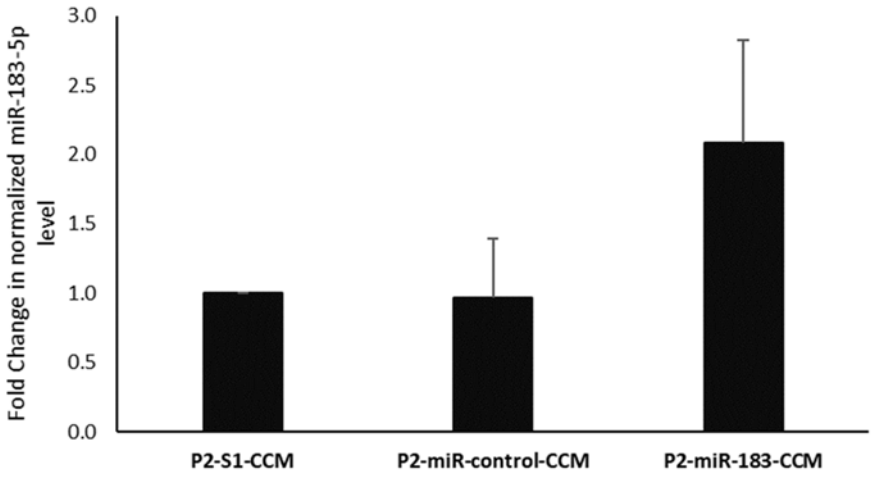


Figure 7 SCp2 (P2) treated with miR-183-5p-S1 conditioned media show elevated miR-183-5p expression compared to controls.Fold change in miR-183-5p expression was assessed between SCp2 in growth medium (GM), and SCp2 treated with culture conditioned medium (CCM) collected from S1-uninfected cells, S1-miR-183-5p infected cells, and S1-miR-control infected cells. miR-183-5p expression was highest in SCp2 treated with CCM from S1-miR-183-5p infected cells compared to those treated with S1-CCM and S1-miR-control-CCM, and to those in GM. Bars represent technical duplicate analysis of ± SEM.

C. Treatment with S1 conditioned media does not induce increase in proliferation in SCp2 cells

Data from our lab suggests that miR-183-5p overexpression is associated with tumor initiation phenotypes in S1 cells. Therefore, we suspected that the increased miR-183-5p expression in SCp2 upon treatment with CCM could lead to enhanced SCp2 proliferation. Therefore, we conducted a proliferation assay to assess the effect of CCM on SCp2 proliferation. SCp2 cells were first starved to slow their proliferation rates before spiking them with either CCM collected from S1-uninfected cells, S1-miR-183-5p-infected cells, S1-miR-control infected cells, freshly prepared S1 media (H14), or complete growth media. One well was kept under starvation conditions to ensure that the cells were starved and the growth rate was nearly constant. SCp2 cells were later

counted 48 hours and 72 hours after treatment with conditioned media or complete growth media to quantify the proliferation rates. SCp2 treated with complete growth media showed a significant increase in cell number after 48 and 72 hours. Cells treated with CCM showed no significant difference in cell counts after 48 hours and 72 hours (Figure 7). This suggested that treatment with CCM does not increase SCp2 proliferation rates.

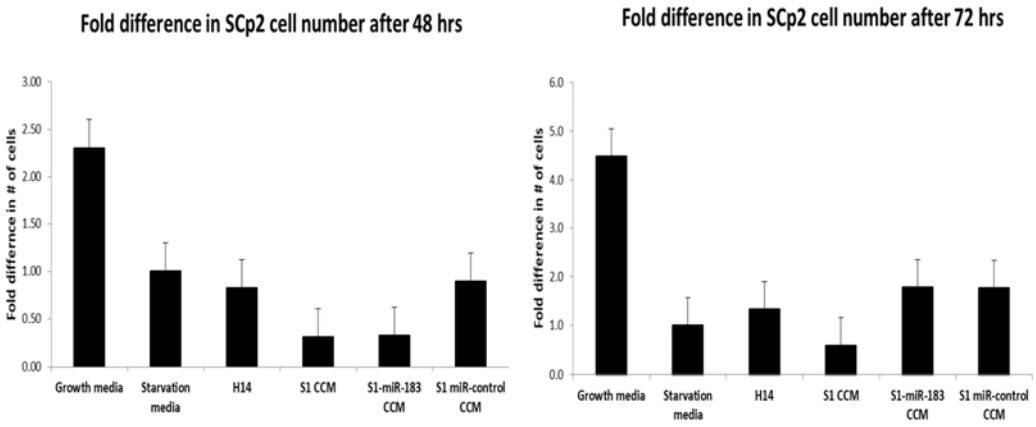
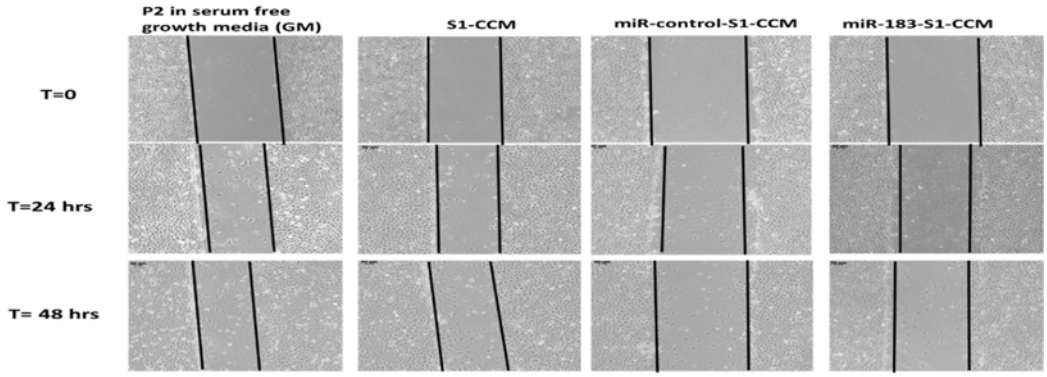


Figure 8 No significant difference in SCp2 proliferation was detected upon treatment with S1 culture conditioned media.SCp2 cells were first starved then kept in growth media or starvation media, or treated with either fresh H14 (S1 growth media), culture conditioned media (CCM) collected from S1 uninfected cells (S1 CCM), or from miR-183/miR-control infected S1 cells (S1-miR-183 CCM and S1 miR-control CCM respectively). After 24 and 72 hours, SCp2 cells showed a slight increase in cell number upon treatment with growth media compared to starvation conditions. The increase in SCp2 cell number after 48 and 72 hours of CCM-treatment was not significant in all treatment conditions as compared to starvation conditions. The experiment was repeated three times using different batches of cells. One-way ANOVA was used for statistical analysis of the difference between the aforementioned treatments. Bars represent triplicate analysis of ± SEM.

