Role of Alpha 6 Integrin in Cancer Stem Cell Plasticity

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Abstract

Stem cells are characterized as having high plasticity since they are able to change their phenotype in response to extracellular signals. Interestingly, most human tumors contain a minor subpopulation of high malignant cells with stem cell characteristic and high plasticity as well. These cells are known as cancer stem cells (CSC) or tumor initiating cells because of their strong capability to originate and maintain tumors. The phenotype of normal and cancer stem cells is tightly controlled by α-6 Integrin dependent pathways. α-6 Integrin, a stem cell marker, is expressed in two splicing forms, variant A and B. The former has been identified to play a role in tumor growth while the latter has been found to be responsible for cell stemness. We hypothesize that α-6 Integrin plays an important role in CSC plasticity. To test our hypothesis, we cultured MB-231 breast cancer cell line, which contains a high percentage of CSC. We treated MB-231 cells with conditioned media from induced pluripotent stem cells (iPSC) assuming that soluble factors secreted by normal stem cells (iPSC) could induce changes in CSC phenotype. Breast CSC are characterized as $CD44^{+}/CD24^{-/low}/CD49f^{high}$ and thus were able to evaluate potential changes in cell phenotype by FACS analysis. The gene expression of α-6 Integrin was analyzed by RT-qPCR. Our results have shown that indeed iPSC conditioned media was able to modify the CSC phenotype to a potentially less tumorigenic type. These modifications in CSC phenotype were accompanied with changes in α-6 Integrin expression. We also analyzed by using mass spectrometry putative secreted factors present in conditioned media of induced pluripotent stem cells. The identified proteins are compatible as members of exosomes. Indeed exosomes have been recently identified as mediators of intercellular communication in tumor microenvironment. α-6 integrin as cell membrane receptor could play an important role in mediating exosomes signaling and be regulated by exosomes as well. In conclusion, our results have shown that it is possible to induce changes in the CSC phenotype and that α-6 Integrin is a good molecular marker for tracking these modifications.

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List of Abbreviations

A6IT	Alpha 6 Integrin
CM	Conditioned Media
CSC	Cancer Stem Cells
ESC	Embryonic Stem Cells
EV	Extacellular Vesicles
HIF	Hypoxia-inducible Factors
HUVEC	Human Umbilical Vein Endothelial Cell
iPSC	Induced Pluripotent Stem Cell
MV	Microvesicles
PCR	Polymerase Chain Reaction
ROS	Reactive Oxygen Species
TIC	Tumor Initiating Cell
TNF	Tumor Necrosis Factor

1 Introduction

1.1 Stem Cells

1.1.1 Normal Stem Cells

Stem cells can be classified into two major groups: embryonic and adult stem cells. Embryonic stem cells (ESC) are pluripotent and divide symmetrically. Hence, ESCs can develop into all kind of cells. The symmetric division of stem cells allows the generation of two ESC from one parent ESC.

The recent discovery of induced pluripotent stem cells provides good alternatives to human-derived stem cells. These were created by de-differentiation of somatic cells and are very similar to ESC regarding pluripotency and symmetric cell division.

Another group of stem cells is known as adult stem cells, which are stem cells that originated from ESC but have the capacity to generate into different types of cells that can form each organ of the body. This type of stem cells undergoes asymmetric division, in which one adult stem cell divides into two daughter cells. One daughter cell has the capacity to differentiate to the corresponding cell of each tissue and the other has the same stem cell characteristic as the mother cell. The latter mechanism is called “self-renewal”. Adult stem cells are multipotent but not pluripotent. Some examples of adult stem cells are mesenchymal stem cells, hematopoietic stem cells in bone marrow, and limited number of stem cells in different tissues such as the heart, the brain, and the liver.

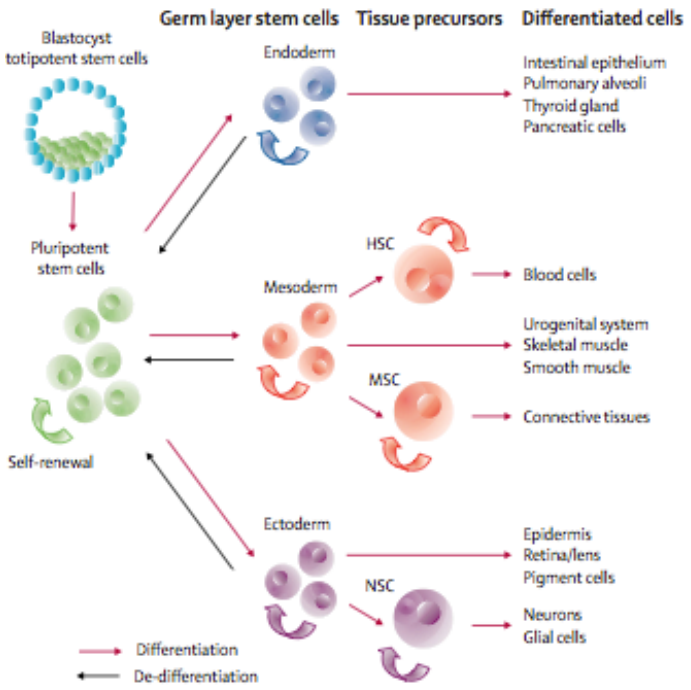

Figure 1 Classical hierarchical model of stem cell differentiation. A scheme of the asymmetric division of stem cells, producing both self-renewed stem cells and differentiated mature cells. The reverse arrows show the recently discovered ability of committed cells to dedifferentiate.

1.1.2 Cancer Stem Cells

Over the past few years, the understanding of cancer formation and growth has increased. Now it is understood that tumors do not consist of a homogenous mass of proliferating cells with same genetic mutations, but is an abnormal heterogeneous tissue consisting of a hierarchy of cells with different levels of capacity to generate tumor cells. Cells with high capacity to generate new tumor cells are called “tumor initiating cells” (TIC). Since TIC presents several similarities to adult stem cells of the corresponding tissue, they may also be called Cancer Stem Cells (CSC) (Tirino et al., 2013). Findings have shown that these proliferating cells with stem cell properties play a role in the development and perpetuation of many forms of human cancer (Singh, Hawkins, Clarke, & Squire, 2004; Tannishtha, Morrison, Clarke, & Weissman, 2001). Eradicating the stem cell character of the tumor may lead to a stable and lifelong remission and even a cure of cancer.

Normal stem cells are unique for their combination of capabilities to self-renew, to develop into multi-lineages and also to proliferate expansively. The balance of self-renewal is vital since abnormally increased self-renewal combined with the intrin-

sic growth potential of a stem cell may be considered a malignant phenotype (Al-Hajj & Clarke, 2004; Pardal, Clarke, & Morrison, 2003). These malignant cells are the cancer stem cells, which have the ability to self-renew, to develop into any cell in the overall tumor population and proliferate extensively to expand the population of malignant cells (Figure 2). Hence, we find properties that are closely parallel to these three features found in the normal stem cells. Knowing this, it is assumed that cancer stem cells may arise by mutation from normal stem cells. Yet some studies have shown that cancer stem cells may develop from mutated progenitor cells (Cozzio et al., 2003; Huntly et al., 2004; Jamieson et al., 2004; Krivtsov et al., 2006). This type of cells has the capacity to replicate but does not have the ability to self-renew like stem cells. To be converted to a cancer stem cell, a progenitor cell must mutate in a way that it receives the ability to self-renew. Although this is still highly discussed, the development of cancer stem cells might also be engaged in the following events: firstly, a change in the microenvironment of the stem cell niche within a tissue, secondly, mutations in cellular metabolism, cell cycle, signaling pathways or lastly, amplification of cell populations with genetic mutations that can give rise to heterogenic primary tumors and metastasis (Klonisch et al., 2008).

The key clinical problems of these malignant cells are that they are known to be drug-resistant, responsible for relapse after cancer treatment, and that they can give rise to metastases. It is very clear that cancer therapy that fails at eradicating cancer stem cells may lead to the regrowth of the tumor. This is so because traditional cancer therapies normally target normal cancer cells but spare cancer stem cells (Liu, Chen, Yang, Pan, & Zhang, 2011). So in cases in which cancer cells were eliminated via treatment yet a relapse takes place, it can be concluded that the cancer stem cells have not been entirely killed. Therefore the cancer stem cell hypothesis suggests that developing and finding targeted therapies that aim cancer stem cells should lead to a more effective treatment of cancer and reduce the risk of relapse and metastasis. Numerous studies of different cancer types support the concept that cancer development and propagation is lead by a minor subpopulation of highly malignant cancer stem cells within a particular tumor (Huang, Heidt, Li, & Simeone, 2007).

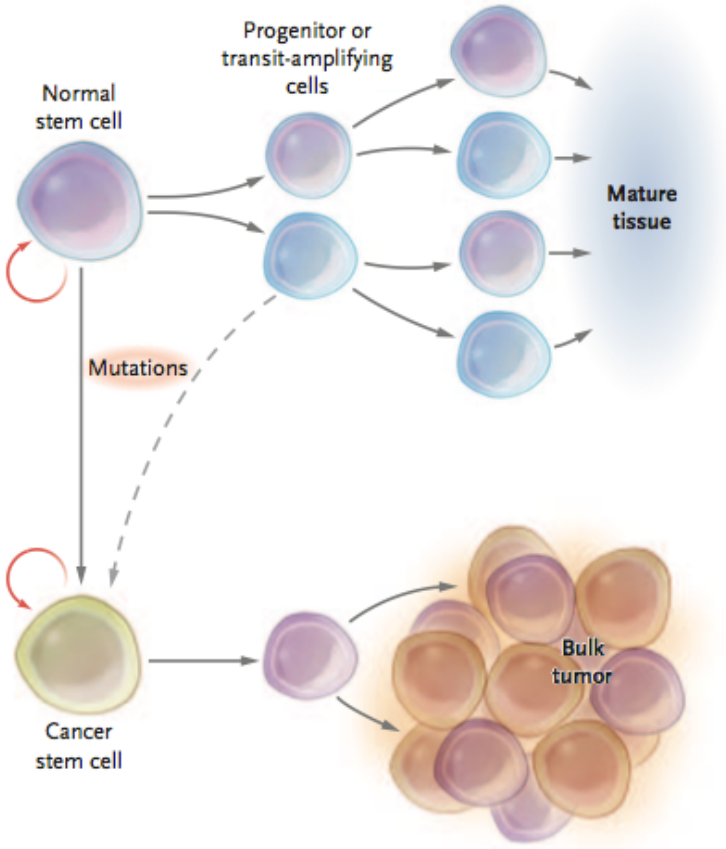

Figure 2 Stem Cell-Systems. Normal tissues are developed from a normal stem cell that differentiated to create progenitor and mature cells. Normal stem cells are known to self-renew, to have multilineage potential and also extensive proliferation. Cancer stem cells may subsequently grow and differentiate to create tumors. Cancer stem cells are known to be the malignant phenotype of the stem cells (Jordan, Guzman, & Noble, 2006) (Jordan et al., 2006).

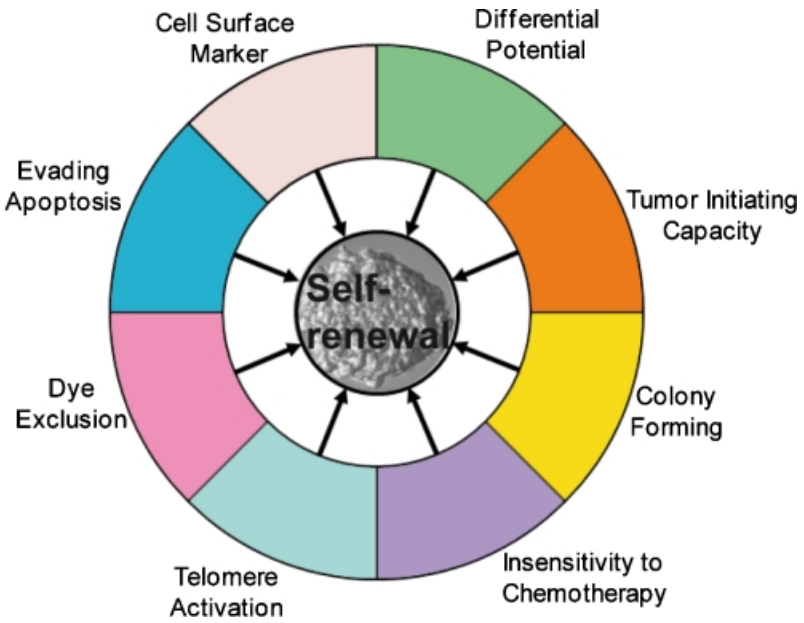

Figure 3 Acquired capabilities of CSCs. It is suggested that most if not all CSCs acquire the same set of functional capabilities during their development, albeit through various mechanistic strategies (Regenbrecht, Lehrach, & Adjaye, 2008).

1.1.3 Cell Plasticity

In the past few years, groundbreaking advances in stem cell biology have been shown. Due to “reprogramming”, for instance, a differentiated cell could be converted to an induced pluripotent stem cell by engineering a few transcription factors. Lately, a new form of “reprogramming” was identified, when a conversion from one differentiated cell to another differentiated cell was possible without going through an undifferentiated and pluripotent state (Bonfanti, Barrandon, & Cossu, 2012).

Cell plasticity is defined as the ability of a cell to change its fate in response to extracellular signals. This type of reprogramming is known as environmental or extrinsic factor-mediated, e.g. the reprogramming was a result of experimental manipulations in vitro or therapies in vivo. In contrast, there is the transcriptional or intrinsic factor-mediated reprogramming. An example would be the generation of induced pluripotent stem cells, where genetic engineering has to take place for “reprogramming” (Bonfanti et al., 2012).

ESCs are known to have very high plasticity, due to the fact that they can self-renew and differentiate into every somatic cell. The differentiation of ESCs leads to permanent cell cycle arrest and loss of cellular plasticity. Cancer cells preserve high plasticity, in which they can switch between epithelial and mesenchymal phenotypes (Strauss, Hamerlik, Lieber, & Bartek, 2012).

In solid tumors, the microenvironment can control cancer cell plasticity. This is why high plasticity and heterogeneity of cancer stem cells can make treatment strategies more complicated, as some signaling pathways are only active in certain cellular phenotypes. Additionally, due to the cancer stem cells’ ability to proliferate while cell cycle repressors are absent, mutations occur that escape targeted therapies (Strauss et al., 2012).

1.2 Cancer cells

Over the past few years there were a lot of takes on how the process of carcinogenesis works. In the early 1990s scientists believed that cancer was a somatic cell disorder, however, Tyzzer quickly presented the notion of somatic mutation in

connection with cancer. Later, Boveri presented the cancer genetic hypothesis, in which he talks about chromosomal abnormalities being necessary in carcinogenesis. Decades later, Nowell introduced the multistep genetic model of tumorigenesis and in 2000, Hanahan and Weinberg explained the somatic mutation theory, which is now a very well-known classical model of carcinogenesis (Figure 4). Lately, Feinberg et al added the aspect of epigenetics, which deals with the mutations in global DNA methylation that may lead to abnormal activation of proliferation genes and the silencing of tumor suppressor genes. This group also suggests that tumor progenitor genes induce epigenetic disruption of stem/progenitor cells and that the epigenetic plasticity combined with genetic injuries play a big role in tumor cell heterogeneity and tumor progression (Ciurea et al., 2014). Due to the broad diversity of cancer types, it is very difficult to find common characteristics. However, they share at least one feature, which is the excessive and uncontrolled growth of abnormal cells that penetrate and destroy tissues (O'Brien, Kreso, & Dick, 2009). Cancers are not only a group of identical neoplastic cells, but is a tumor that has its own system consisting of vasculature, inflammatory cells and (extra-) cellular components (Rich & Eyler, 2008). Progress in understanding new concepts on cancer discussed in this thesis has led to improvements in diagnosis and treatment of cancer patients.

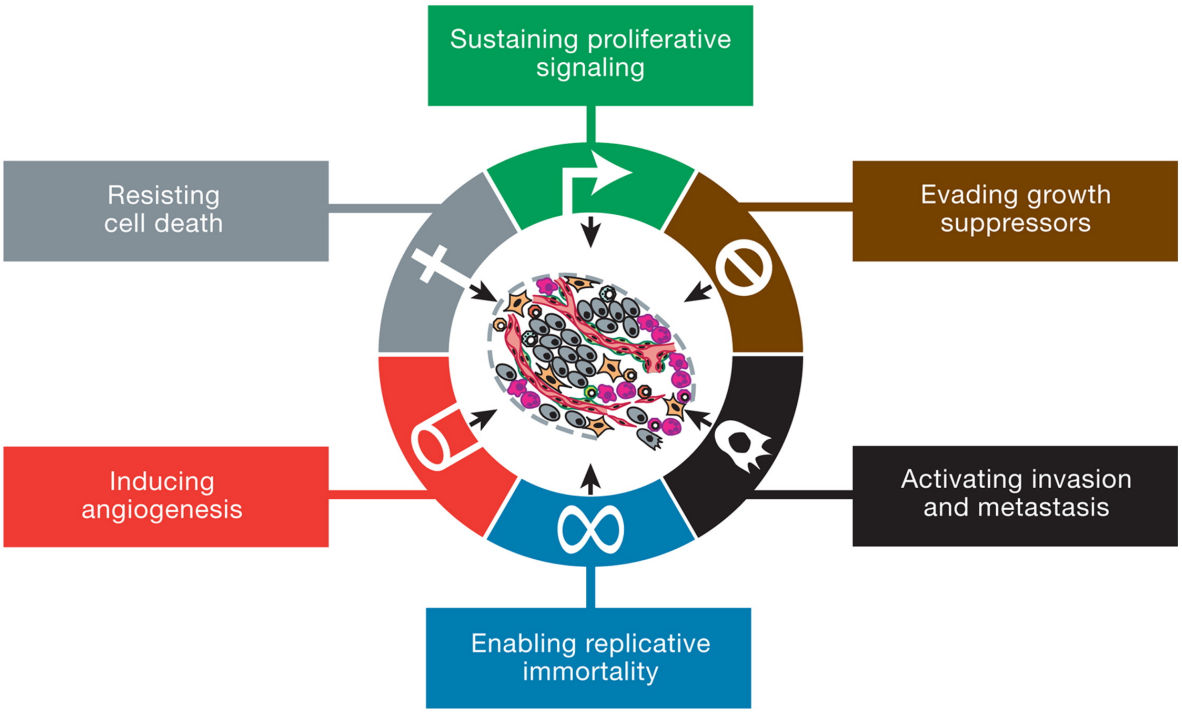

Figure 4 The hallmarks of cancer (Hanahan & Weinberg, 2011).

1.3 Integrins

Integrins in a general context are known to be heterodimeric, transmembrane glycoproteins that form a non-covalent bond to an alpha and a beta chain. Known functions of integrins are the transmission of chemical and mechanical signals, induction of changes in cytoskeleton during cell migration and cell adhesion. They also play a vital role in cell proliferation and multiple survival mechanisms. The integrin proteins associate with the extracellular matrix by linkage of the cytoskeleton through the short cytoplasmic tail. During metastasis, cancer cells interact with their proximate environment through soluble factors and interaction with other cells and extracellular matrices. Abnormal expression of integrins involved in these interactions have been found to be engaged in malignant cell growth and in the process of metastasis (Juliano, 1994).

1.3.1 Alpha 6 Integrin

Alpha 6 integrin is part of the alpha chain family of proteins. The alpha 6 subunit may associate with either beta 1 or beta 4 subunit to form a complete integrin that function as laminin receptors. They mediate signals from the basal membrane and have been used as a marker for enhancement of adult stem cells, progenitor cells, and cancer stem cells (Stingl et al, 2006; Yoshioka et al, 2013). Alpha 6 integrin combined with a beta 4 subunit may induce tumorigenesis and are surprisingly low expressed in cancer stem cells. On the other hand, alpha 6 combined with beta 1 may be responsible for a negative regulation of erbB2/HER2 signaling and has been found dominant in cancer stem cells (Goel et al, 2013; Lathia et al, 2010). Although associations of alpha 6 integrin to breast and other cancer stem cells have been found, not enough studies have been made to understand the contribution of alpha 6 integrin in cancer stem cell genesis. In addition, due to alternative splicing, two transcript variants that encode different isoforms have been found: alpha 6 integrin variants A and B.

Studies have shown that cancer lines expressing high levels of Alpha 6 integrin have shown an aggressive metastatic growth in nude mice. Examples of breast cancer cell lines with high expression of Alpha 6 integrin are MDA-MB231 cells, classified as triple negative because its growth is independent of estrogen, proges-

terone and epidermal growth factor-2 receptors. Conversely, cell lines with low levels of alpha 6 integrin have shown to be less tumorigenic and non-metastatic in nude mice (Mukhopadhyay, Theriault, & Price, 1999).

1.3.2 Alpha 6 Integrin variants A and B

Alpha 6 integrin has been identified with two cytoplasmic variants: variant A and variant B. When studying their genetic sequence, it is established that variant B is shorter and that their difference lies in their cytoplasmic tails, which ultimately causes the activation of different signaling pathways. Recent studies have tried to identify whether alpha 6 integrin is engaged in tumor stemness and plays a role in tumor genesis (Visvader & Lindeman, 2012). Goel et al have reported cancer stem cells with $CD44^{+}/CD24^{-/low}$ in triple negative breast cancer, which consist of a minor stem-like population. This population is composed of certain epithelial and mesenchymal subpopulations that can be identified by their total alpha 6 integrin expression, especially the prevalence of alpha 6A in comparison to alpha 6B, respectively. This same group, using knock down of the expression of the alpha 6B variant, found that that this variant plays a critically vital role in driving mammosphere formation and anchorage-independent growth enriched in CSC, a hallmark of cancer progression, implicating that alpha 6B drives the cancer stem cell behavior, promoting self-renewal and tumor genesis in triple negative breast cancer cells. The counterpart alpha 6A has been identified being engaged in signaling involved in cell adhesion, proliferation and survival ultimately leading to tumor growth (Figure 6) (Goel et al., 2014).

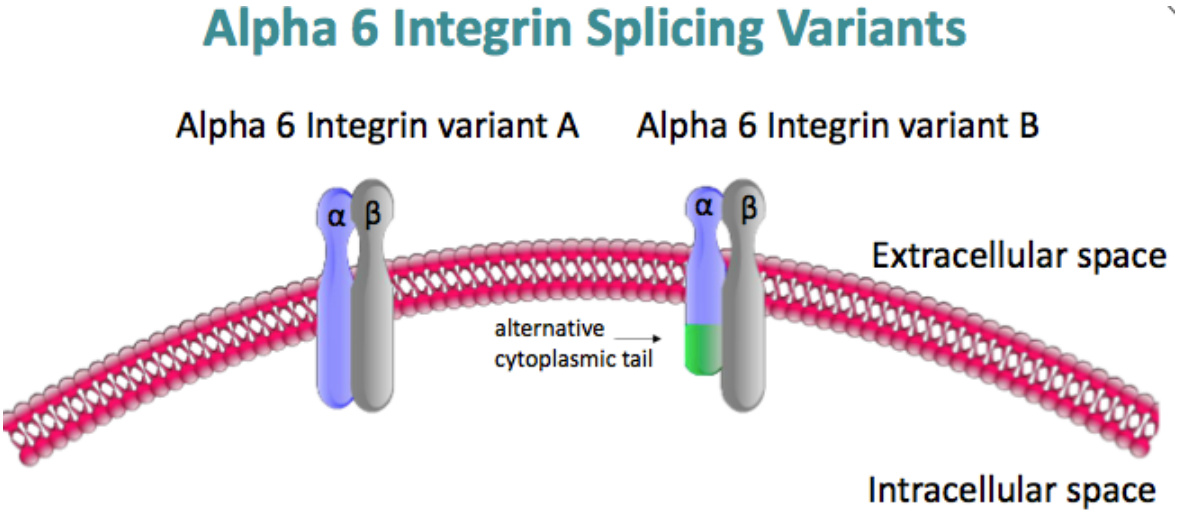

Figure 5 Structural differences between Alpha 6 integrin variants A and B

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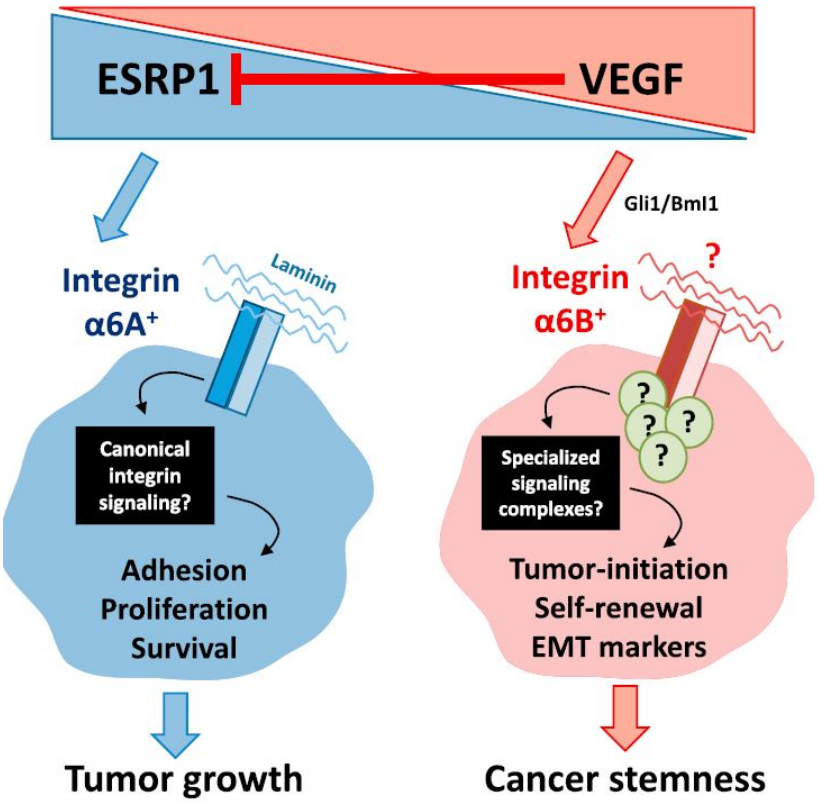


Figure 6 Contribution of each variant of Alpha 6 integrin (Seguin, Weis, & Cheresh, 2014).

1.4 Cell Microenvironment

1.4.1 Normal Stem Cells and their Microenvironment

The stem cell is surrounded by a sheltering microenvironment that consists of factors that enable stem cells to maintain tissue homeostasis (Moore & Lemischka, 2006). Normal stem cells actively interact with and influence their microenvironment, which in return are also regulated by signaling from the same niche (Sundar et al., 2014). Their dependence on the niche is a given property, since this specialized microenvironment needs to maintain their quiescent and undifferentiated state, while at the same time maintain their capability to proliferate and differentiate (Ailles & Weissman, 2007). Hence, the behavior of the normal stem cells is regulated by the factors found in the stem cell niche (Kasai et al., 2014). In the figure below (Figure 7), the composition of mTeSR-1 stem cell media is depicted, which lists factors that enable the stem cells to expand and maintain their stemness.

Component	mTeSR™1
DMEM/F12	●
$NaHCO_3$	●
L-Ascorbic Acid	●
Selenium	●
Transferrin	●
Insulin	●
FGF2	●
TGF-β	●
BSA	●
Glutathione	●
Trace Elements	●
BME	●
Pipecolic Acid	●
GABA	●
Lithium Chloride	●
Defined Lipids	●
Total Protein (g/L)	~13

Figure 7 Composition of stem cell media mTeSR-1

1.4.2 Tumor Microenvironment and Cellular Stress

Just like how normal stem cells need input from their microenvironment to be in optimal condition, a very similar concept may hold for cancer stem cells (Medema, 2013). This minor subpopulation within the tumors is surrounded by a specialized niche that consists of many microenvironmental factors such as autocrine signaling and stimuli released from stromal fibroblasts, immune cells and extracellular matrix (ECM) and also physiochemical factors such as oxygen, nutrient supply and tissue pH (Cojoc, Mäbert, Muders, & Dubrovska, 2014). This microenvironment can control the amount of stem cells, their proliferation, and their fate of determination. A deeper understanding of the biology of stem cells and their microenvironment will lead to new strategies in treatments of a variety of diseases, including malignant tumors (Honoki, Fujii, & Tsujiuchi, 2006). It has been found that the niche has epigenetic effects on stem cells due to the interactions between stem cells and the rest of the surrounding cell populations or due to the secreted factors from the surrounding cells in the niche. Various studies have proven that the tumor microenvironment does not only maintain carcinogenesis, but also actively helps in tumor initiation, progression, and metastasis. During tumor progression, the cells within the tumor go through phenotypic and epigenetic changes (Hu & Polyak, 2008).

Recent studies have shown that a cancerous niche that consists of growth promoting signals rather than growth inhibiting signals may induce the genesis of cancer stem cells (Yan et al., 2014). Numerous studies have demonstrated that the ESC microenvironment could have a significant effect on the phenotype of aggressive cancer cells by suppressing its malignant phenotype via alternative expression of miRNAs and by epigenetic regulation. Furthermore, studies have reported that secreted factors from tumors can inhibit p53 induction, which raised the hypothesis that the secreted factors from cancer cells might confer cancerous characteristics to the adjacent stem cells (Kasai et al., 2014).

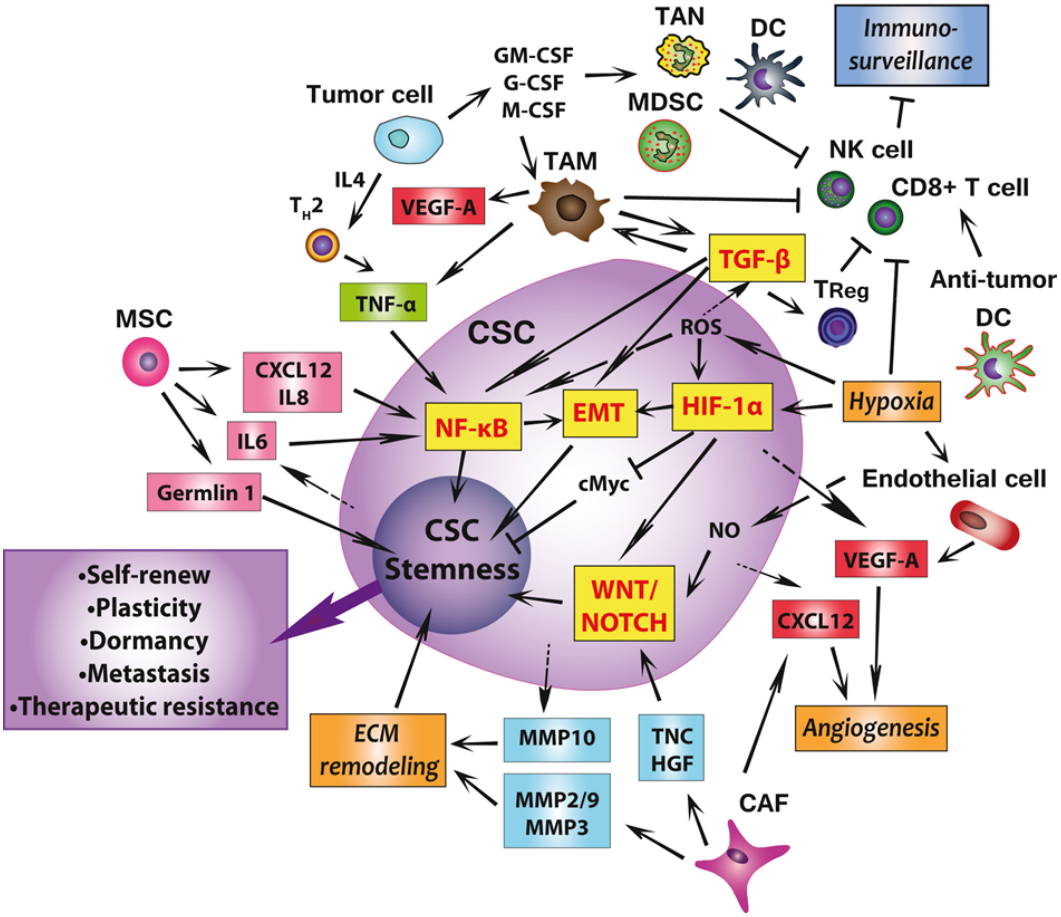


Figure 8 The molecular and cellular basis of the cross talk between CSCs and their niche (Plaks et al, 2015).

1.4.3 Secretory Factors

Niches are physically diverse microenvironments within the whole tumor microenvironment. Cancer stem cells, for instance, have factors in their niche that promote cancer stem cell self-renewal, angiogenesis as well as recruitment of immune and other stromal cells that secrete extra factors to stimulate tumor cell invasion and metastasis (Plaks, Kong, & Werb, 2015). It has been found that cells in a cancerous niche produce factors that include VEGF, TGF-β, MMPs, FGF, HGF, EGF, SDF-1, IGF, PDGF, Wnt, Notch and Hedgehog ligands, which stimulate CSC self-renewal, induce angiogenesis, and recruit tumor-associated macrophages, neutrophils, and mast cells; which secrete additional growth factors that promote tumor cell invasion and metastasis (Ye, Wu, Wu, Chen, & Huang, 2014). Studies have shown that secreted factors by cancer stem cells have been able to induce a conversion of normal fibroblasts to cancer-associated fibroblasts (Plaks et al., 2015).

1.5 Microvesicles and Exosomes

Membrane vesicles of endosomal and plasma membrane origin are called exosomes and microvesicles, respectively. These extracellular vesicles (EV) are released by cells into the extracellular environment and play an important role in intracellular communication by serving as vehicles that transfer between the cells of the membrane and cytosolic proteins, lipids, and RNA (Raposo & Stoorvogel, 2013). These vesicles have been known to only shed from apoptotic bodies, but recently it has been found that healthy cells also release them. These vesicles are also termed ectosomes, shedding vesicles, or microparticles among others. Circulating vesicles are likely composed of both exosomes and microvesicles and up-to-date purification methods cannot separate exosomes and microvesicles. However, it has been shown that a single cell type releases both exosomes and microvesicles have been demonstrated in platelets (Heijnen, Schiel, Fijnheer, Geuze, & Sixma, 1999), endothelial cells (Deregibus et al., 2007) and breast cancer cells (Muralidharan-Chari et al., 2009).

1.5.1 Exosomes and their effect on stem and cancer cells

Stem cells maintain their stemness through interaction with secreted factors that enhance the stem-like phenotypes in resident cells. Studies have shown that stem cells release exosomes, which serve as mediators in communication between cells and also deliver factors to recipient cells that are needed to maintain their stemness, differentiation, self-renewal, and repair (Ma et al., 2014). Secreted factors are important between local stroma cells and stem cells, whereby the cells may regulate themselves in a given tumor niche and may exchange cytoplasmic and genetic material. The fusion and exchange of biological material play an important role in cancer development and hence could show several characteristics of cancer stem cells (Bjerkvig, Tysnes, Aboody, Najbauer, & Terzis, 2005). Exosomes transfer their contents to proximate cells and therefore can induce phenotypic changes, which ultimately leads to modulation of the microenvironment (Camussi et al., 2011). Recent data have shown that exosomes play an important role in the development and progression of cancer (Webber, Yeung, & Clayton, 2015). They can promote cancer by suppressing immune surveillance mecha-

nisms and by inducing drug resistance and thus can be potential clinical applications in the future (Iero et al., 2008).

Data have shown that pluripotent transcription factors like Nanog, Oct-4, HoxB4, Rex-1, Wnt-3 protein all found in stem cells derived exosomes that may represent features of stemness. Other studies have proven that WNT, Hedgehog, and β-catenin which are expressed on stem cell-derived exosomes may play a potential role in maintaining stemness. Importantly, several other reports have clearly indicated that stem cell signatures expressed on exosomes play a vital role in maintaining stem cell characteristics, such as differentiation, self-renewal, and maturation (Bauer et al., 2011).