D. Treatment with S1 culture conditioned media does not impose any difference in cell migration on SCp2 cells

Knowing that miR-183-5p overexpression triggers cell invasion in S1 cells (Naser el din et al, 2021-manuscript under review), we sought to assess the effect of conditioned media treatment on the migration of SCp2 using wound healing assays. Upon full confluence, SCp2 cells were treated with either serum free growth media, or conditioned media collected from either S1 cells, S1-miR-183-5p infected cells, or S1-miR-control-infected cells. A straight wound was made, and the scratch site was monitored throughout time. The closure of the wound or scratched surface was measured after 24 and 48 hours from wounding. Closure of the scratched surface was not significantly different in cells treated with conditioned media from infected and uninfected S1 cells as compared to the untreated control (Figure 8).

A)



B)

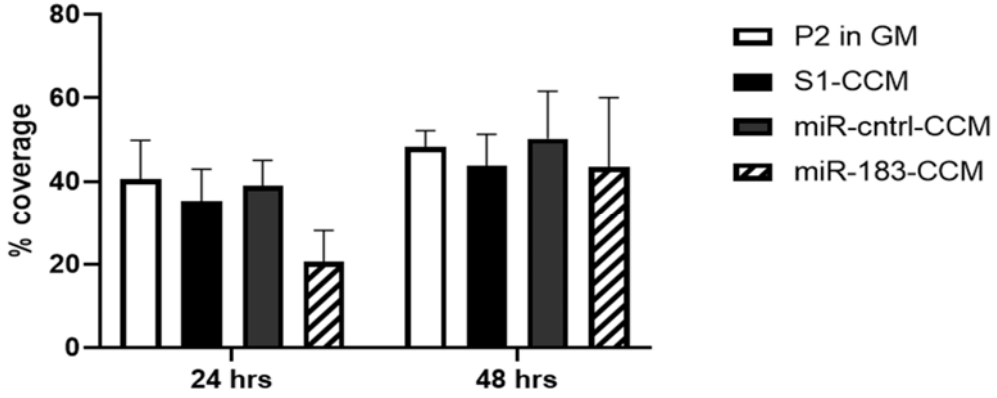


Figure 9 **A)** Treatment with S1 conditioned media has no significant effect on SCp2 cell migration.SCp2 cells show no enhanced migration after treatment with conditioned media collected from S1-uninfected, S1-infected with miR-183-5p, S1 infected with miR-control as compared with controls of SCp2 not treated with conditioned media as assessed by wound healing assay. **B)** Cell migration was measured as the percentage of cells covering the wound area after 24 and 48 hours post wounding. The experiment was repeated three times. One-way ANOVA in Graph pad prism v.8.4 was used for statistical testing of the differences between the conditions. Bars represent triplicate analysis of ± SEM.

E. Additives of conditioned media inhibit the expression of β-Casein by SCp2 cells in EHS drip conditions

The effect of miR-183-5p overexpression on differentiation of SCp2 has not been investigated yet. Therefore, we aimed to determine the influence of conditioned media-which possibly induces miR-183-5p overexpression in SCp2- on the expression of the differentiation marker β-Casein by SCp2 cells. Quantitative RT-qPCR was performed on SCp2 cells induced to differentiate using EHS drip, and treated with conditioned media collected from miR-183-5p infected S1 cells, miR-control-S1-collected cells, and uninfected S1 cells. SCp2 cells treated with differentiation media on drip were used as a positive control, while cells grown on plastic with non-

differentiation media were used as a negative control (not shown in the figure). The results showed that the expression of β-Casein was diminished in all samples treated with conditioned media collected from either infected on uninfected S1 cells (Figure 9).

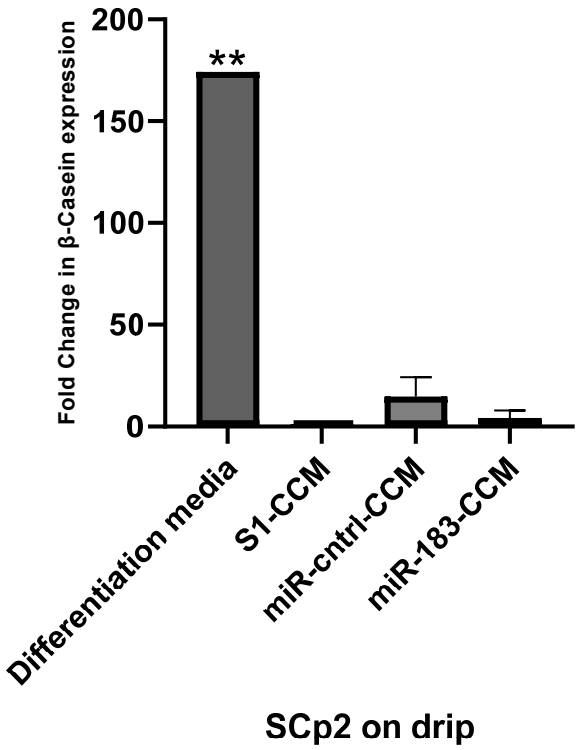


Figure 10 Conditioned media treatment disrupts β-Casein expression in SCp2 despite being induced to differentiate by EHS drip.

EHS matrix was dripped onto SCp2 to trigger differentiation after treatment with either differentiation media, or conditioned media collected from infected and uninfected S1 cells. SCp2 cells grown on plastic and in non-differentiation media (lacking prolactin) were used as a negative control for β-Casein expression (not shown in graph). Despite being under differentiation permissive conditions, SCp2 that were treated with conditioned media from both infected and uninfected S1 showed minimal expression of β-Casein compared to the positive control that showed over 150-fold

increase in β-Casein expression. The experiment was repeated twice using separate batches of cells. One-way ANOVA with multiple comparisons was used for statistical analysis of the difference among the conditions. Bars represent duplicate analysis of ± SEM.