Exosomes have been found to be potential vectors for cancer immunotherapy and could be used in clinical settings and in the development of vaccination. They could be engineered in a way to deliver a potent immunogen that can induce effective immune responses in recipient cells. Studies have demonstrated that mesenchymal stem cells secrete immunologically active exosomes that can serve as a basis for stem cell based cancer immunotherapy.

1.5.2 Exosomes and Integrins

Although there are some controversies on what proteins the exosomes actually contain, several studies have shown that integrins are found in exosomes from different cell types. Alpha 2, 3, 6 and beta 1 and 4 integrin subunits have been found in microvesicles from pancreatic cancer cells. In addition, a recent study has shown that alpha 3 in pancreatic cancer interferes with non-cancerous prostate cell functions (Bijnsdorp et al., 2013). Clayton et al. have shown that B cell-derived exosomes express beta-1 and beta-2 integrins that can mediate anchorage to the extracellular matrix (Clayton et al., 2004). Also, $alpha_v beta6$ has been shown to be expressed in exosomes and when co-expressed with ovalbumin in gut epithelial cell-derived exosomes, it induces activation of different immune system cell types (Chen et al., 2011).

Human macrophrages-derived exosomes negatively regulate endothelial cell migration through control of integrin trafficking. These exosomes have been discovered to internalize integrin beta-1 in primary HUVECs, which is not recycled back

to the plasma membrane, ultimately leading to a corresponding degradation of the integrin (Lee, Kim, & Kim, 2014). This observation supports our assumption that exosomes could (down) regulate integrin in target cells; not only in beta-1 but we could also extrapolate that other intgerins share a similar fate.

1.6 Hypoxia

1.6.1 Hypoxia and Metabolism in Cancer

Hypoxia is defined as reduced oxygen levels and is associated with different types of tumors and can enhance the characteristics of cancer stem cells (Ye et al., 2014). Hypoxia-inducible factors (HIFs) belong to the family of transcription factors and play a vital role in controlling cellular response to hypoxia (Chi et al., 2006). Hypoxia Inducible Factor-1-alpha (HIF-1alpha) is induced at low oxygen levels and lead to a response in a genetic program, while in normoxia, at normal oxygen level conditions HIF1alpha is degraded. High levels of HIF1alpha is typical in cancer development; especially in metastatic cancers (Diaz, Nguewa, Redrado, Manrique, & Calvo, 2015).

Studies have shown that hypoxia supports the undifferentiated and quiescent state of different stem cell populations. HIFs are directly associated with the expression of transcription factors associated in stem cell self-renewal and multipotency (Mathieu et al., 2013). Hypoxia has been found to play a vital role in metabolic re-programming of tumor cells and is considered to be a hallmark of cancer (Hanahan & Weinberg, 2011). However, not much is known about the different types of responses of different cell types to hypoxia and how this variation is associated with tissue- and cell-specific diseases (Chi et al., 2006). Furthermore, it is important to mention that although results of many studies have shown that hypoxia promotes potency, even small shifts in oxygen concentration can lead to differentiation. This result may depend on the different culture conditions. Hence a lot of studies still have to be made to understand the optimal oxygen content for the maintenance of self-renewal and potency (Abdollahi et al., 2012).

1.6.2 Hypoxia and Regulation of Cancer Stemness

Stem cells are cultured in the stem cell niche, which contains important factors that can keep the balance between their self-renewal, differentiation and stem cell quiescence. Recent studies have shown that stem cells are usually found in and benefit from low oxygen thus showing the importance of hypoxia for the undifferentiated phenotype of stem/precursor cells (Mathieu et al., 2011). A hypoxic environment can protect cancer stem cells from environmental influences, including chemotherapy and irradiation. Since oxygen is a potent radio-sensitzer and is needed for radiation dependent cancer cell damage through the generation of reactive oxygen species (ROS), hypoxia can make cells more resistant to irradiation. Therefore, the hypoxic niche can protect the cancer stem cells by low oxygen levels.

Recent studies have shown that elevated levels and activation of HIFs often happen during cancer progression and is associated with acquiring a more malignant behavior that make cells resistant to treatment (Mimeault & Batra, 2013). Furthermore, current findings have shown that HIF-1alpha and HIF-2alpha play vital roles in highly tumorigenic cancer stem cells in the acquisition of more malignant phenotypes (Mimeault & Batra, 2013).

1.7 Goal of our project

We hypothesize that cancer stem cells (CSC) in culture can change their phenotype to a less malignant one by incubating cancer cells in the presence of conditioned media of normal stem cells (iPSC). Since CSC shows higher plasticity in comparison to non-CSC in a tumor, we expect to see major changes in the CSC cell population of cancer cells.

For our project we set **two specific aims**:

Aim 1: to reduce the percentage of cancer stem cells in human breast cancer cell line MB-231 by incubating these cells with conditioned media of induced pluripotent stem cells (iPSC). MB-231 cells contain over 80% of CSC. Conversely, we plan to increase the percentage of CSC in human breast cancer cell line MCF-7 by incubating these cells with conditioned media of MB-231 cells. MCF-7 cells have a very low percentage of CSC, usually less than 1%. We hypothesize that Alpha 6 Integrin plays an important role in the plasticity of these breast cancer cells.

Aim 2: to analyze the composition of iPSC conditioned media in order to identify putative proteins responsible for inducing phenotypic changes in CSC.

2 Materials and Methods

2.1 Materials

2.1.1 Cell Culture Cell Lines

Human breast cancer cell lines MDA-MB-231 and MCF-7 were both obtained from the Frederick National Laboratory for Cancer Research Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD, USA). They were cultured in 6-well plates, 25cm^2 and/or 75cm^2 using the RPMI-1640 (Mediatech Incorporation Cat# 10-040-CV) cell culture media. For the normal stem cell line (iPSC), Riv9 (Stem Cell Core Facility, University of California, Riverside) was used and was cultured in 6-well plates with mTeSR-1 (Stem Cell Technologies Cat# 05851, 05852). All cell cultures were incubated at 37 °C and 5% CO_2.

2.1.2 Antibodies

The antibodies were used to stain cell cultures for Flow Cytometric Analysis (FACS). Antibodies used were CD44 (FITC) (eBioscience, Cat# 45-0441-80), CD24 (PE) (Becton Dickinson Pharmingen, Cat# 555428) and CD49f (APC) (eBioscience 17-0495-80) and were all stored at 4 °C.

2.2 Methods

2.2.1 Cell Culture

The cells were stored in cryotubes in liquid nitrogen and thawed at 37 °C. The breast cancer cells were added to their corresponding cell culture media to reduce DMSO concentration from the freezing medium, were centrifuged at 300xg for 5 minutes, and were subsequently re-suspended in the same cell culture media. Cell culture media was changed every 2-3 days. Normal stem cells were first cultured with ROCK Inhibitor (10 μM) and were then treated with cell culture media on a daily basis.

The cells were passaged at a ratio of 1:3-1:6. PBS was used to wash the cells and Trypsin (Caisson Laboratories Incorporation Cat# TRL01-100ML) or non-enzymatic medium Lonza-7 (for iPSC) (BioWhittaker Cat# FP-5013) were used to detach breast cancer cells.

2.2.2 Conditioned Media (CM)

Conditioned media from breast cancer cells were obtained by culturing cells in 75cm^2 flasks. For iPSC-conditioned media, stem cells were cultured in 6-well plates. Cell culture media were collected every day, filtered at 0.45μm to eliminate residual cells and were stored at -20°C.

2.2.3 Microscopy

The morphology of the respective cell cultures was taken before and after exposure to different experimental conditions. An Inverted Olympus Microscope was used to observe cells. The software taking and analyzing pictures was Spot Advanced Program.

2.2.4 RNA Purification

RNA extraction is performed using the Zymo Research Quick RNA Mini Prep Kit Catalog# 11-327. The RNA sample obtained was in DNAse/RNAse-free water at 30μl and was stored at -80°C.

2.2.5 RNA Absorbance Measurement

RNA absorbance was measured with a Spectrophotometer. RNA was diluted 1:60 (1μl RNA + 59μl water). Absorbance was measured at 260/280.

2.2.6 Reverse Transcription

RNA was transformed to cDNA using the MAXIMA Reverse Transcriptase Enzyme Catalog #EP0742 and Thermal Cycler as incubator. The program used was 25°C for 10 minutes, 50°C for 30 minutes and 85°C of 5 minutes. The samples were stored at -20°C.

2.2.7 End point Polymerase Chain Reaction (PCR)

cDNA samples were amplified using Denville Hot Start Taq Polymerase Catalog #CB4080 and were loaded into the Thermal Cycler (Applied Biosystem 2700). A 50µL total volume for each sample was prepared and the following program was executed in 35 cycles: 94°C for 15 minutes, 55°C for 30 seconds, 72°C for 45 seconds, 72°C for 10 minutes, then 4°C until removed. 15µl of amplified product were mixed with 3µl 6X DNA Loading Dye and were added to the corresponding wells in the agarose gel with the exception of the sample(s) amplified with the GAPDH primer as positive control, from which 10µl of the amplified product was mixed with 2µl Loading Buffer and added to the corresponding well(s) in the gel. For the DNA Ladder Mix, 5µl was loaded to the corresponding well in the gel. The 2% agarose gel was prepared using 0.9g of agarose powder combined with 45ml of TAE 10X Buffer, and 2.25µl 1%EtBr as DNA staining solution. The solidified agarose gel was submerged in the electrophoresis box filled with TAE 1X Buffer prior to the loading of samples in their corresponding wells. The electrodes were then attached and electrophoresis was carried out for approximately 20 minutes at 150V̊. Pictures were taken of the gel under UV-light using the BioSpectrumTM 500 Imaging System.

2.2.8 Flow Cytometric Analysis (FACS)

The Flow Cytometer was used to measure fluorescent signals of the samples prepared. Cells were detached by trypsinization and were stopped by media containing 10% FBS. Cells were then centrifuged at 300g for 5 minutes, re-suspended in PBS/1.5% BSA twice, and centrifuged after each interval. Cells were then suspended in “x” amount of PBS/1.5% BSA for 15 minutes at room temperature after which 10µl was used in the preparation of each sample to be stained with the respective antibody solutions. However, to prepare the unstained samples, cells were added directly to 500µL PBS in FACS tubes. The stained samples were then incubated at 4°C for 15 minutes, washed with PBS/3% FBS, centrifuged at 1400rpm for 1 minute, mixed with 200µl PBS, and transferred into FACS tubes containing 300µl PBS.

2.2.9 Quantitative Polymerase Chain Reaction (qPCR)

cDNA samples were amplified using the iQTM SYBR® Green Supermix Catalog #170-8880 and loaded into the C1000TM Thermal Cycler (BioRad). A 20µl total volume for each sample was prepared and the following program was executed in 40 cycles: 95°C for 3 minutes, 95°C for 10 seconds, 55°C for 15 seconds, and then 72°C for 30 seconds.

2.2.10 Magnetic Bead Separation

Samples were magnetically labeled and separated using the CD24 MicroBead Kit human (MACS Miltenyi Biotec, Order# 130-095-951). Degassed buffer used was phosphate-buffered saline (PBS) pH 7.2, 0,5% bovine serum albumin (BSA), and 2mM EDTA. Cell pellet (up to 10^7 cells) of the sample was resuspended in 40µl PBS and 10µl of CD24 biotin added. This was incubated for 15 minutes in 2-8C. The cells were washed with PBS, centrifuged at 300xg for 10minutes, and aspirated the PBS. The cell pellet was resuspended in 80µl of buffer and 20µl of anti-biotin was added. This was mixed well and incubated for 15 minutes in 2-8°C. Depletion was completed with LD columns (MACS Miltenyi Biotec, Order# 130-042-901) in a suitable magnetic field.

2.3 Experimental Outline

2.3.1 Plasticity of Cancer Stem Cells

MB231 and MCF-7 cell cultures were prepared in 6-well plates and exposed to iPSC conditioned media for 3.5 and 4 weeks, respectively. Positive and Negative Controls were also prepared; mTeSR1 media was used as control. Cell culture media was changed every second day. When the respective timeframes elapsed, the methodological flow outlined in the method section was executed.

2.3.2 Identification of Proteins in iPSC conditioned media by Mass Spectrometry

CM samples were centrifuged at 15000xg for 10 minutes. The samples were loaded into cartridges (Waters, Part No. Wat094266 HLB 3cc). This was washed with 5% MeOH in water and afterwards eluted with 30% MeOH, 60% MeOH, and 90% MeOH. Afterwards the sample was speed vacuumed (Thermo Scientific Speed Vac) with the vacuum pressure of 30torr/min (ramp) until sample was dried. For trypsin digest of proteins, samples were treated as follows: 50µl 8M urea, 0.4M NH_4HCO_3 to the dry proteins, then 5µl of 45 mM dithiothreitol were added and incubated at 50°C for 15 minutes. Later added 5µl of 100mM iodoacetamide were added after cooling to room temperature. This was incubated for another 15 minutes at room temperature, and then 140µl water was added. Afterwards 1/25 enzyme (trypsin) to the protein (w/w) ratio of trypsin in a volume of 5µl was added. This was incubated at 37°C for 24 hours. Later, the samples were acidified with TFA by adding 1-4µl of 0.1% TFA to acidify to pH <4. 10µl of wetting solution was aspirated (100% ACN) with “ZipTips” twice, then with equilibration solution 5 times. 10µl of equilibrium solution (0.1% TFA and water) was aspirated. The sample was aspirated and dispensed 10 times and eluted with wash solution (5% methanol in 0.1% TFA in water) the sample 5 times. The samples were analyzed in collaboration with the Mass Spec Core Facilty at Loma Linda University. The analysis was performed with LTQ-Orbitrap Velos with ETD.

3 Results

3.1 Plasticity of Cancer Stem Cells

Cancer stem cells are characterized as $C44^{+}/CD24^{-/low}$. As such, fluorescent labels were bound to MB-231 and MCF-7 human breast cancer cells to determine if they were acquiring new surface marker expression to non-cancerous conditioned media (iPSC-CM). Figure 9 shows the negative control of breast cancer cell lines MB231 and MCF-7 showing that MB231 has high percentage of CSC while MCF-7 has a low one. Furthermore, in figures 16 and 22 we can see a reduction in CSC percentage linked to the treatment with iPSC-CM in both normoxia and hypoxia (1% O2). CSC is also known to express elevated levels of CD49f (alpha 6 integrin) (Figures 10 and 11) and in figures 17 and 23 we can see the reduction in alpha 6 integrin levels.

3.1.1 Cancer Stem Cells in Breast Cancer Cell lines

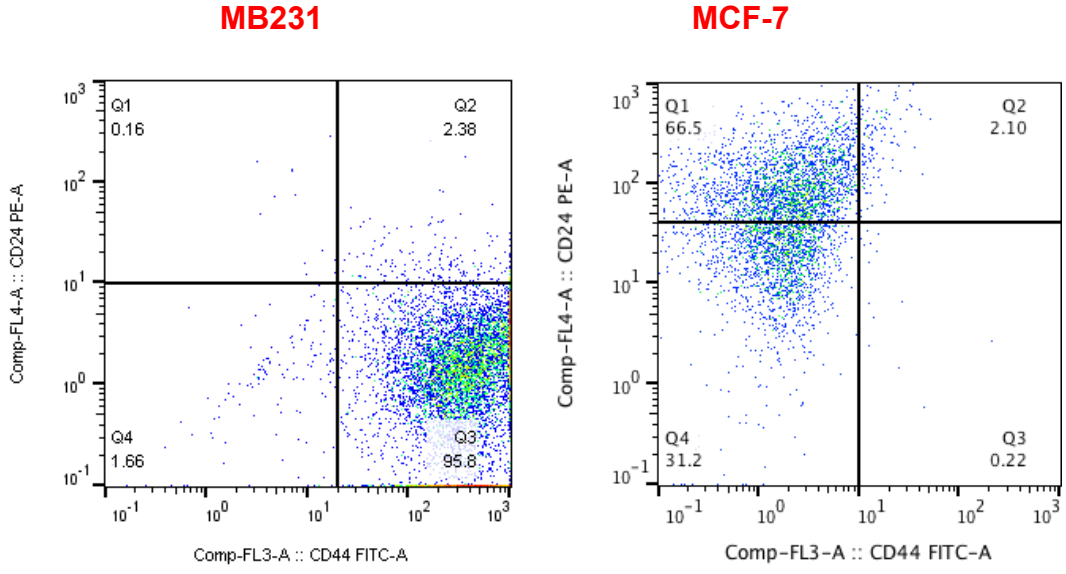


Figure 9 FACS analysis indicating percentage of cancer stem cells (CD44+/CD24-/low) in MB231 and MCF-7. Results indicate that MB231 has higher levels of cancer stem cells than MCF-7.

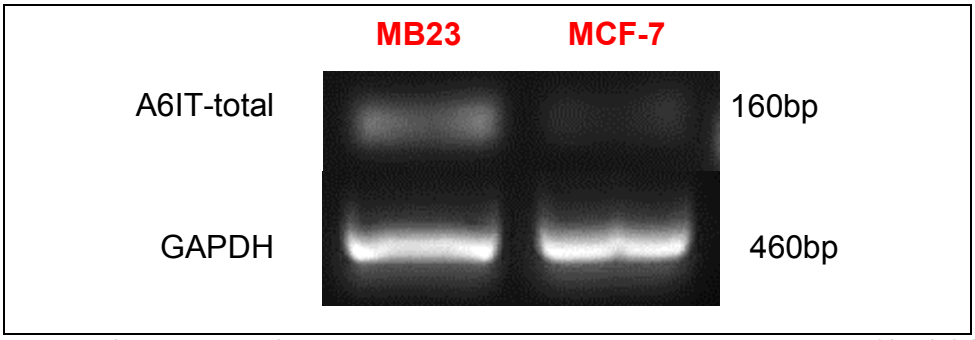


Figure 10 RT-PCR analysis for A6IT-total and variant B in MB231 (higher % of CSC) and MCF-7 (lower % of CSC).

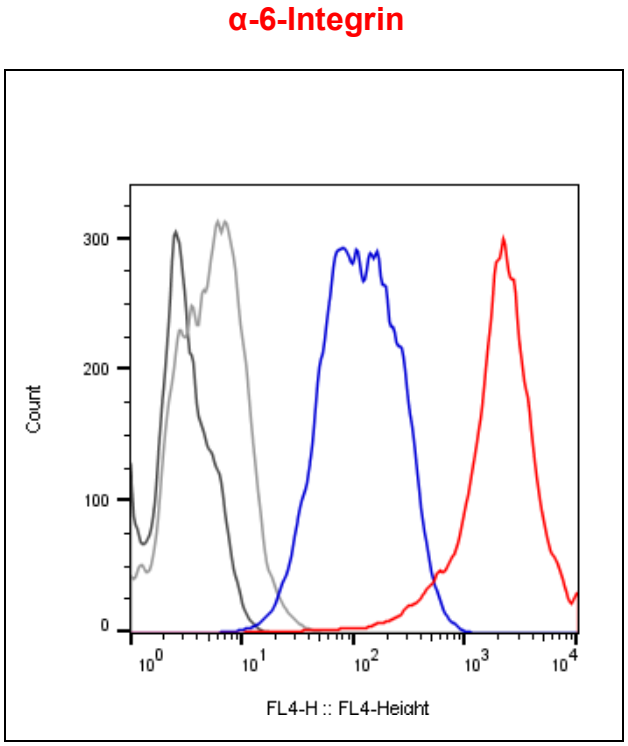


Figure 11 FACS analysis indicating levels of alpha 6 integrin (CD49f) for (red) MB231 and (blue) MCF-7 cells and (grey) controls. This shows that MB231 has high level of Alpha 6 Integrin.

3.1.2 Cell Morphology (Microscopy)

For the microscopic images, MB-231 cells and MCF-7 cells were cultured in RPMI media until they reached 80% confluency. Cell cultures were exposed to induced pluripotent stem cell conditioned media (iPSC-CM) for three and a half weeks in normoxia and for four weeks in hypoxia. As a control, MB-231 and MCF-7 cells were also exposed to stem cell media (mTeSR-1). When both cell lines were exposed to iPSCs-CM in normoxia and hypoxia, the typical cell structure characterizing these cells was visually altered.

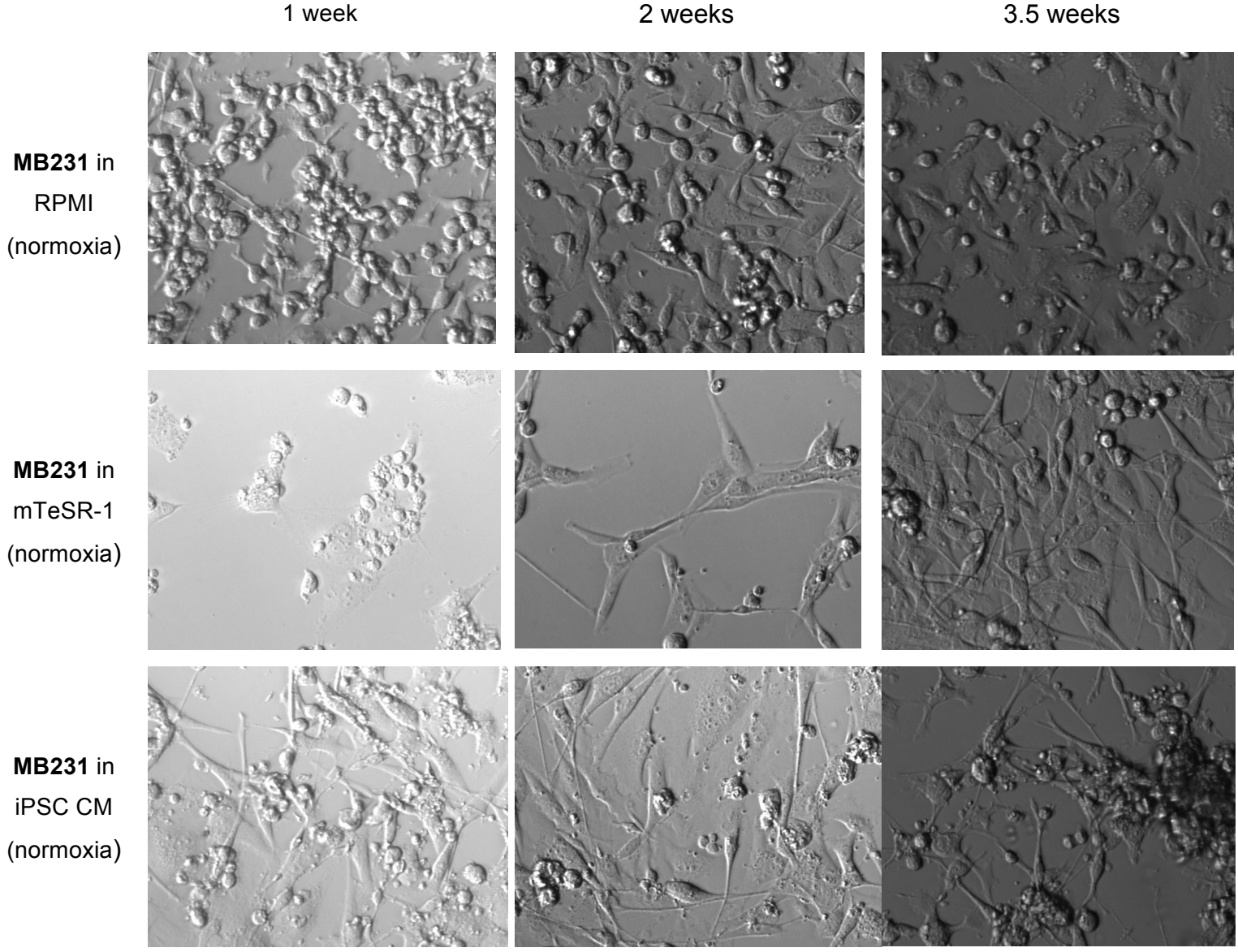


Figure 12 Microscopic view of MB231 cells exposed to induced pluripotent stem cells conditioned media under normoxia after one week, two weeks and three and a half weeks.

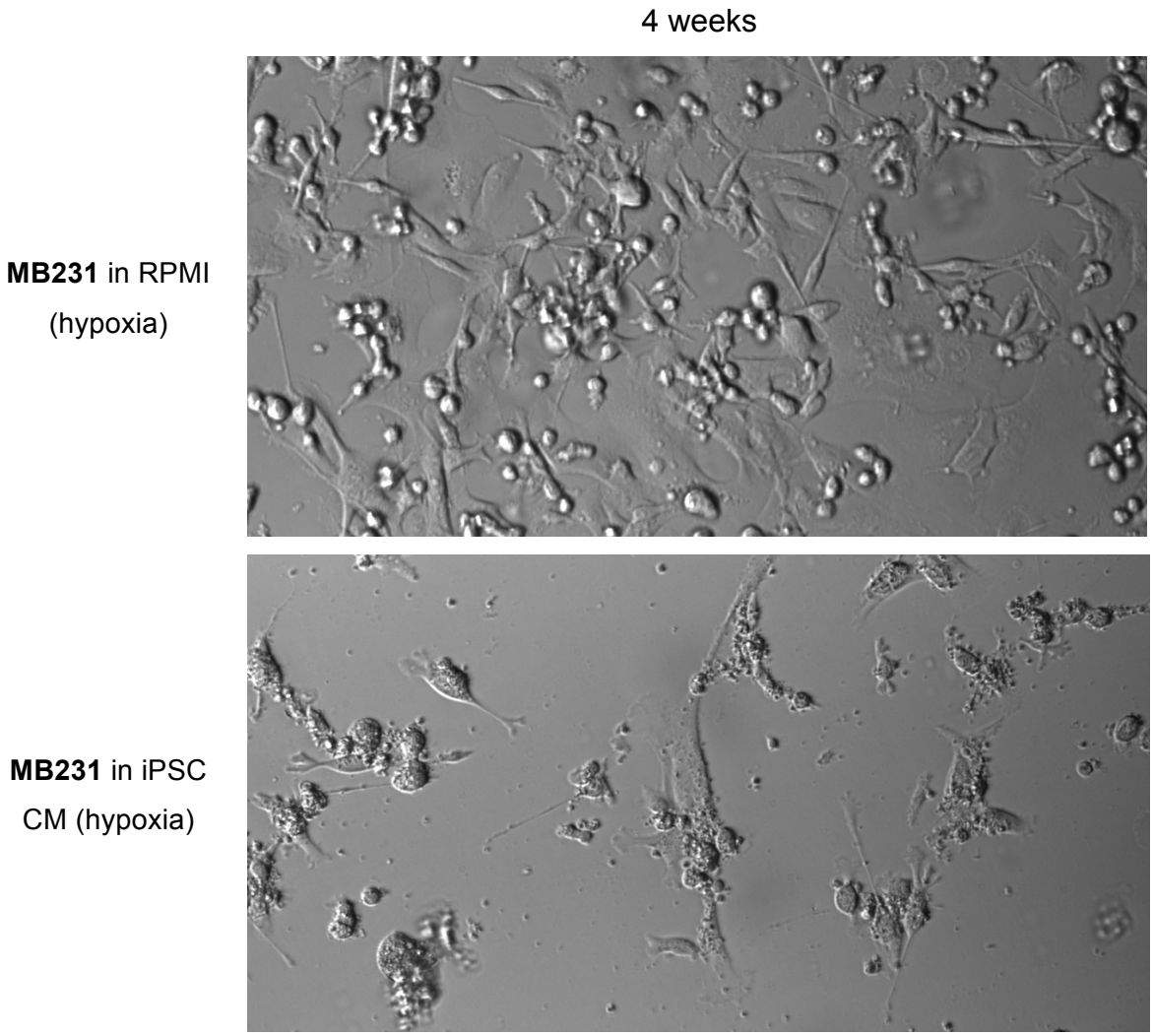


Figure 13 Microscopic view of MB231 cells exposed to induced pluripotent stem cell conditioned media under hypoxia after four weeks.

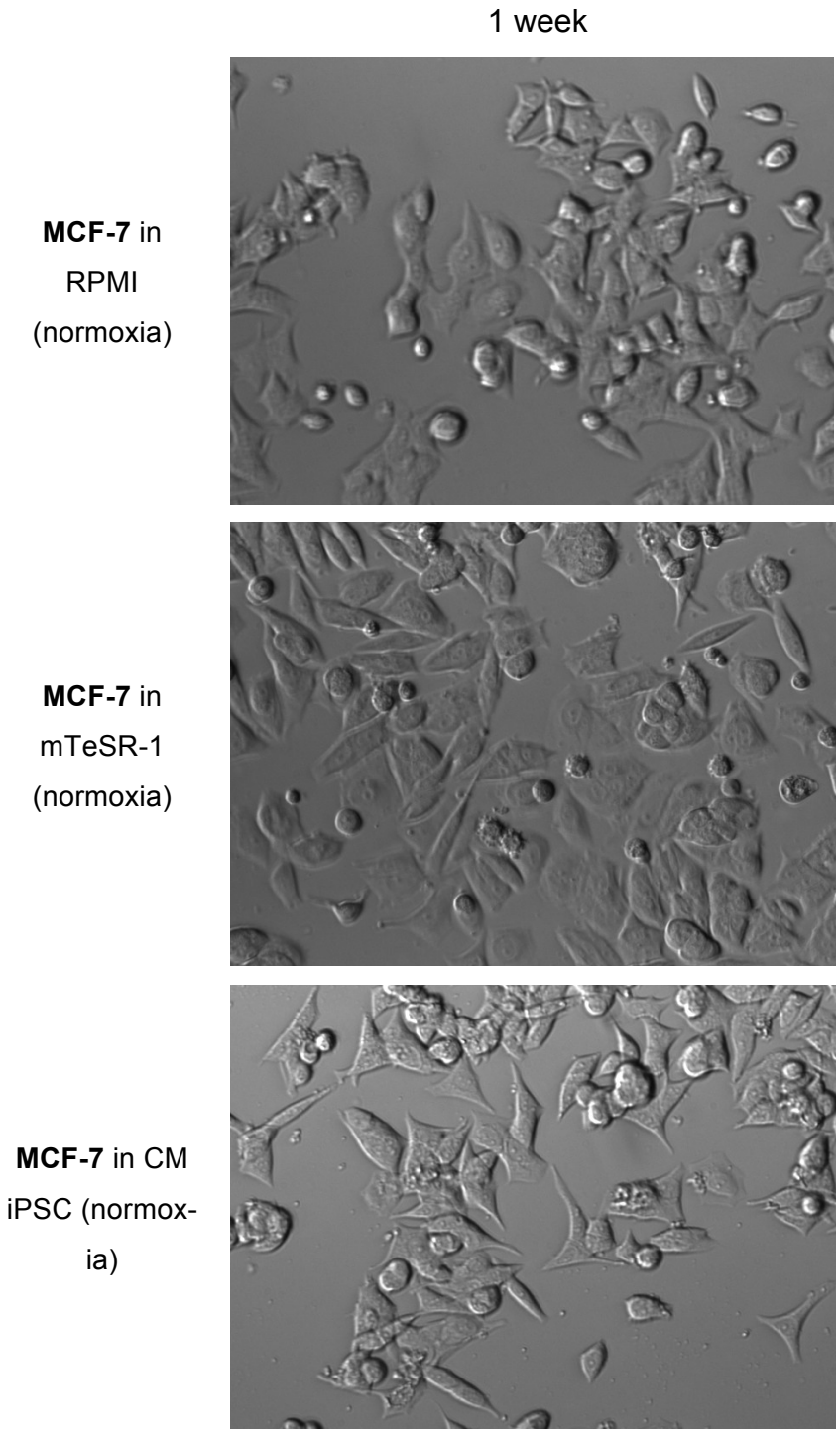


Figure 14 Microscopic view of MCF-7 cells exposed to iPSC CM under normoxia after one week.

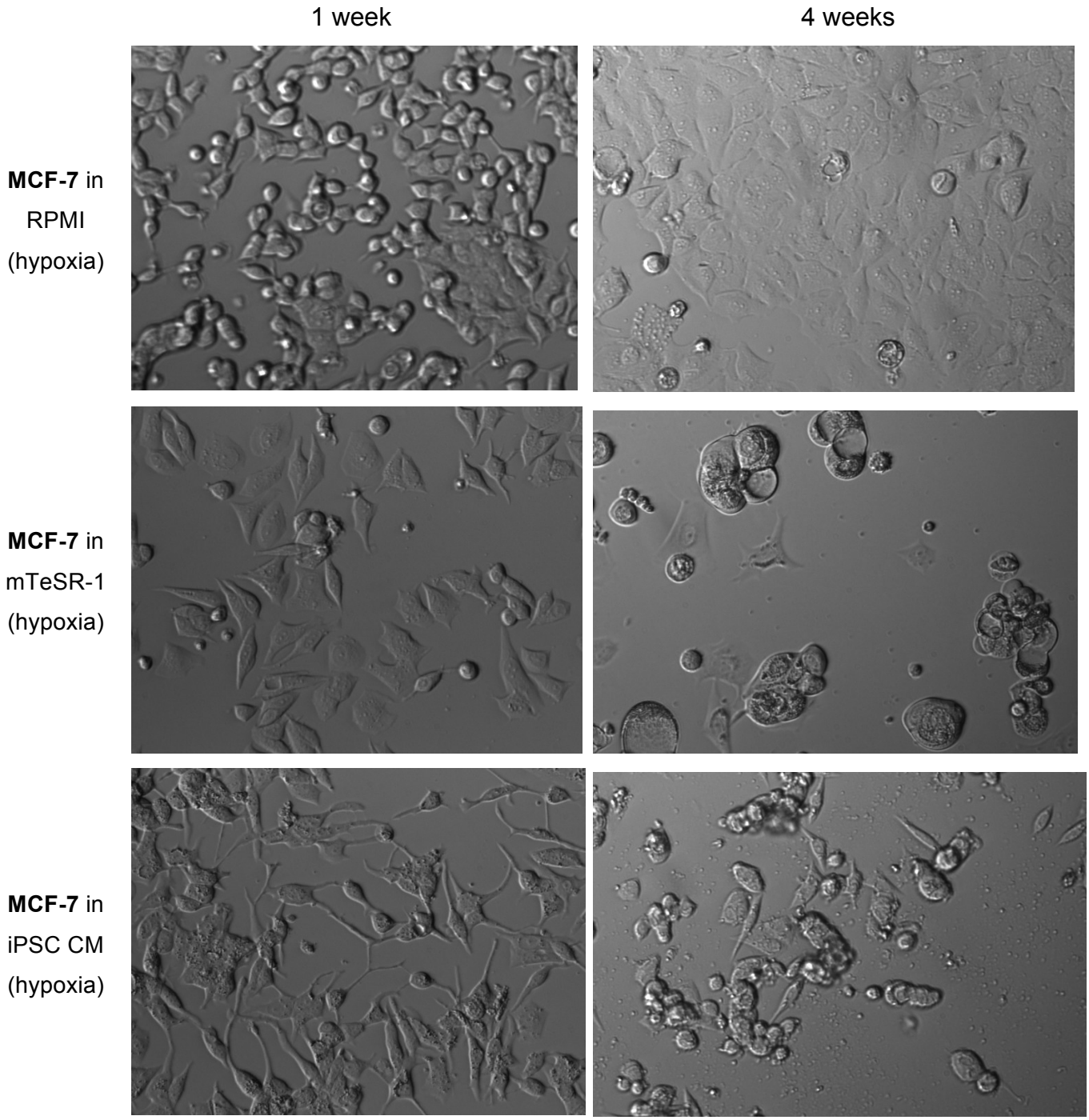
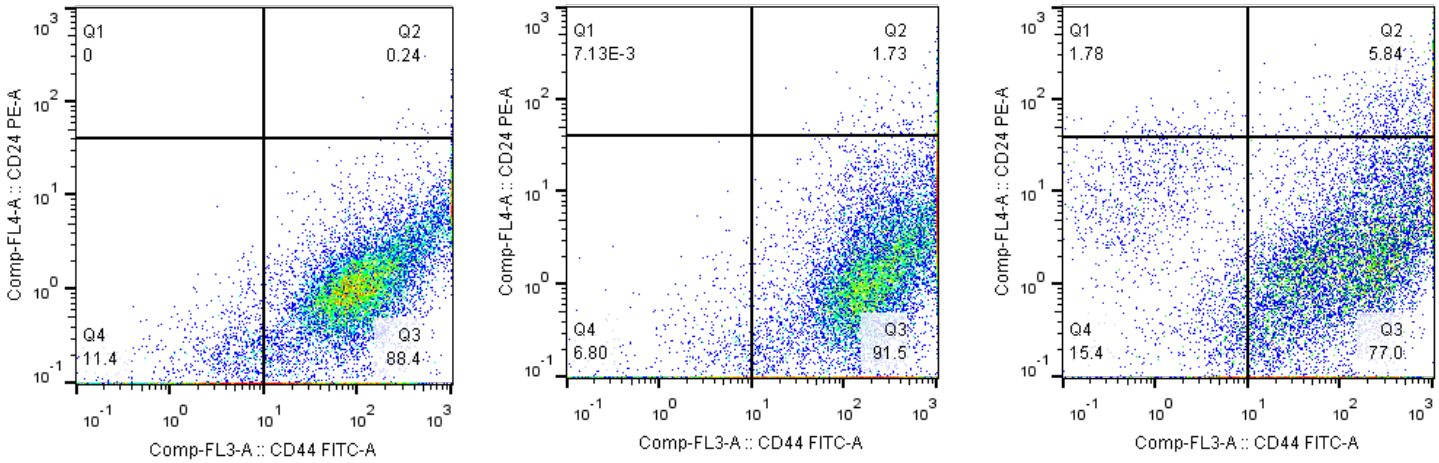


Figure 15 Microscopic view of MCF-7 cells exposed to iPSC CM under normoxia after one week, two weeks and three and a half weeks.