S1-derived conditioned media normally contains a set of media additives that are required by S1 cells to survive and grow. In order to determine whether one of those media components is responsible for modulating β-Casein expression in SCp2, differentiation media was prepared and supplemented with each of the S1 additives alone, then the cells were induced to differentiate by EHS drip. The media additives supplemented were either apo-transferrin, sodium selenite, or β-estradiol. After performing RT-qPCR and quantifying β-Casein expression levels, it was shown that adding either one of the additives alone reduces β-Casein expression. Importantly, adding sodium selenite alone almost abolishes β-Casein expression in SCp2 on drip (Figure 10). To overcome this issue, SCp2 should be induced to differentiate by drip in S1 conditioned media lacking sodium selenite, before assessing the levels of β-Casein and miR-183-5p in them. Alternatively, exosomes-that might possibly contain miR-183-5p- could be extracted from miR-183-5p-infected-S1 conditioned media and directly supplied to SCp2 cultured on drip.

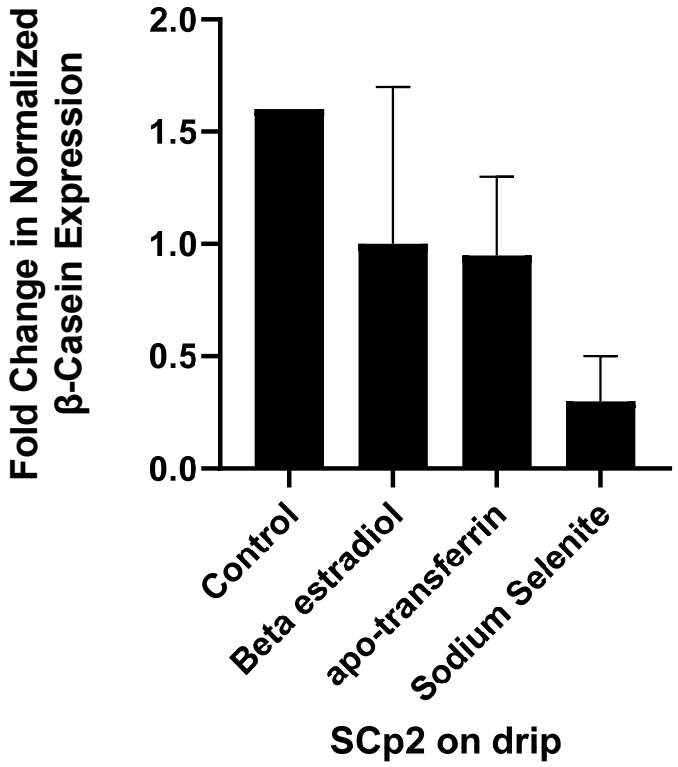


Figure 11 Sodium Selenite treatment leads to down regulation of β-Casein expression in SCp2 on drip.β-estradiol, Apo-transferrin, and Sodium selenite are cell culture media additives supplemented in S1 media for optimal cell growth, and therefore are present in the conditioned media used to treat SCp2. It was suspected that one of those additives could be responsible for blocking β-Casein expression by SCp2 cells as previously observed. To determine which of those additives is influencing β-Casein expression, SCp2 in differentiation media was supplemented with each additive alone on drip and the fold change in β-Casein expression was quantified by RT-qPCR. Although not significantly shown, SCp2 supplemented with either one of the additives show reduced β-casein expression compared to SCp2 in differentiation media on Drip without additives supplementation (DM drip), however, sodium selenite treatement showed the most decrease in β-Casein expression as compared to the non-treated control. Statistical analysis was done using one-way ANOVA with multiple comparisons via GraphPad prism v8.4 software. This experiment was repeated twice and error bars were plotted to represent duplicate analysis of ± SEM.

F. miR-183-5p is upregulated in exosome-containing extracts of conditioned media collected from S1-miR-183-5p infected cells

Knowing that miRNAs could be present in cell culture media within exosomes [116, 117], we sought to extract exosomes from conditioned media of infected and uninfected S1 cells. Differential ultracentrifugation was carried out on conditioned media collected from S1-miR-183-5p infected cells, S1-miR-control infected cells, and S1 uninfected cells. The level of miR-183-5p were then quantified by RT-qPCR. The results showed a high level of miR-183-5p in exosome extracts originating from miR-183-5p-infected S1 conditioned media but not from S1-miR-control nor S1 uninfected conditioned media (Figure 11).

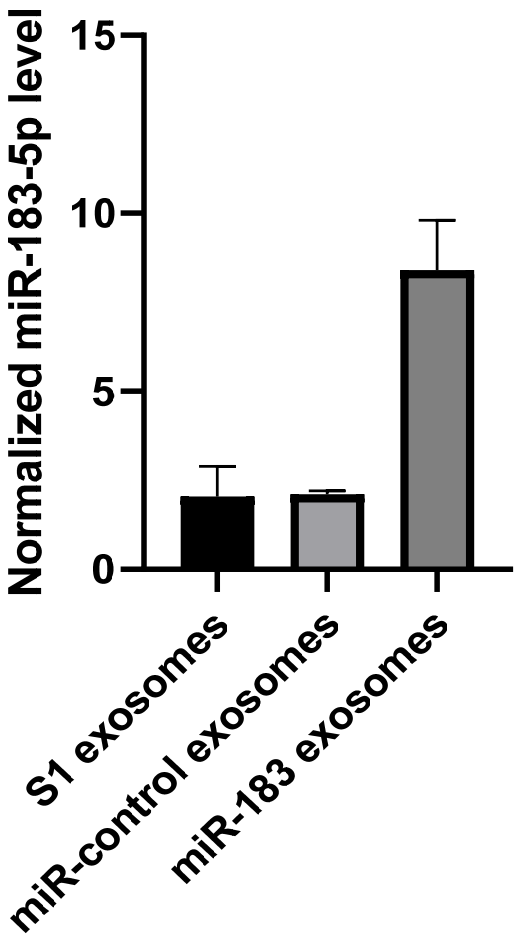


Figure 12 miR-183-5p level is elevated in exosome extracts collected from conditioned media collected from miR-183-5p-infected-S1 cells.Exosome extracts were obtained by differential ultracentrifugation carried out on conditioned media of infected and uninfected S1 cells. RT-qPCR was done to assess the levels of miR-183-5p in those extracts. The results revealed an elevated level of miR-183-5p in exosome extracts from S1-miR-183-5p conditioned media but not in those deriving from miR-control and uninfected S1 conditioned media. The graph was plotted via GraphPad prism v8.4 software. This preliminary experiment was done once and error bars were plotted to represent duplicate analysis of ± SEM.