3.1.3 Treatments in Hypoxia (1% O_2)

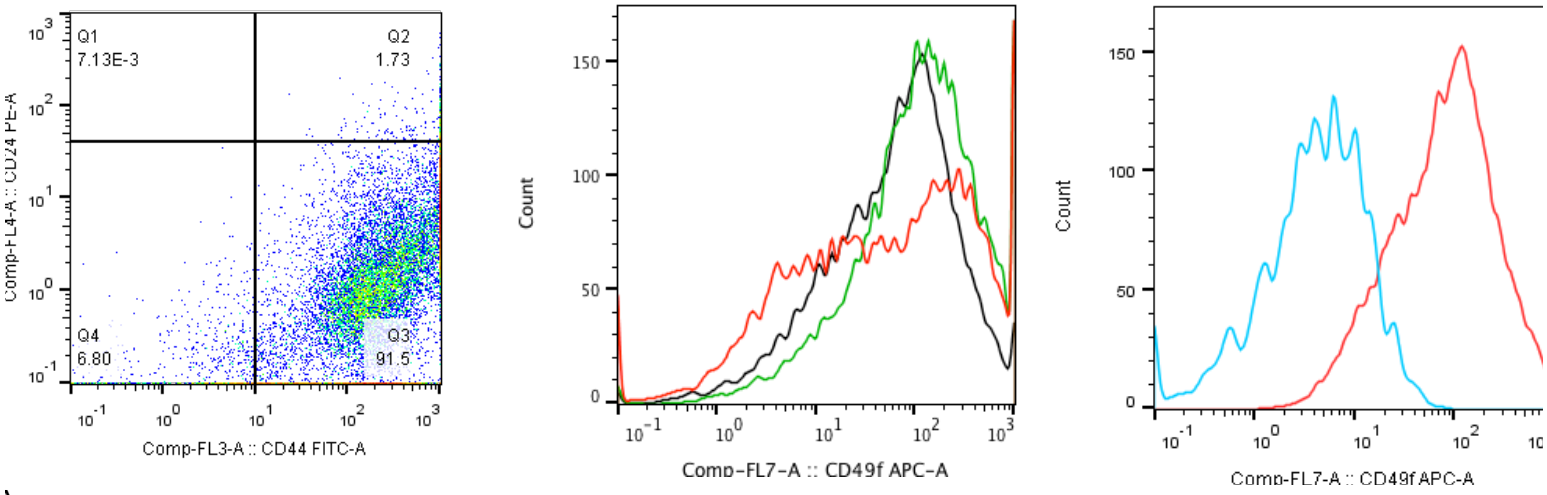
MB231 in Conditioned Media of induced Pluripotent Stem Cells



A B C

Figure 16 Flow Cytometric Analysis (FACS) of the $CD44^{+}/CD24^{-/low}$ surface marker expression in MB231 under (A) Normoxia (B) Hypoxia (C) in conditioned media of iPSC (Riv9) after three weeks. A reduction from 91.5% CSC to 77.0% CSC can be observed.

MB231 in Conditioned Media of induced Pluripotent Stem Cells



A

B

(black) control in normoxia

(green) control in hypoxia

(red) iPSC-CM

C

(red) CD49f in Quadrant 3

(blue) CD49f in Quadrant 4

Figure 17 Flow Cytometric Analysis (FACS) of alpha 6 integrin levels expressed in MB231 under (A) Hypoxia and (B)-(C) in conditioned media of iPSC (Riv9) after three weeks. A reduction in alpha 6 integrin level (CD49f) can be observed, especially in quadrant 3 ($CD44^{+}/CD24^{-/low}$).

MCF-7 in Hypoxia

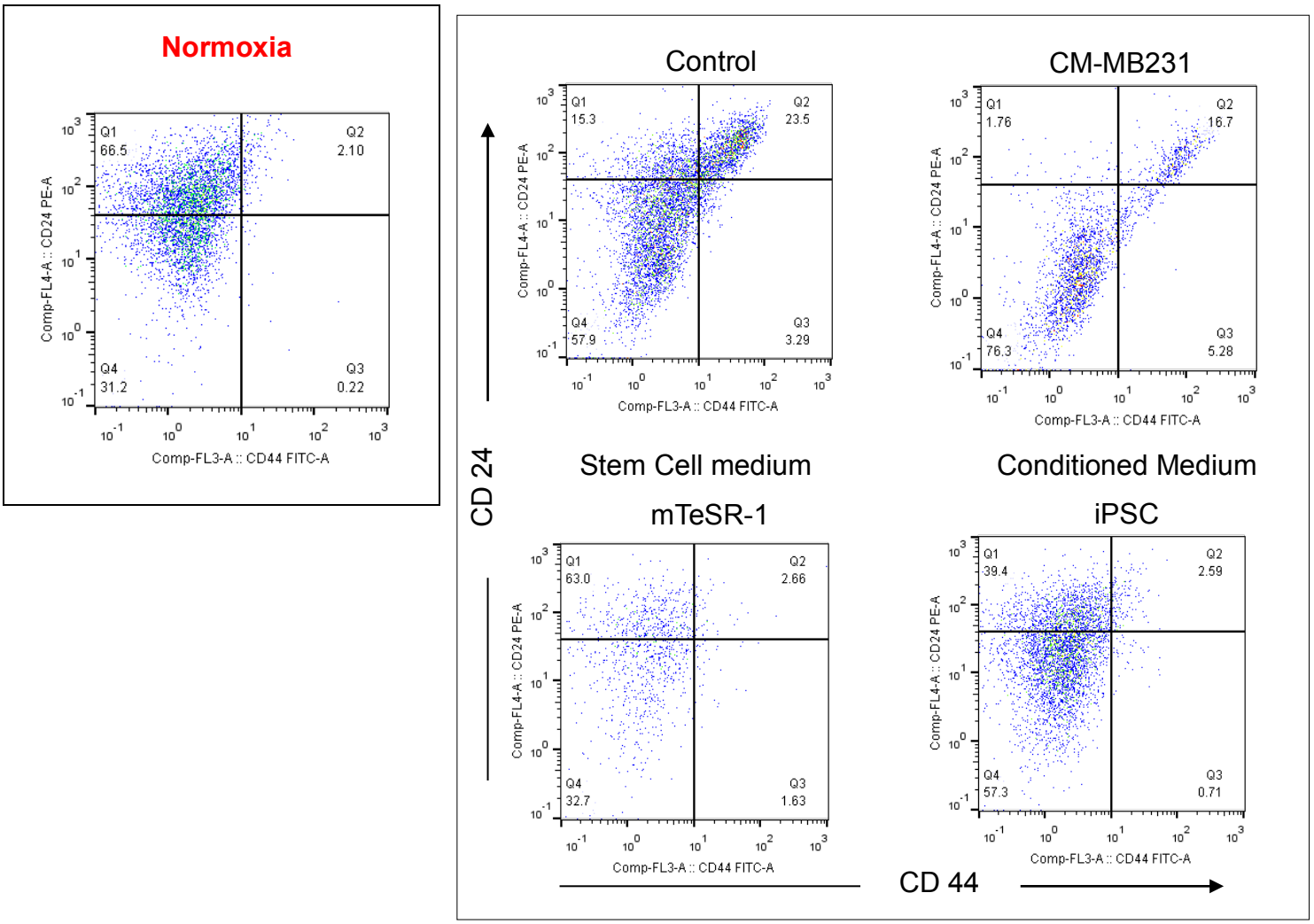
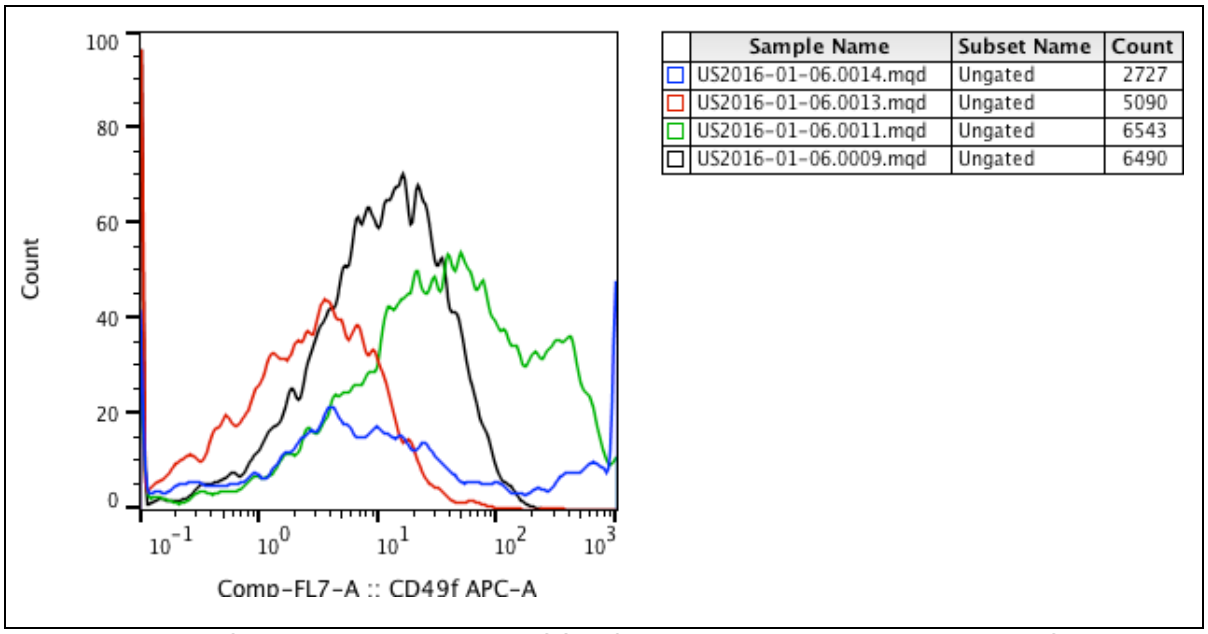


Figure 18 Flow Cytometric Analysis (FACS) of the $CD44^{+}/CD24^{-/low}$ surface marker expression in MCF-7 under normoxia and under hypoxia in RPMI, mTeSR-1, conditioned media of MB231 and conditioned media of iPSC. MCF-7 treated with MB231 CM shows an increase in CSC%, while MCF-7 treated with iPSC CM exhibits a decrease in CSC%.

MCF-7 in Hypoxia



	Sample Name	Subset Name	Count
□	US2016-01-06.0014.mqd	Ungated	2727
□	US2016-01-06.0013.mqd	Ungated	5090
□	US2016-01-06.0011.mqd	Ungated	6543
□	US2016-01-06.0009.mqd	Ungated	6490

Figure 19 Flow Cytometric Analysis (FACS) of alpha 6 integrin expression in MCF-7 under (black) normoxia control, (green) hypoxia control, (red) iPSC conditioned media and (blue) MB231 conditioned media. Compared to hypoxia control, there is an increase in the level of alpha 6 integrin (CD49f) when MCF-7 is treated with MB231 CM. On the other hand, when MCF-7 is treated with iPSC-CM, a significant reduction of alpha 6 integrin level is observed.

MCF-7 in Conditioned Media of induced Pluripotent Stem Cells in Hypoxia

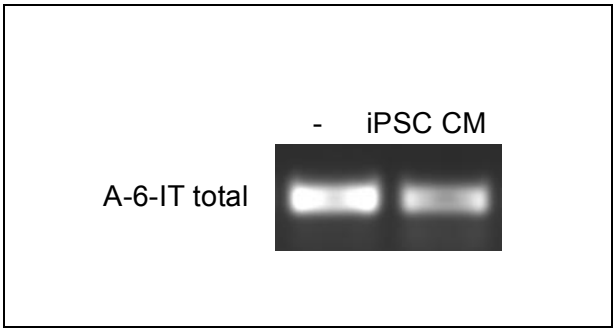


Figure 20 RT-PCR of alpha 6 integrin expression total in (-) MCF-7 control and (CM iPSC) in induced pluripotent conditioned media after four weeks. A reduction of alpha 6 integrin expression in MCF-7 can be observed after treatment with iPSC CM.

MCF-7 in Conditioned Media of MB231 in Hypoxia

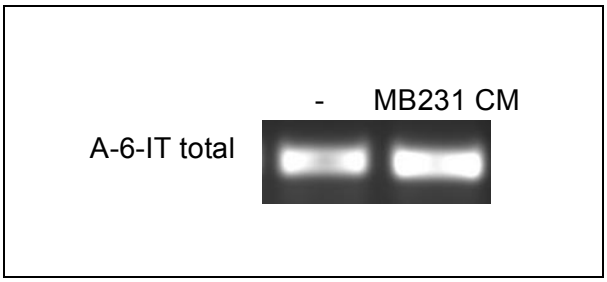
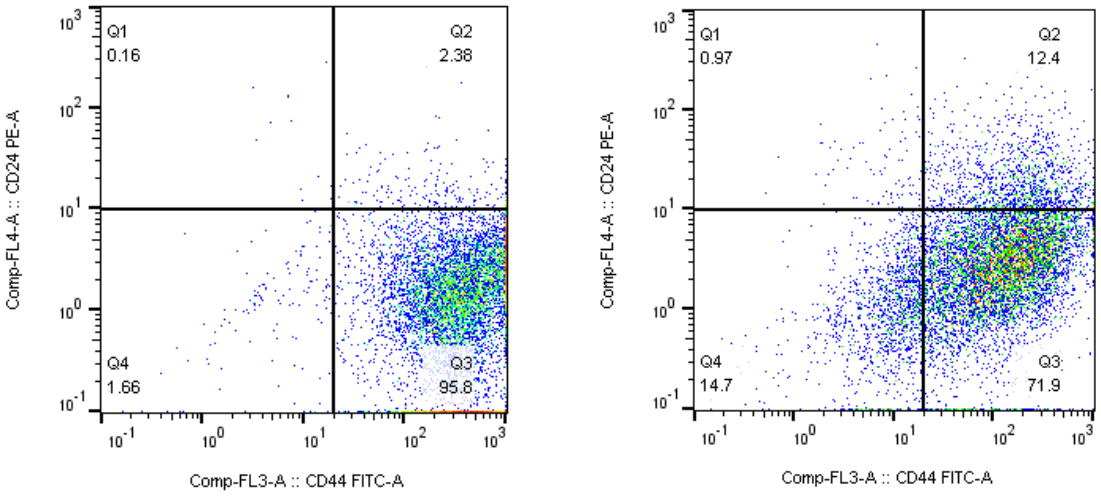


Figure 21 RT-PCR of alpha 6 integrin expression total in (-) MCF-7 control and (CM MB231) in MB231 conditioned media after four weeks. An increase of alpha 6 integrin expression in MCF-7 can be observed after treatment with MB231 CM.

3.1.4 Treatments in Normoxia

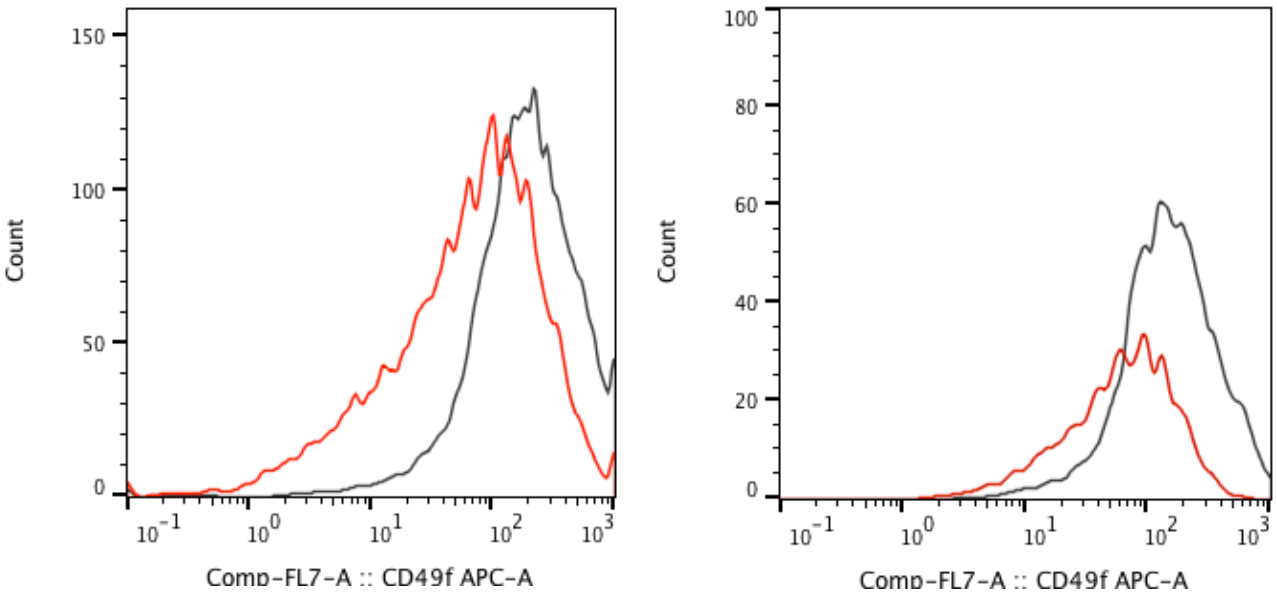
MB231 in Conditioned Media of induced Pluripotent Stem Cells



A B

Figure 22 Flow Cytometric Analysis (FACS) of the CD44+/CD24- surface marker expression in MB231 under normoxia (A) control and (B) in conditioned media of iPSC (Riv9) after three weeks. A reduction of CSC% in MB231 from 95.8% to 71.9% can be observed after treatment with iPSC CM.

Alpha 6 integrin expression in MB231 in Conditioned Media of induced Pluripotent Stem Cells



A (pool)

(grey) normoxia control

(red) CM iPSC

B (Quadrant 3: $CD44^{+}/CD24^{-/low}$)

(grey) normoxia control

(red) CM iPSC

Figure 23 Flow Cytometric Analysis of alpha 6 integrin levels expressed in (A) MB231 pool and (B) in quadrant $CD44^{+}/CD24^{-/low}$, stem cell surface markers. MB231 treated with iPSC CM show a reduction in alpha 6 integrin expression (CD49f), especially in quadrant 3 ($CD44^{+}/CD24^{-/low}$).

MB231

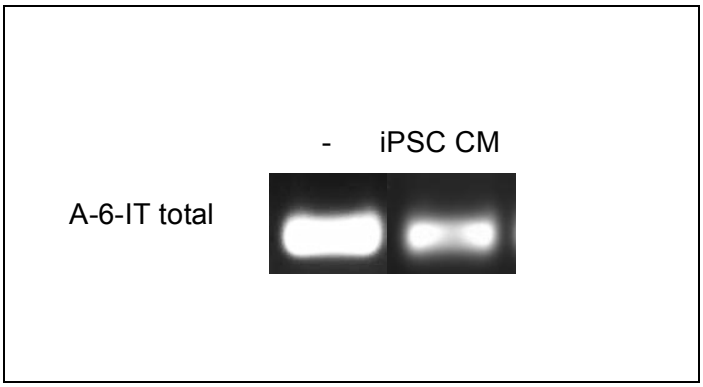


Figure 24 RT-PCR of alpha 6 integrin expression total in (-) MB231 control and (CM iPSC) in induced pluripotent conditioned media after 3.5 weeks. MB231 cells treated with iPSC CM show a reduction in alpha 6 integrin expression.

MB231 in iPSC CM

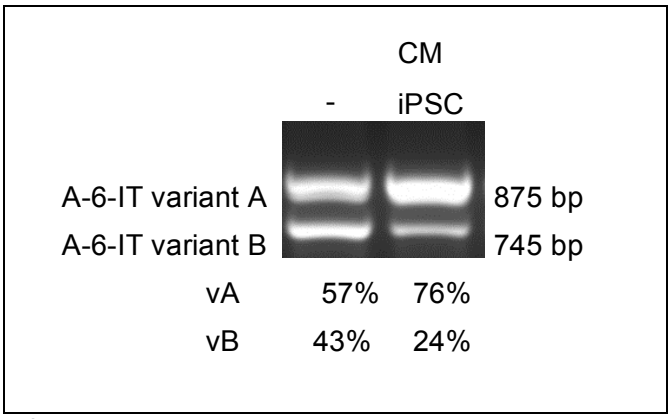


Figure 25 RT-PCR of alpha 6 integrin expression variants A and B in (-) MB231 control and (CM iPSC) in iPSC conditioned media after 3 weeks. After treatment of MB231 with iPSC CM, an increase in alpha 6 integrin variant A (associated with normal cancer cells) and a decrease in alpha 6 integrin variant B (associated with CSC) is observed.

MCF-7 in Conditioned Media of induced Pluripotent Stem Cells and MB231

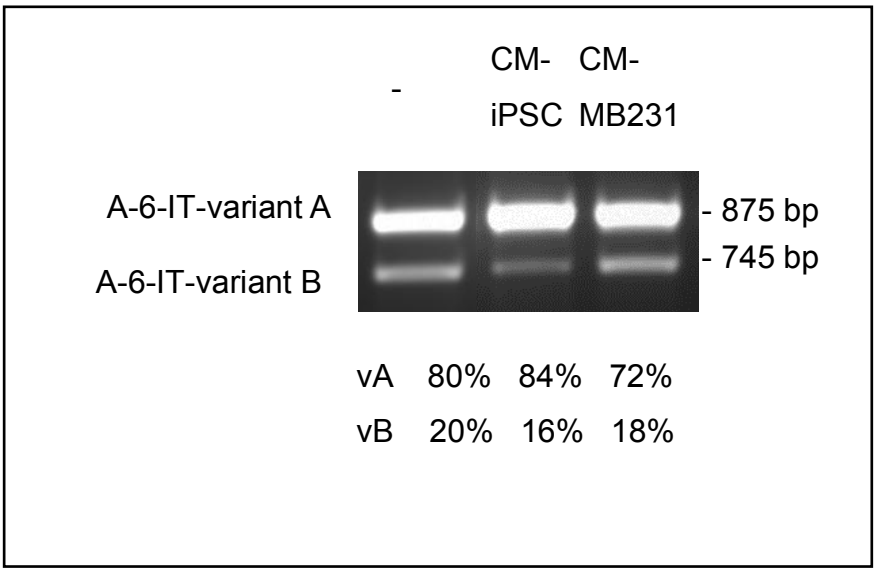


Figure 26 RT-PCR of alpha 6 integrin expression variants A and B in MCF-7 (-) control and (CM iPSC) in iPSC conditioned media after 3 weeks. MCF-7 treated with iPSC CM shows an increase in alpha 6 integrin variant A (associated with normal cancer cells) and a decrease in alpha 6 integrin variant B (associated with CSC). On the other hand, when treated with MB231 CM, a decrease in alpha 6 integrin variant A and an increase in alpha 6 integrin variant B is observed.

Induced Pluripotent Stem cells – Riv9 cells

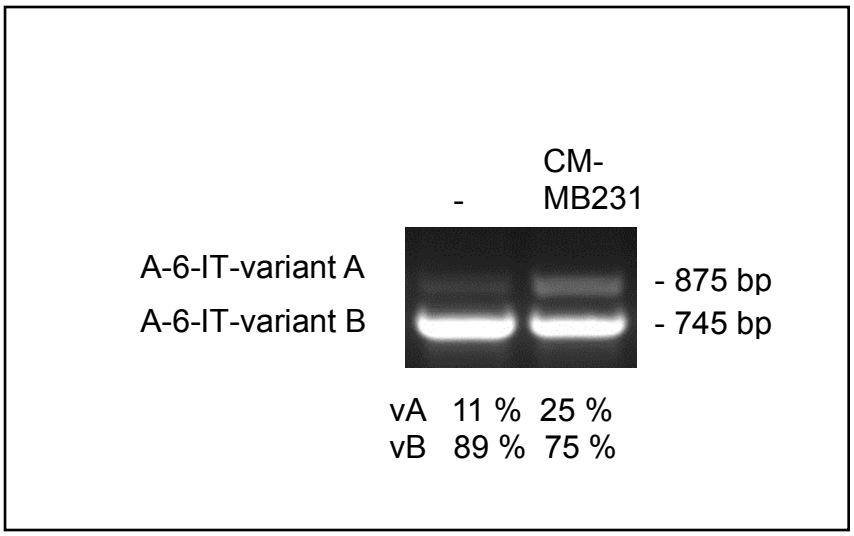
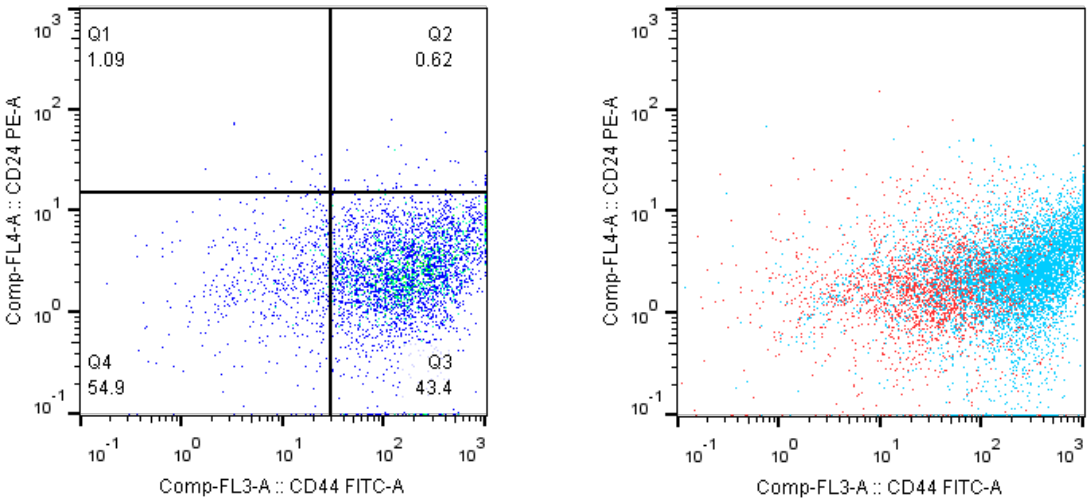


Figure 27 RT-PCR of alpha 6 integrin expression variants A and B in induced pluripotent stem cells (-) control and (CM iPSC) in iPSC conditioned media after 3.5 weeks. After treatment of iPSC with MB231 CM a increase in alpha 6 integrin variant A (associated with normal cancer cells) and a decrease in alpha 6 integrin variant B (associated with CSC) is observed.

MB231 (Magnetic Bead Separation)



A

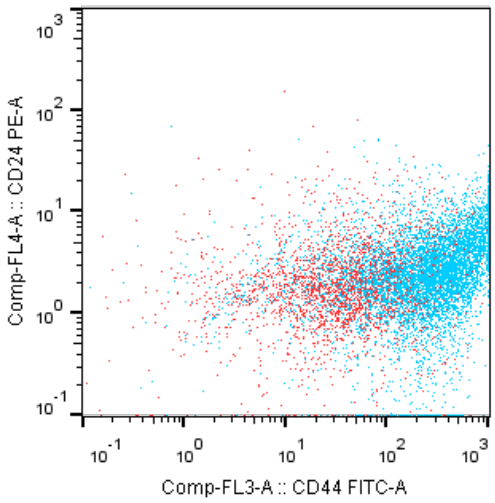
B

(blue) CD 24^{-}

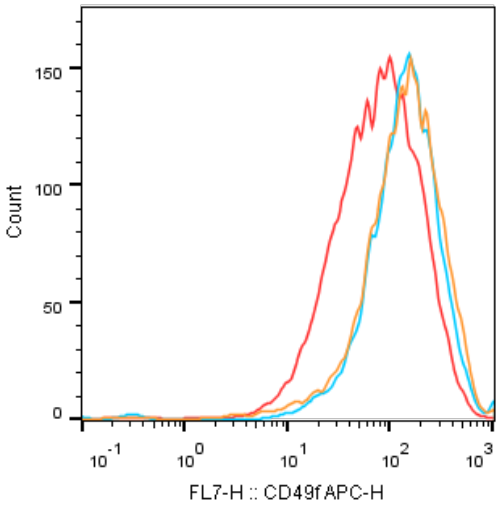
(red) CD 24^{+}

Figure 28 Flow Cytometric Analysis (FACS) of MB231 (A) before and (B) after separation by CD24 magnetic beads. This graph shows the distribution of CD24^{-} and CD24^{+}.

$CD24^{+/-}$ MB231 Alpha 6 Integrin expression



A



B

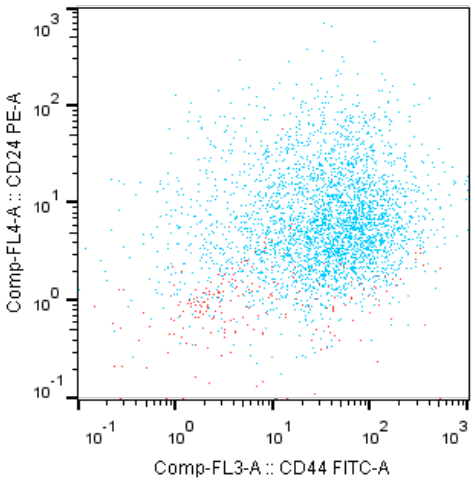
(orange) control

(blue) CD24-

(red) CD24+

Figure 29 Flow Cytometric Analysis (FACS) of MB231 **after separation** using CD24 magnetic beads. (A) CD44+/CD24- surface marker expression in MB231 and (B) CD49f expression in MB231. Results show that cells with marker $CD24^{+}$ have lower expression of alpha 6 integrin (C49f) than cells with marker $CD24^{-}$.

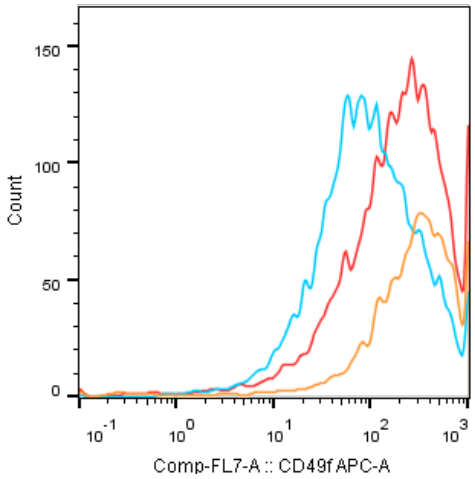
$CD24^{+/-}$ surface marker expressions in MB231



MB231 in mTeSR1 (control)

(blue) CD24+

(red) CD24-

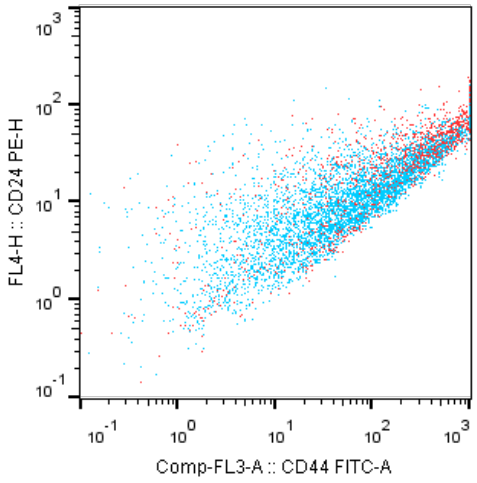


MB231 in mTeSR1 (control)

(red) MB231 pool

(blue) CD24+

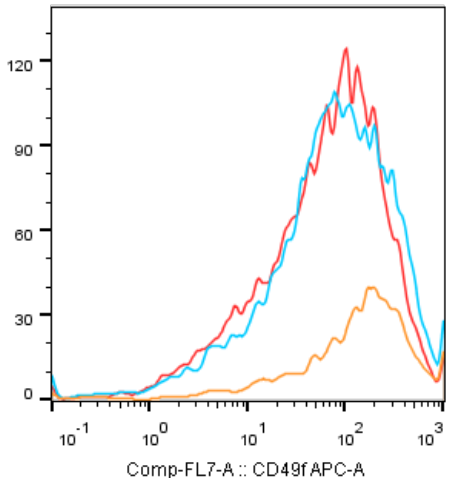
(orange) CD24-



MB231 in CM iPSC

(blue) CD24+

(red) CD24-



MB231 in CM iPSC

(red) MB231 control (pool)

(blue) MB231 CD24+

(orange) CD24-

Figure 30 Flow Cytometric Analysis (FACS) of MB231 control and CM iPSC after separation using CD24 magnetic beads.

3.2 Identification of Proteins in iPSC conditioned media

We analyzed mTeSR-1 (Control) and conditioned media of induced pluripotent stem cells via mass spectrometry to identify any putative factors in iPSC CM. We performed trypsin digest and the samples were cleaned by extraction Cartridge (Waters, Milford, MA) and eluted at 3 different acetonitrile concentrations (30%, 60% and 90%) to obtain a mixture of peptides.

The upper graphs (Figures 31-36) correspond to the elution pattern of peptides of HPLC in the Orbitrap. The samples analyzed at the Orbitrap consist of a mix of peptides that were obtained after the trypsin digest and isolation in reverse phase chromatography (FPLC) at certain acetonitrile concentrations.

The bottom graphs (Figures 31-36) correspond to an example representing a Mass Spectrometric profile of peptides eluted at a certain time (retention time (RT)). On top of these graphs they state a selected retention time of the mass spectrum analysis of peptides eluted at that time. The x-axis states the “m/z” that corresponds to the mass and charge number of ions.