G. SCp2 show higher expression of miR-183-5p after treatment with exosome extracts from S1-miR-183-5p conditioned media

One approach to induce miR-183-5p overexpression in SCp2 was to treat the cells with exosome extracts and check whether they will differentially express the miR-183-5p. This hypothesis was based on the previous results showing that the different extracts themselves have different miR-183-5p levels based on the type of conditioned media they were initially harvested from. SCp2 cells were treated with exosome extracts from infected and non-infected S1 conditioned media, and the levels of miR-183-5p in those cells were assessed by RT-qPCR. The results show that miR-183-5p is approximately 200 fold higher in SCp2 treated with exosomes from miR-183-5p-S1 derived conditioned media than those derived from miR-control-S1, and S1-uninfected conditioned media. This experiment is preliminary and error bars were plotted according to technical duplicate analysis of SEM for each condition (Figure 12). The next step is to determine the effect of exosome treatment on β-Casein expression. SCp2 should be cultured in differentiation media on drip and β-Casein expression assessed after treatment with miR-183-5p-S1-conditioned-media-derived exosomes, miR-control-S1-conditioned media-derived exosomes, or S1-conditioned media-derived exosomes.

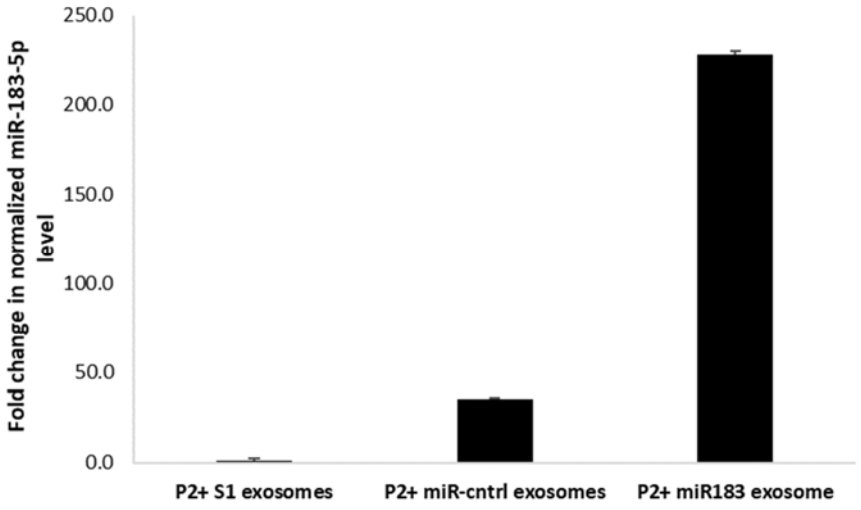


Figure 13 miR-183-5p levels are highest in SCp2 treated with miR-183-5p derived exosome extracts compared to exosomes from uninfected S1, and miR-control-infected S1 conditioned media.SCp2 treated with miR-183-5p derived exosomes show an approximate 200-fold increase in miR-183-5p compared to those treated with exosome extracts from uninfected S1, or miR-control-infected conditioned media. The graph was plotted via GraphPad prism v8.4 software. This preliminary experiment was done once and error bars were plotted to represent duplicate analysis of ± SEM.

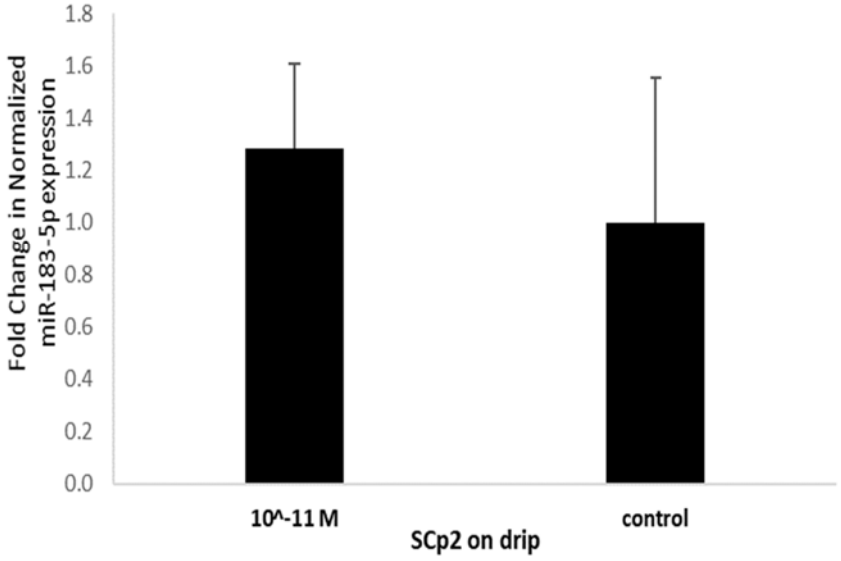
H. Triggering miR-183-5p overexpression by treating SCp2 cells with glyphosate (potential carcinogen)

A previous study has highlighted the role of glyphosate as potential tumor initiators in combination with miR-182-5p overexpression in mammary cells [118]. Further experiments at our lab showed that treating SCp2 cells with glyphosate for twenty-one days results in enhanced cell migration. Moreover, treating S1 cells with glyphosate triggers cell invasion and leads to disruption of lumen formation -associated with cell polarity and differentiation- in 3D cultures (unpublished data).

Due to their potential for triggering cancer-like phenotypes in SCp2 and S1 cells, and their link to miR-182-which is a member of the miR-183 cluster family [119], we aimed to check whether glyphosate influence onco-miR-183-5p in SCp2 cells. We

also hypothesized that glyphosate treatment might interfere with the differentiation of SCp2 and reduce β-Casein levels under differentiation permissive conditions. In order to answer these questions, SCp2 cells were treated with glyphosate at 10^{-11} M concentration for 21 days then induced to differentiate by EHS drip. The levels of miR-183-5p and β-Casein were assessed by RT-qPCR. The results revealed an estimated 1.4-fold increase in miR-183-5p expression in SCp2 cells treated with glyphosate as compared to a non-treated control (Figure 13-A). On the contrary, β-Casein expression was minimal in SCp2 cells on drip after treatment with glyphosate compared to an untreated control (Figure 13-B). Both experiments were repeated once and error bars were plotted according to technical duplicate analysis of the SEM.

A)



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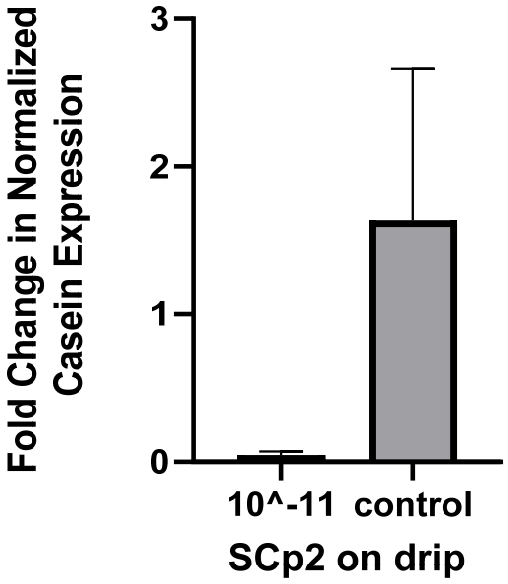


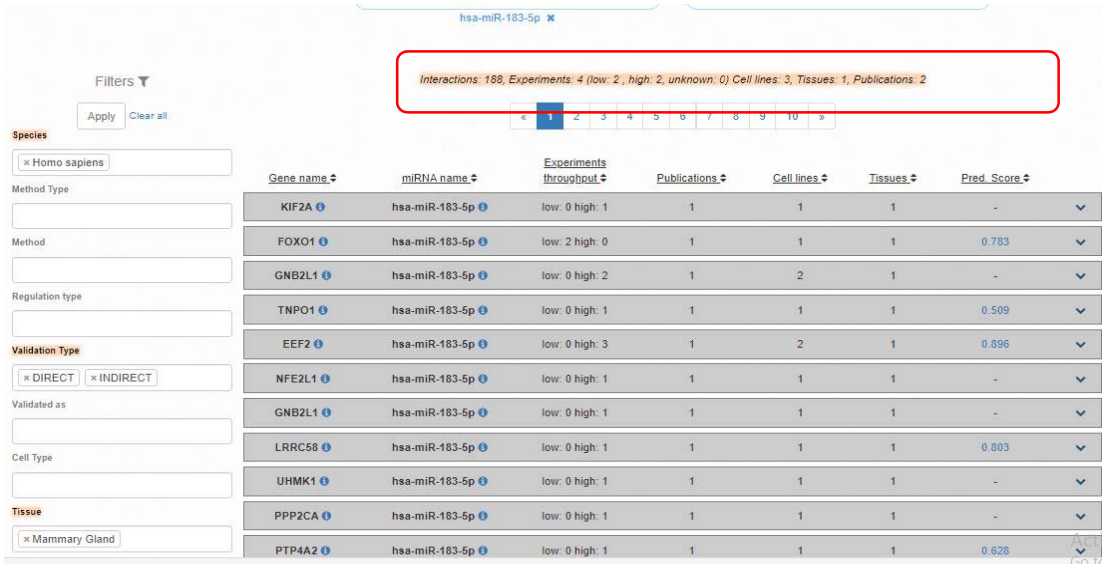
Figure 14 **A)** Treatment of SCp2 with 10-11 M glyphosate triggers elevated miR-183-5p expression, and **B)** hinders β-Casein expression in SCp2 induced to differentiate by EHS drip.SCp2 cells were treated with glyphosate for 21 days before being cultured with EHS matrix for four days to induce their differentiation. miR-183-5p expression increased under differentiation permissive conditions when the cells were treated with glyphosate 10^{-11}M showing an approximate 2-fold increase in expression compared to untreated controls. β-Casein expression in SCp2 on drip was diminished upon treatment with glyphosate as compared to the untreated positive control. As a negative control for drip in both experiments, SCp2 cells were seeded on plastic and supplied by non-differentiation media (NDM) lacking prolactin (not shown). The graphs were plotted via GraphPad prism v8.4 software. This preliminary experiment was done once and error bars were plotted to represent duplicate analysis of the SEM.

I. miR-183-5p has 188 experimentally validated target genes in humans, and 17 experimentally identified targets in mice

DIANA tools Tarbase V.8 was used to identify experimentally validated miR-183-5p targets in human and mouse tissue. That results showed that miR-183-5p interacts with 188 targets from various tissues in humans, among which 17 are restricted to breast cancer cell lines MCF-7 and MDA-MB-231 (Figure 14-A and B). In mouse, 9 experimentally validated targets were identified to interact with miR-183-5p (Figure 14-

C), none of which was validated in breast tissue. Using TragetScan, integrin β-1 which is an important signaling component leading to β-Casein expression, was found to be a predicted target for miR-183-5p (Figure 14-D).

A)



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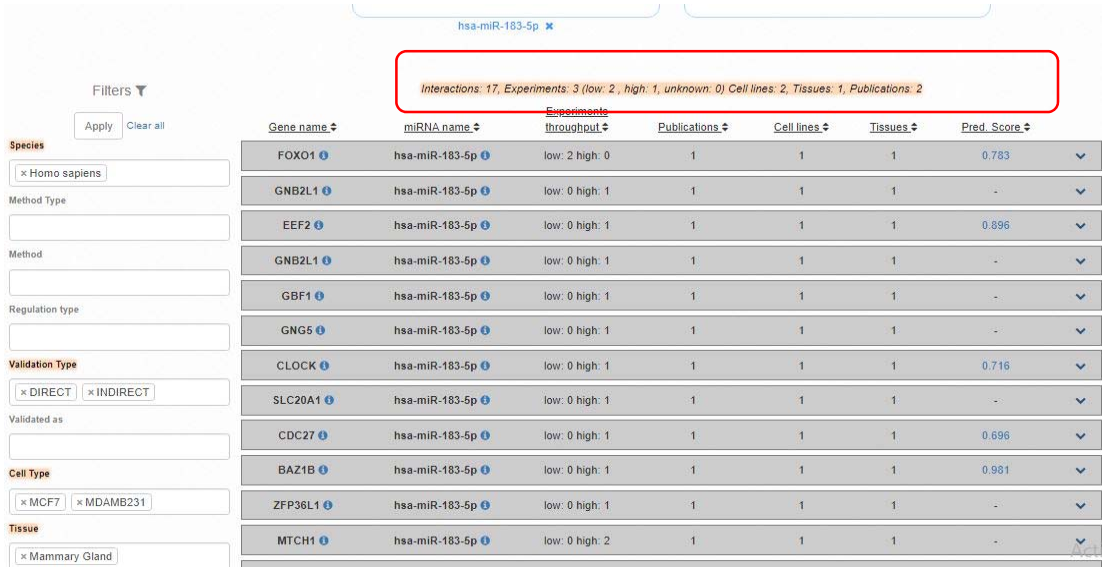