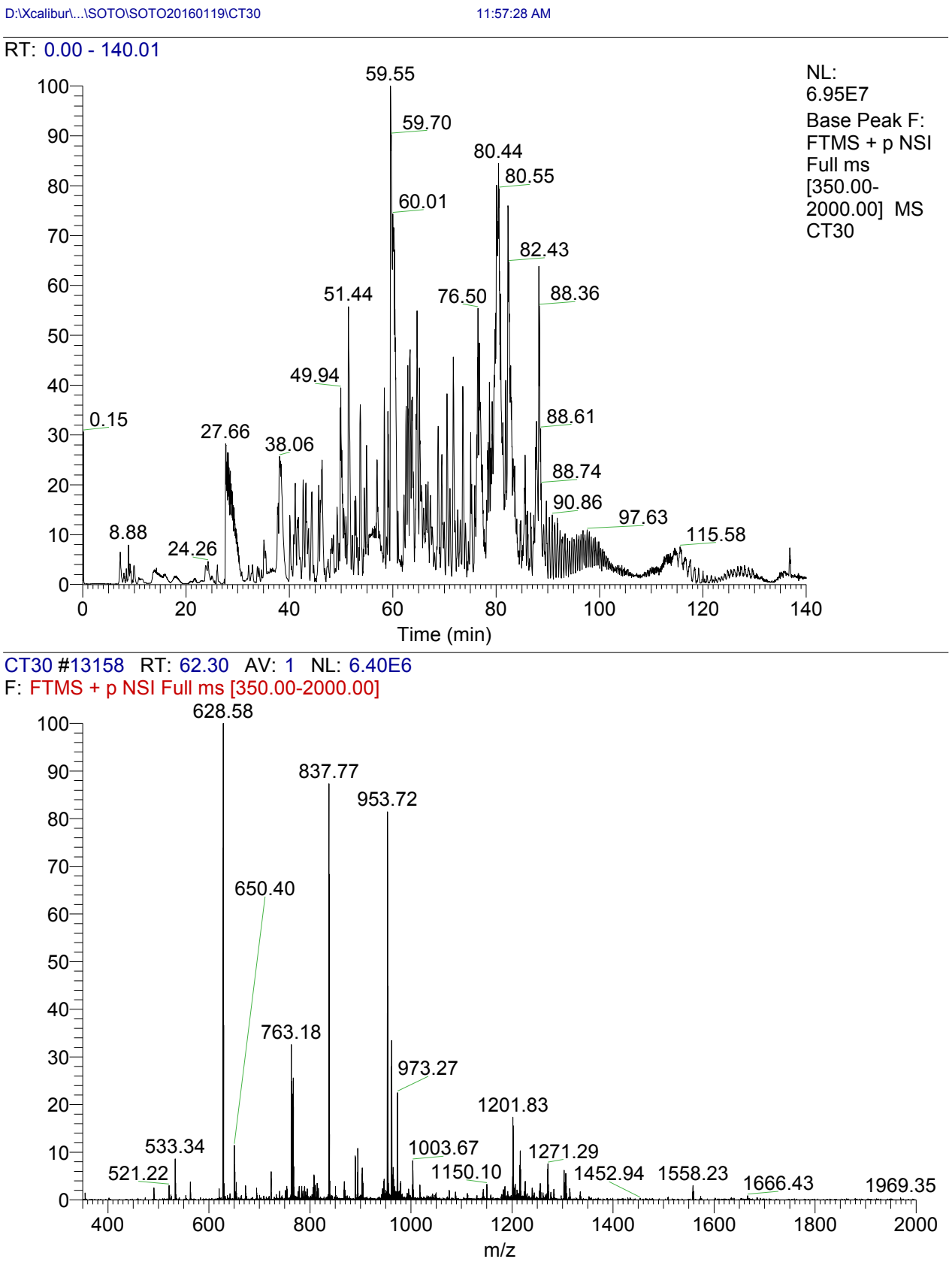


Figure 31 Control at 30% acetonitrile concentration.

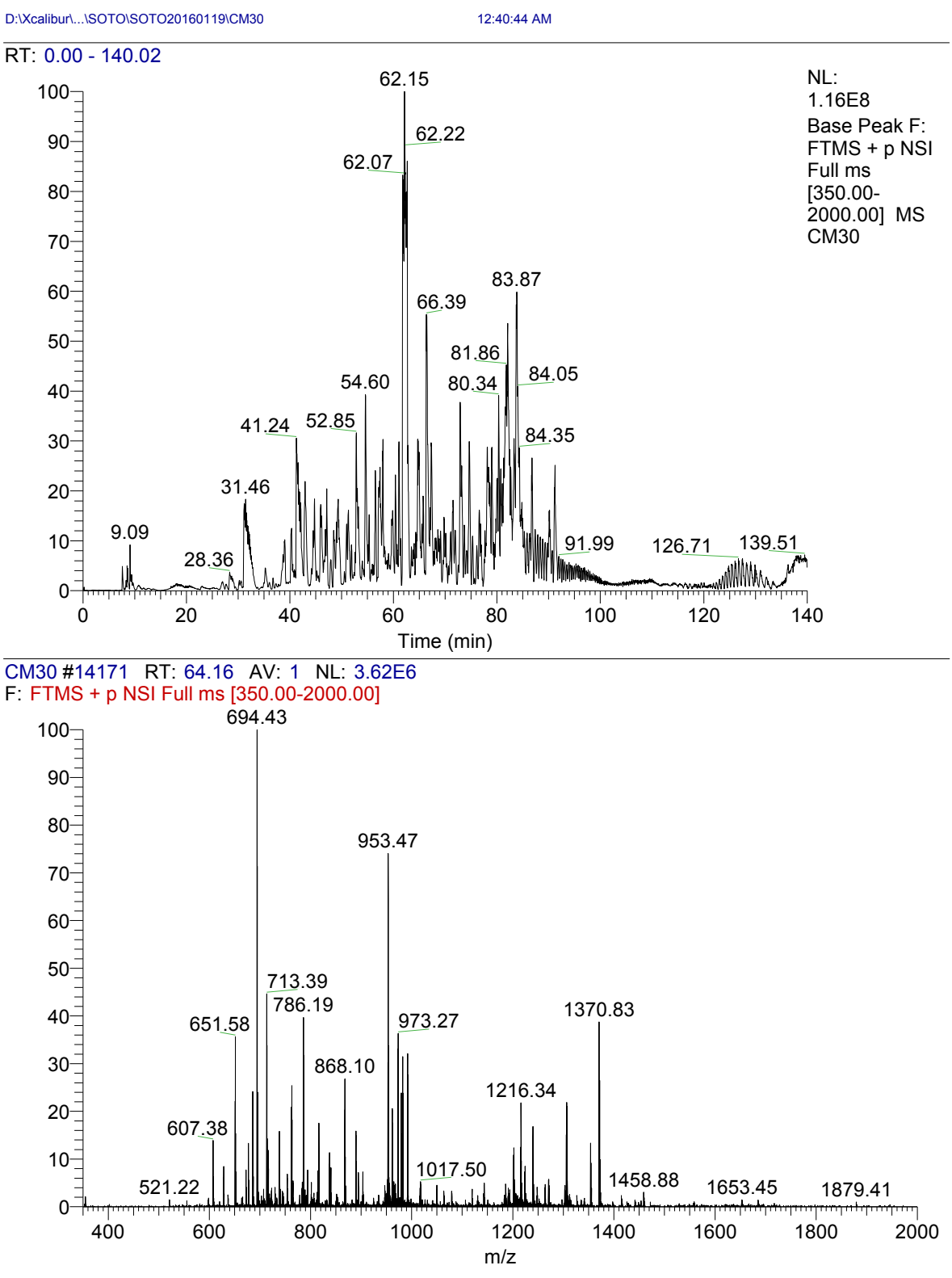


Figure 32 Conditioned media at 30% acetonitrile concentration.

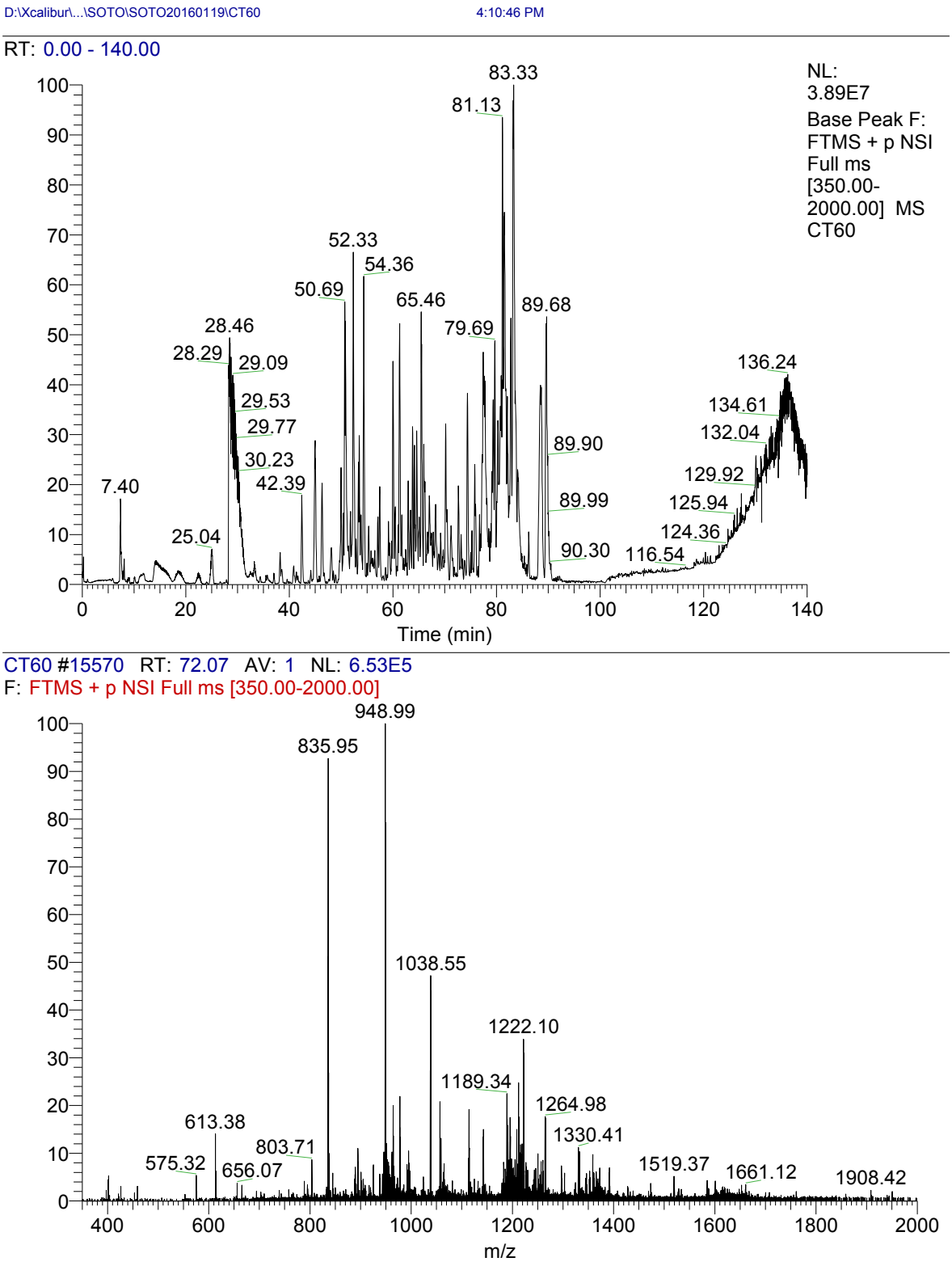


Figure 33 Control at 60% acetonitrile concentration

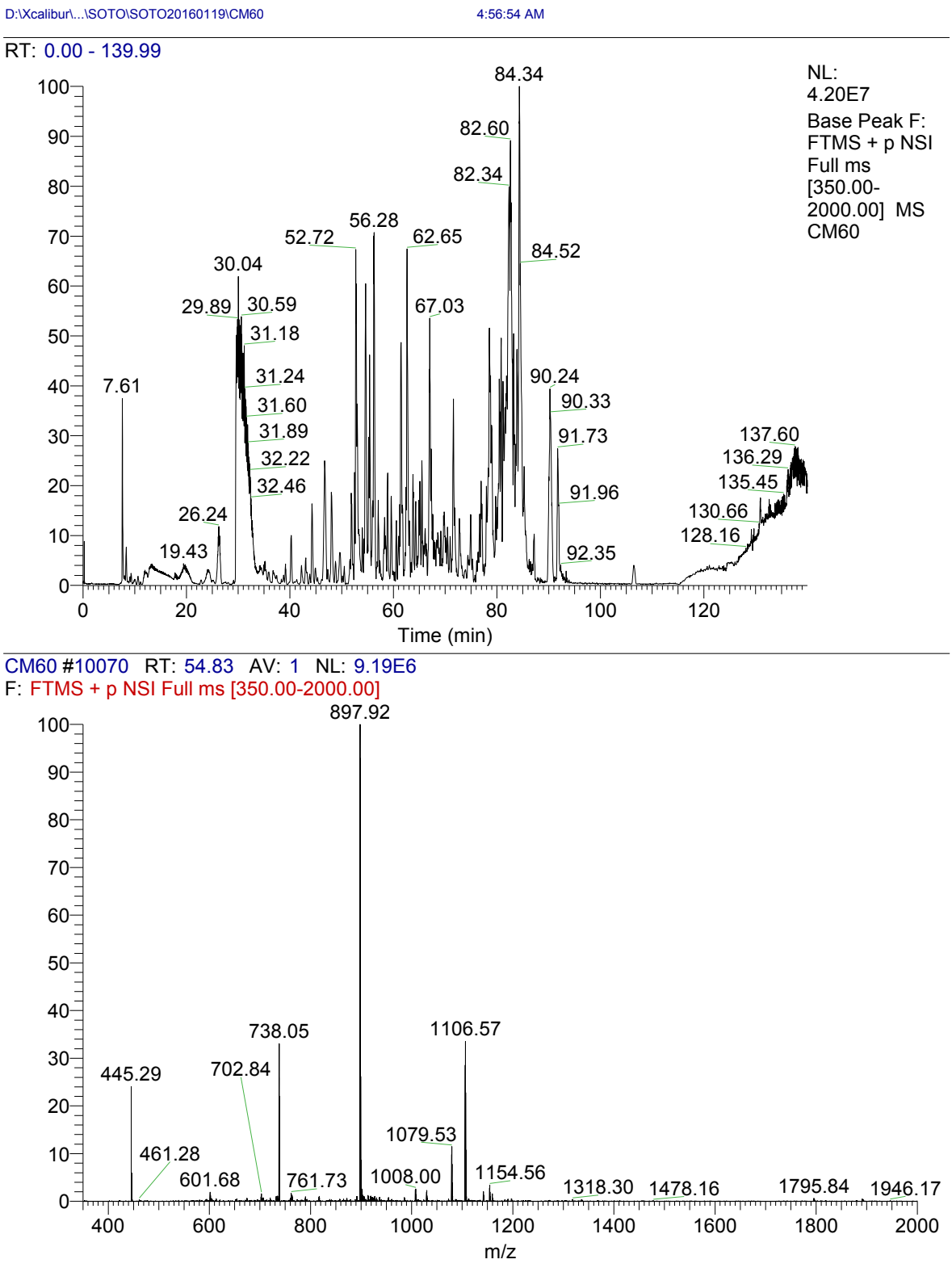


Figure 34 Conditioned media at 60% acetonitrile concentration

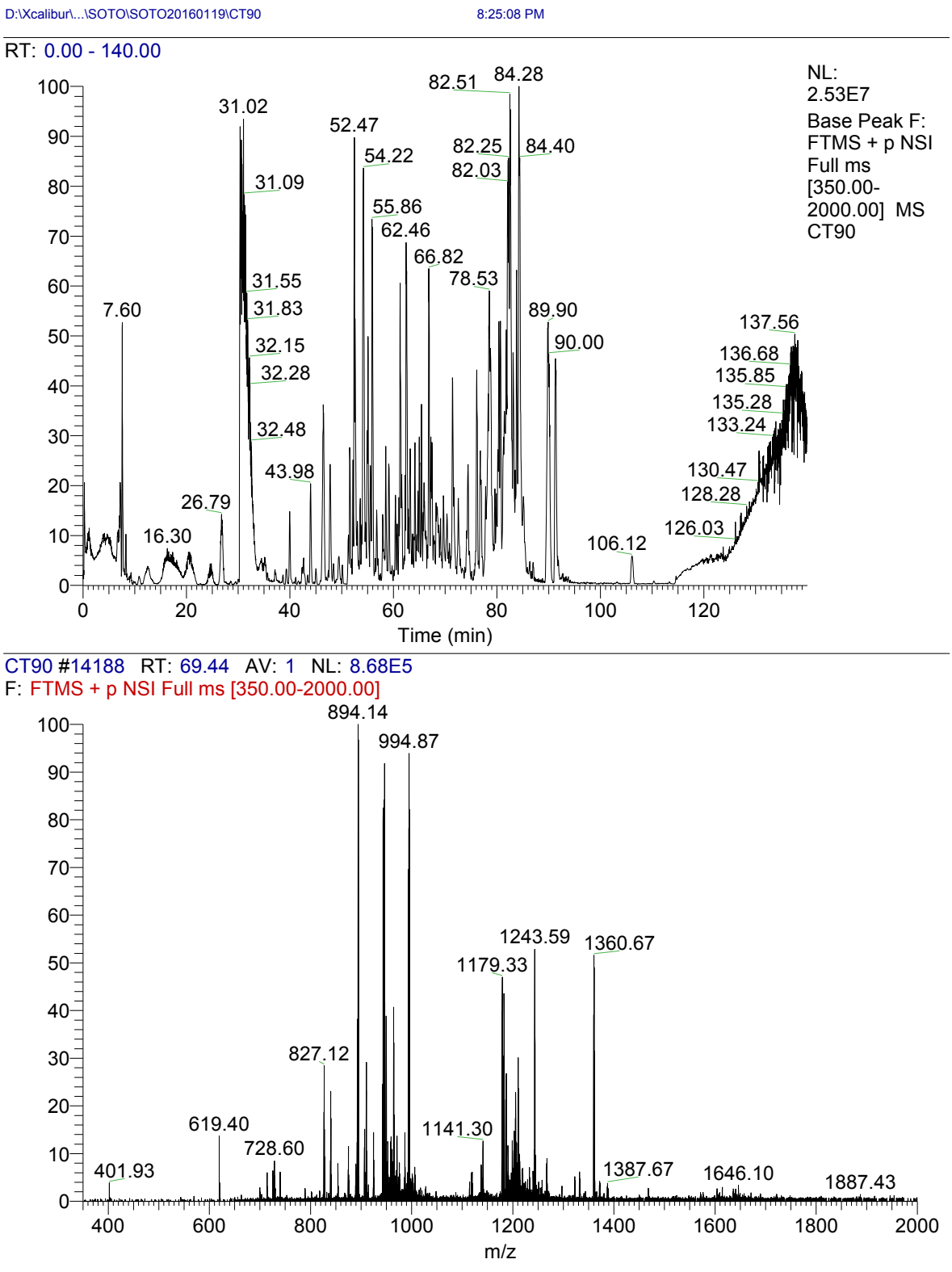


Figure 35 Control at 90% acetonitrile concentration.