C)

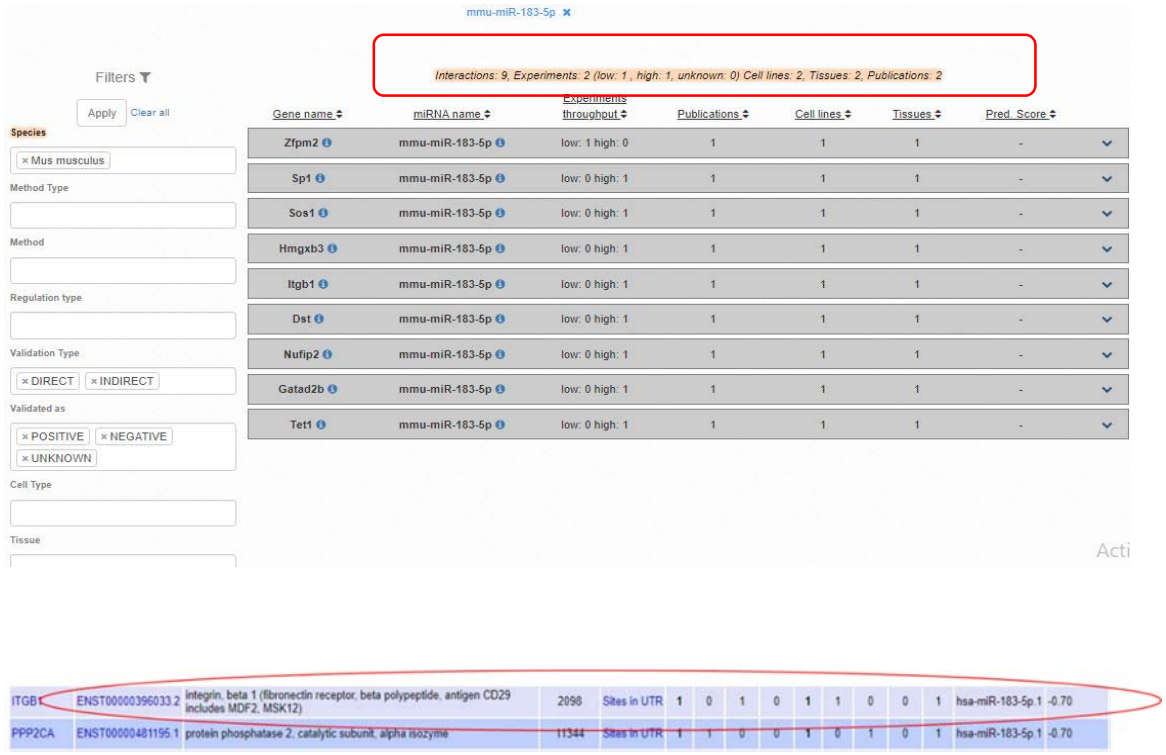

Figure 15 **A)** 188 experimentally validated targets of miR-183-5p were identified in various tissue in humans.**B)** 17 targets interact with miR-183-5p in breast cancer cell lines MDA-MB-231.**C)** In mouse, miR-183-5p interacts with 9 targets in different tissue. **D)** According to the bioinformatics tool TragetScan, β-1 integrin -a mjor contributor to β-Casein expression- is a predicted target for miR-183-5p.

CHAPTER V

DISCUSSION

Dysregulated patterns of miRNA expression have been reported in many cancer types [53, 54], including breast cancer [3, 55, 56]. One example of a cancer-associated miRNA is miR-183. miR-183 is a member of the miR-183-96-182 cluster located on the 7q31-34 locus of the human chromosome and is highly conserved among species. The expression of miR-183 varies between different normal and tumor tissues, and some studies reported opposite miR-183 expression patterns even in the same tumor type [4].

A previous study at our lab confirmed the association of miR-183-5p overexpression with a tumor initiated phenotype in normal ductal breast epithelial cell lines (S1 cells) induced to overexpress miR-183-5p by viral infection (Naser el dine et al, manuscript in preparation). Our observations were consistent with other studies showing the involvement of miR-183 in the initiation and development of various tumors including breast [120], hepatic [121], and endometrial tumors [122]. Furthermore, the contribution of miR-183 to breast tumorigenesis has been extensively studied in the context of ductal epithelial neoplasia [12], and despite being found to be overexpressed in invasive lobular carcinoma and lobular carcinoma in situ [12], the role of miR-183 in the development of lobular neoplasia remains uninvestigated. Therefore, we aimed to understand the molecular signaling targets of miR-183-5p involved in breast lobular tumor initiation, with special focus on tumor initiating targets that modulate Cell-ECM and Cell-Cell communication. This was based on studies showing that miR-183-5p targets integrin-β1 (ITGB1)- an essential mediator of cell ECM communication- in several tumor tissue such as melanoma tissue [123], endometrial

stromal cells [124], and cervical cancer cell lines [80]. In breast cancer cell lines, miR-183 overexpression promotes cell growth and migration by targeting RAB21 which is an essential regulator of integrin internalization and recycling [78]. Similarly, studies done by our team have shown that a breast cancer-like phenotype could be initiated in non-tumorigenic S1 cells by either overexpressing miR-183-5p (Naser el dine et al, manuscript in preparation), or by knocking out Cx43, a major mediator of gap junction intercellular communication, through non-canonical Wnt signaling pathway [125]. In this study, we speculated that miR-183-5p overexpression could initiate a lobular cancer-like phenotype, and lead to loss of differentiation in a model that recapitulates functional cell-cell and cell-ECM interactions. Our model consists of SCp2 lobular mammary epithelial cells that express β-casein as a differentiation marker in response to prolactin stimulation and in the presence of an exogenously provided extracellular matrix through ITGB1 signaling [126, 127]. Alternatively, SCp2 can differentiate in an ITGB1 independent, gap junction intercellular communication (GJIC)-dependent mechanism. Treating SCp2 with the gap junction inducer cAMP in the absence of exogenously provided basement membrane is sufficient to trigger differentiation [15, 127]. Co-culturing SCp2 with SCg6-myoepithelia-like cells also results in differentiation and β-casein expression through the assembly of a gap junction complex comprising connexin43, α-catenin,β-catenin, and ZO-2 [14]. Therefore, determining the effect of miR-183-5p overexpression on β-casein expression in SCp2 cells might provide insight into the effect of miR-183-5p on cell-ECM and GJIC-mediated lobular tumor initiation targets.

To determine the effect of miR-183-5p overexpression on the differentiation and possible tumor initiation in lobular mammary epithelial cells, we sought to stably infect

both SCp2 and SCg6 (myoepithelia-like) cells with miR-183-5p. We proposed that miR-183-5p overexpression could be sufficient to impede differentiation and downregulate β-Casein expression in SCp2 cells. Furthermore, we aimed to determine the effect of miR-183-5p overexpression in SCg6 myoepithelia-like cells on the differentiation of SCp2 in co-cultures. Knowing, and miRNAs can be transferred between neighboring cells via gap junction channels in multiple tissue types [128-130], we speculated that miR-183-5p in SCg6 could either directly lead to dysfunctional GJIC, or could be transferred from miR-infected SCg6 cells to uninfected SCp2 cells. In case the latter was valid, we hypothesized that miR-183-5p could possibly induce transcriptional repression of downstream targets leading to loss of differentiation, and tumor initiation in SCp2. To induce miR-183-5p overexpression, SCp2 and SCg6 were stably infected with a viral vector carrying miR-183-5p gene. The infected cells showed minimal expression of miR-183-5p after infection despite showing resistance to Puromycin selection. The results obtained could be explained by the possible low expression efficiency of the human CMV (HCMV) promoter upstream of miR-183-5p gene within the viral vector that was used. CMV promoters have shown high levels of transgene activation post transduction in many cell lines [131] including our previously reported non-tumorigenic S1 breast epithelial cell line (Naser el dine et al, manuscript in preparation). However, several studies have also reported low transduction efficiency when CMV promoters were used to drive transgene expression in multiple cell types. For example, CMV promoter resulted in less than 1% transduction efficiency compared to EF promoter in mouse embryonic stem cells (ES). It was postulated that ES cells do not express the transcription factors necessary for driving CMV promoter activity [132]. The CMV promoter may also show variations in transduction efficiency among cells

derived from different species. In fact, Ghaneialvar et al showed that mesenchymal stem cells (MSC) isolated from mouse and goat tissue showed low CMV transduction efficiency-as observed by low GFP expression- compared to human and ovine -derived cells exhibiting high CMV promoter activity [133]. On the other hand, infecting murine adenocarcinoma cells with viral vectors in which the transgene expression is under the control of a human HCMV promoter resulted in 10-100-fold lower transgene expression compared to human cells infected with the same vectors. Infecting the same cells with viral vectors having murine CMV promoters lead to 5-30-fold increase in transgene expression compared to the HCMV promoters [134]. These results, in addition to a previous observation of poor CMV promoter efficiency specifically in SCp2 and SCg6 (unpublished data) suggests that the low CMV promoter activity might be leading to low miR-183-5p and GFP expression in infected SCp2 and SCg6 cells.

miRNAs are present in many extracellular body fluids such as plasma, breast milk, saliva, cerebrospinal fluid, and urine [66]. Cell lines in culture also release miRNAs into their culture media [116, 135]. Studies revealed that the released miRNAs remain stable due to them being either incorporated within exosomes upon release, or bound to RNA-binding proteins such as Ago2 [135, 136]. For example, a study by Pegtel et al showed that B cells infected with EBV release EBV-associated-miRNAs into exosomes, and that target cells internalizing the EBV-exosomes accumulate the EBV-associated miRNAs and trigger specific repression of target genes [137]. Thus, as an alternative to the failed viral transduction approach, we sought to increase the levels of miR-183-5p in SCp2 cells by treatment with either conditioned media collected from S1-miR-183-5p-infected cells, or exosome extracts isolated from them. We suggest that miR-183-5p is possibly being released by S1-miR-183-5p-infected cells into the

conditioned media in exosomes and is being taken up by SCp2 cells. Alternatively, other media and exosome components could be directly influencing the expression of miR-185p in SCp2 cells. Nevertheless, treatment with conditioned media derived from both infected and uninfected S1 cells inhibited β-Casein expression, which suggested the presence of inhibitors in the conditioned media deriving from S1 cells. S1 derived media contains multiple additives that result in a relative decrease in β-Casein expression when added separately to SCp2 in differentiation media on drip.

Importantly, sodium selenite treatment alone leads to a near abolishment of β-Casein expression in SCp2. Sodium selenite is usually added to some cell culture media-including S1 cells- to protect the cells from oxidative damage and inhibit lipid peroxidation [138-140]. However, in some reports selenite treatment was associated with cytotoxic and genotoxic effects [141, 142]. For example, sodium selenite treatment inhibited cell growth and induced quiescence and hypervacularization of the COMMA-D mouse mammary epithelial cell line TM6 [141]. Thus, sodium selenite could be the factor leading to diminished β-Casein expression upon conditioned media treatment. On the other hand, exosome miRNA uptake has been previously reported, therefore, delivering miR-183-5p-containing exosomes to cells might provide and alternative way for triggering miR-183-5p abundance in SCp2 without the need for transduction methodologies. For example, Guo et al found that miR-183-5p was transferred into macrophages within exosomes derived from breast cancer 4T1-cell lines. miR-183-5p targeted *PPP2CA* leading to the expression of pro-inflammatory cytokines by the macrophages, thus contributing to breast cancer progression in 4T1 breast tumor model [143]. Furthermore, Shnag et al showed that miR-183-5p was significantly expressed in colorectal cancer cell (CRC)-derived exosomes, and that it promotes angiogenesis

through regulating the expression of VEGF and ANG2. miR-183-5p also increased proliferation, migration of CRC cells by targeting FOXO1 [144]. We hypothesized that miR-183-5p delivered in exosomes could influence gene expression in SCp2 and negatively regulate β-Casein expression by targeting members of ITGB1/STAT5 signaling. This was based on a previous study showing that miR-183 decreases β-Casein expression in goat mammary epithelial cells [145]. Similarly, β-Casein expression decreases in cow mammary epithelial cells after being transfected with miR-183 mimic [146]. Notably, the two aforementioned studies, focused on the association between the upregulation of miR-183 and milk fat metabolism, and aimed to enhance milk quality rather than studying miR-183 targets in the context of mammary tumor initiation and cell communication. For further support of our above mentioned hypothesis, we looked into the experimentally validated breast-specific targets of miR-183-5p via the bioinformatics tool "Tarbase V.8 DIANA tools", 188 targets of miR-183-5p are identified in the human breast, 17 of which were identified in the breast cancer cell lines MDA-MD-231 and MCF7. In mice, mmu-miR-183-5p interacts with 9 validated targets from different tissue types. No validated targets for mmu-miR-183-5p were found in mouse mammary cells. Interestingly, miR-183-5p targets β1-integrin by binding to its 3'UTR in breast cancer cells [147], HeLa cells[148],and endometrial stromal cells [124]. Therefore, it is possible that miR-183-5p inhibits β1-integrin in SCp2 cells, which might indirectly lead to the downregulation of β-Casein expression. An additional experiment involving treatment of SCp2 with exosomes under differentiation permissive conditions then assessing β-Casein expression should be done as a first step to validating the proposed link between miR-183-5p and ITGB1/STAT5 pathway.

miRNA expression in cancer is influenced by multiple factors that act at the post transcriptional and transcriptional levels. Chemical compounds being either endogenous (hormones, chemokines), or exogenous (xenobiotics) may interfere with the processing and stability of miRNAs, thereby, regulating their expression at the post transcriptional level [62]. For instance, Malliot et al showed that a wide set of miRNAs are repressed upon treatment with estrogen, and that the re-expression of those repressed miRNAs reduced estrogen-dependent cell growth of breast cancer cell lines [65]. Glyphosate –an active ingredient in many herbicides- has been linked to miRNA dysregulation in various models. For instance, glyphosate exposure leads to the upregulation of 55 and downregulation of 19 miRNA in the pre-frontal cortex of post-natal mice offspring [149]. As another alternative to viral transduction, we therefore aimed to investigate whether glyphosate treatment increases miR-18-5p expression leading to the downregulation of tumor suppressing signaling pathways involved in cell communication and differentiation in SCp2. In breast cancer, glyphosate treatment in combination with miR-182-5p overexpression lead to tumor initiation in mice, and the breast epithelial MCF10A cell line. In the latter study, Duforestel et al showed that non-tumorigenic MCF10A treated with glyphosate, and induced to overexpress miR-183-5p adopted a tumor-initiated phenotype manifested as an increase in the cells' invasion and migration potential. Those results were attributed to a global DNA hypo-methylation induced by glyphosate exposure that influenced TET pathway related genes [118]. The role of glyphosate in promoting altered DNA methylation patterns and cancer progression has been also reported in other models such as in human peripheral blood mononuclear cells [113] and uterine tissue of female rats [110]. Notably, epigenetic modifications such as promoter hypo- and hyper-methylation, and mutations in the

miRNA genes also affect miRNA expression [62]. Methylation of the CpG islands in the 5'upstream region of the miR-183/96/182 cluster leads to its transcriptional inactivation. In fact, hypo-methylation of the miR-183 cluster has been documented in hepatocellular carcinoma (NBNC-HCC) cells, and was correlated with the manifestation of its tumor phenotype [150]. Therefore, we propose that miR-183-5p expression might be upregulated in SCp2 cells due to a glyphosate-induced hypo-methylation of the promoter regulating miR-183-5p or any of its transcriptional regulators. In addition to direct promoter methylation, glyphosates may induce methylation of genes that indirectly influence miRNA expression. Intergenic miRNAs genes have their own promoters, and thus are regulated by the transcription factors that directly interact with their promoter regions. For instance, the activation of Wnt/β-Catenin signaling pathway has been correlated with miR-183 overexpression in hepatocellular carcinoma. It was shown that the downregulation of Wnt/β-Catenin signaling occurs concomitantly with the downregulation of miR-183 expression.

Furthermore, β-Catenin-TCF-LEF-1 bind to the promoter of miR-183 and activates its transcription in human gastric cancer cells. miR-183 overexpression in breast cancer may be regulated by HSF2 transcription factor. Similarly, ZEB1 suppresses the transcription of miR-183, however, miR-183 can also target ZEB1 and downregulate its expression in breast cancer [4]. Moreover, a study by Chang et al showed that ectopic expression of P53 in MCF12A and HMEC significantly upregulated the miR183 expression. Co-immunoprecipitation analysis showed that P53 interacts directly with miR-183 promoter and activates its expression [151]. Therefore, further investigation is required to determine whether glyphosate treatment could directly affect the expression of either miR-183, or any of its regulatory factors.

CHAPTER VI

CONCLUSION

Viral transduction of miR-183-5p using a CMV promoter in SCp2 and SCg6 mouse-derived mammary epithelial and myoepithelia-like cells respectively, results in low transgene expression efficiency. We expect that alternatively, miR-183-5p might be released into cell culture conditioned media from S1 cells previously infected with miR-183-5p possibly through exosomes. Treating SCp2 mouse lobular epithelial cells with either conditioned media derived from miR-183-5p infected S1 cells, or exosome extracts isolated from those conditioned media might lead to an increase in miR-183-5p in the target SCp2 cells. This could possibly be attributed to either direct uptake of miR-183-5p from conditioned media or exosomes by SCp2, or other media and exosome components might be influencing miR-183-5p expression by SCp2 cells. Further studies are needed to determine the exact mechanism by which miR-183-5p abundance is triggered in SCp2 upon treatment with conditioned media and exosomes. An additional experiment should be done to determine the effect of miR-183-5p on β-casein expression after conditioned media/exosome treatment. This could be done by inducing differentiation of SCp2 on drip and then assessing the levels of β-casein expression.

Glyphosate treatment at low concentration (10^{-11} M) might also increase miR-183-5p expression in SCp2 cells. Concomitantly, glyphosate exposure might result in the downregulation of β-casein expression by SCp2 cells cultured under differentiation permissive conditions. These suggestions could possibility occur due to the well-established role of glyphosate in inducing genome-wide DNA aberration. However, further studies are needed to confirm the association between glyphosate exposure,

miR-183-5p expression, and cell-cell/cell-ECM-dependent tumor initiation as assessed by β-casein expression in our SCp2 model of mammary epithelial differentiation.

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