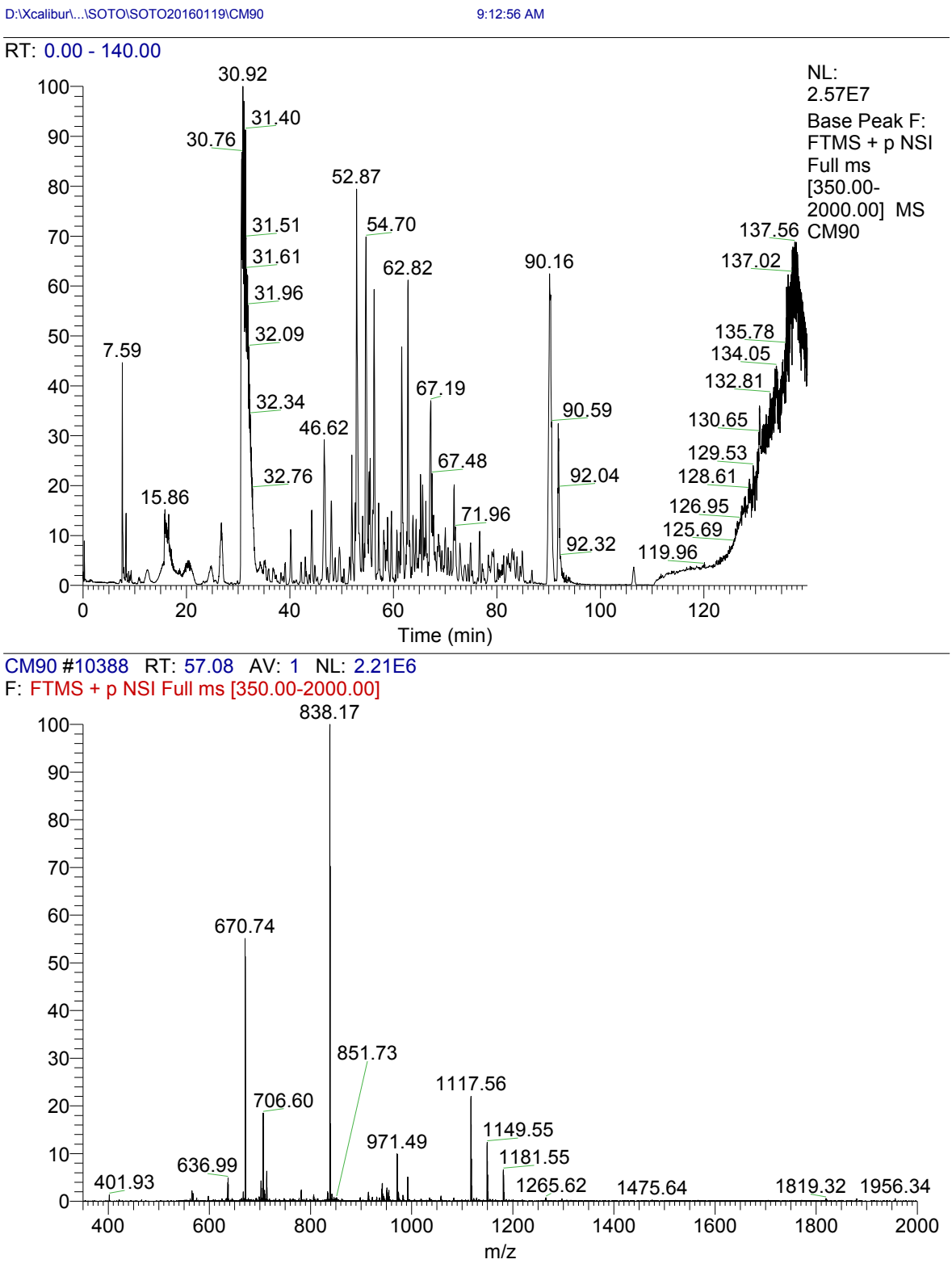


Figure 36 Conditioned media at 90% acetonitrile concentration.

Table 1 Protein identified at mTeSR-1 (control) at 30% acetonitrile concentration.

Protein Description	Score	Coverage	MW (kDa)	Calc. pI
Keratin, type II cytoskeletal 1	85,25	46,89	66,0	8,12
Keratin, type I cytoskeletal 9	61,58	44,14	62,0	5,24
Keratin, type I cytoskeletal 10	43,89	41,10	58,8	5,21
Serum albumin	37,92	17,90	69,3	6,28
Serotransferrin	36,32	31,81	77,0	7,12
Keratin, type II cytoskeletal 2 epidermal	32,99	25,35	65,4	8,00
Isoform Numa-m of Nuclear mitotic apparatus protein 1	17,33	9,80	201,3	5,29
Trypsin-1 (Fragment)	15,36	7,04	15,4	7,27
Isoform 4 of Intersectin-1	7,40	12,24	187,7	7,94
Novel protein	4,69	14,12	9,5	10,80
EF-hand calcium-binding domain-containing protein 12	3,67	12,41	66,5	9,47
Protein-arginine deiminase type-4	3,64	7,24	74,0	6,58
Isoform 4 of Ankyrin repeat domain-containing protein 6	2,74	3,17	73,2	9,32

Table 2 Protein Description of conditioned media of iPSC at 30% acetonitrile concentration.

Protein Description	Score	Coverage	MW [kDa]	calc. pI
Keratin, type II cytoskeletal 1	87,56	50,93	66,0	8,12
Keratin, type I cytoskeletal 9	60,51	38,52	62,0	5,24
Keratin, type II cytoskeletal 2 epidermal	56,43	30,83	65,4	8,00
Serotransferrin	51,22	30,09	77,0	7,12
Keratin, type I cytoskeletal 10	37,42	20,03	58,8	5,21
Serum albumin	25,97	12,81	69,3	6,28
* Tumor necrosis factor receptor superfamily member 12A	15,45	25,00	8,7	8,31
* Actin, cytoplasmic 2, N-terminally processed	12,96	25,07	42,1	6,30
Trypsin-1	10,81	39,68	26,5	6,51
Ubiquitin-40S ribosomal protein S27a	10,20	50,64	18,0	9,64
Tubulin-specific chaperone A	10,16	21,43	10,1	4,63
* Profilin 1	10,09	21,15	11,4	9,17
Proadrenomedullin N-20 terminal peptide	9,17	36,96	15,0	10,76
* Cadherin-3 (Fragment)	8,15	60,27	7,8	4,84
Polyadenylate-binding protein 3	7,52	20,60	70,0	9,67
TBC1 domain family member 8	7,38	2,63	130,8	5,52
Far upstream element-binding protein 1	7,33	14,44	67,5	7,61
Fatty acid-binding protein, epidermal	6,25	34,65	11,2	6,07
* Thymosin beta-4	4,96	27,27	5,0	5,06
* Stathmin (Fragment)	4,91	31,40	9,9	7,42
Keratin, type II cytoskeletal 73	4,70	15,74	58,9	7,23
Testis- and ovary-specific PAZ domain-	4,42	5,73	190,8	7,87

containing protein 1				
Vitamin D-binding protein	4,00	17,24	38,8	5,50
* Fibrillin-2	3,37	23,08	314,6	4,86
Hematological and neurological-expressed 1-like protein (Fragment)	3,19	12,17	12,2	8,19
Keratin, type I cytoskeletal 16	2,99	20,72	51,2	5,05
Isoform Er16 of Ankyrin-1	2,88	3,55	166,4	7,27
Uncharacterized protein C17orf78	2,81	29,09	30,5	9,55
Mitochondrial dynamic protein MID49	2,80	53,85	12,4	9,98

Table 3 Protein Description of mTeSR-1 (Control) at 60% acetonitrile concentration.

Protein Description	**Score**	**Coverage**	**MW [kDa]**	**calc. pI**
Keratin, type II cytoskeletal 1	16,83	10,40	66,0	8,12
Trypsin-1	9,83	43,66	15,4	7,27
Keratin, type II cytoskeletal 2 epidermal	9,15	13,62	65,4	8,00
Keratin, type I cytoskeletal 9	8,58	15,09	62,0	5,24
Serum albumin	6,41	8,54	69,3	6,28
Keratin, type I cytoskeletal 16	5,11	16,70	51,2	5,05
Laminin subunit alpha-2	3,69	14,99	343,7	6,40
Transgelin-2	3,58	33,67	22,4	8,25

Table 4 Protein Description of conditioned media of iPSC at 60% acetonitrile concentration.

Protein Description	**Score**	**Coverage**	**MW [kDa]**	**calc. pI**
Keratin, type I cytoskeletal 9	19,68	21,51	62,0	5,24
Mediator of RNA polymerase II transcription subunit 8	17,65	7,09	29,1	7,44
Keratin, type II cytoskeletal 1	13,19	16,15	66,0	8,12
Eukaryotic translation initiation factor 2 subunit 1 (Fragment)	7,17	7,54	29,0	8,48
Trypsin-1	7,11	27,94	26,5	6,51
Kinesin-like protein KIF23	4,42	17,65	109,1	8,57
Proprotein convertase subtilisin/kexin type 6 (Fragment)	4,15	8,66	88,5	7,12

Table 5 Protein Description of mTeSR-1 (Control) at 90% acetonitrile concentration.

Protein Description	Score	Coverage	MW [kDa]	calc. pI
Trypsin-1	14,90	31,17	26,5	6,51
Keratin, type II cytoskeletal 1	8,75	6,52	66,0	8,12
Probable ubiquitin carboxyl-terminal hydrolase FAF-Y	6,17	12,49	290,9	5,86
Keratin, type I cytoskeletal 9	3,92	16,05	62,0	5,24
Lymphokine-activated killer T-cell-originated protein kinase	3,85	13,66	36,1	5,12
SPOC domain-containing protein 1 (Fragment)	3,84	100,00	4,9	4,15
Hornerin	3,49	4,39	282,2	10,04
Isoform 2 of TATA element modulatory factor	3,39	7,12	123,1	4,92
Putative trypsin-6	3,33	14,98	26,5	6,27

Table 6 Protein Description of conditioned media of iPSC at 90% acetonitrile concentration.

Protein Description	Score	Coverage	MW [kDa]	calc. pI
Keratin, type I cytoskeletal 9	20,84	20,71	62,0	5,24
Adenylate kinase 8	10,55	14,41	54,9	6,15
Keratin, type II cytoskeletal 2 epidermal	9,23	9,86	65,4	8,00
Isoform 2 of Serine/threonine-protein kinase Nek1	9,04	9,72	138,0	5,68
SCY1-like protein 2	5,36	15,50	103,6	8,22
Excitatory amino acid transporter 3 (Fragment)	3,36	35,12	26,7	5,02

4 Discussion

4.1 Plasticity of Cancer Stem Cells

Our main research deals with the examination of the plasticity of cell lines MB231 (high percentage of CSC) and MCF-7 (low percentage of CSC) (Figure 9). We found that indeed it was possible to change the CSC phenotype of those cells by incubating them with conditioned media from normal stem cells (iPSC) or high malignant cells (MB-231), respectively. We also identified that the cell line with high percentage of CSC, MB231, has high levels of alpha 6 integrin compared to MCF-7 (Figure 10 and 11). We want to establish a proof of concept that suggests that the phenotype of cancer stem cells can be distorted and reprogrammed via intervention of the cell microenvironment.

The surface marker expression of cancer stem cells is known as $CD44^{+}/CD24^{-/low}$. By using flow cytometric analysis (FACS), we were able to identify that in the cancer cell line MB231 we have 95.8% of cancer stem cells while MCF-7 only contains 0.22% of its population (Figure 9). Therefore we used cancer cell line MB231 to examine the plasticity of cancer stem cells and to use another extreme, we used MCF-7 since it mainly consists of normal cancer cells and contains extremely low CSC. We changed the microenvironment of the cells by placing them into a new niche known as conditioned media. Upon placement into either cancerous or non-cancerous environment, we observe changes in their phenotype.

When the cancer cell line MB231 was exposed to conditioned media of induced pluripotent stem cells (iPSC-CM) for three and a half weeks in normoxia, we observed changes in its phenotype. The first sign of changes was seen under the microscope. As seen on figure 12 and 13, we assess that they acquired a different cellular morphology that is not very typical for this cell line; however, they did not acquire extreme cell structure modifications in comparison to treatments made to iPSC. This is due to the fact that normal cells have higher plasticity compared to cancerous cells. However, the growth of the cells was highly affected since a decrease in confluency in cell cultures was observed. For the examination of changes on protein-level, we used flow cytometric analysis (FACS). As hypothesized, we

found that there is a significant reduction from 95.8% to 71.9% of cell population expression surface markers $CD44^{+}/CD24^{-/low}$ cells and an increase in other markers indicating an acquisition of a non-CSC phenotype, suggesting a less tumorigenic one. This finding shows that by exposing CSC to a positive (iPSC CM) microenvironment containing extracellular solubles secreted by induced pluripotent stem cells, a positive change in the cell's surface marker takes place. We suggest that the extracellular proteins bind to either unknown surface receptors, alpha 6 integrin receptors (CD49f), or both that then relay signals and trigger changes in surface marker expressions. As seen in figure 17, we can observe a reduction from 91.5% to 77.0% in cancer stem cell percentage when put into a hypoxic (1% O_2) environment. We also tested for the marker CD49f, which represents protein alpha 6 integrin. On figure 17 and 23, we can see a reduction in alpha 6 integrin expression compared to control caused by the iPSC conditioned media in both normoxia and hypoxia. This is significant because when CD49f is highly expressed in cancer cell lines, it is a signifying marker for identifying the tumorigenic potential in cancer. Thus when the expression of CD49f decreases, which is an identical reflection of the known oncogenic effect of alpha 6 integrin, it induces the CSCs into differentiation and the tumorigenic potential/state of these cells are diminished. To examine the changes on RNA-level, we performed an RT-PCR. Here, we were also able to prove that there are optimistic changes in alpha 6 integrin levels total as well as in alpha 6 integrin variant B, which is known to be related to malignant cancer stem cells (Figures 24-25).

Moreover, we performed magnetic bead separation to separate MB231 pool into populations of $CD24^{+}$ and $CD24^{-}$ (Figures 28-30). Afterwards, we examined them for alpha 6 integrin (CD49f) to see if phenotypic difference in each population were observed after treatment with iPSC conditioned media. Figure 29 shows that population $CD24^{+}$ has lower signals of alpha 6 integrin (CD49f) in comparison to $CD24^{-}$. This coincides with our belief that cells that do not have the cancer stem cell phenotype ($CD24^{-/low}$) have low expression of alpha 6 integrin.

Breast cancer cell line MCF-7 is typically characterized as $CD44^{-}/CD24^{+}$, as it mainly consists of normal cancer cells and contains only little cancer stem cell percentage (Figure 18-20). We wanted to test their plasticity by exposure of MCF-7 cells to iPSC conditioned media. We noted that changes in cell morphology, es-

pecially in hypoxia (Figures 14-15) already came about around the first week, where they have transformed from round cells to cells that have created a network with each other. The confluency in both normoxia and hypoxia was affected as it decreased during their incubation time. For the analysis of cell surface markers, we have used flow cytometry (FACS). As seen on Figure 18, we see a reduction of CSC percentage from 1.63% to 0.71%. This finding indicates that there was an increase in cell population with surface markers $CD44^{+}/CD24^{-/low}$ hence the little amount of CSC that was present was still affected by the iPSC conditioned media. We exposed MCF-7 cells to MB231 conditioned media, to test if this cancerous niche would increase the amount of CSC percentage. In the same figure, we notice that indeed there was an increase from 3.29% to 5.28%. This result shows that the number of cancer stem cells can not only be reduced but also be increased when put into a microenvironment filled with secreted factors of cancer stem cells. For the examination of alpha 6 integrin, FACS and RT-PCR were used. Our data in figures 20 and 26 confirm the reduction in alpha 6 integrin levels in both normoxia and hypoxia respectively. Figure 25 displays a reduction in alpha 6 integrin variant B, which shows a transformation to a less tumorigenic phenotype. As mentioned before, we additionally cultured MCF-7 cells in a cancerous niche, MB231 conditioned media. In figure 25 we can see an increase in alpha 6 integrin total and variant B.

Induced pluripotent stem cells are known to be rich in alpha 6 integrin. We have exposed iPSC to MB231 conditioned media and were able to see morphological alterations. However, this was expected due to the extremely high sensitivity trademark for pluripotent stem cells to differentiate in response to extracellular signals. We assume that after a treatment of 3 weeks the cancerous-CM obtained from MB231 have induced the iPSCs into differentiation and potentially converting them into cancer cell populations. Carefully assessing the iPSCs population exposed to cancerous-CM revealed a significant decline in the cell population expressing the $CD44^{-}/CD24^{+}$ surface markers, and thus acquiring the CSC phenotype $CD44^{+}/CD24^{-/low}$, and the differentiated phenotype of $CD44^{+}/CD24^{+}$. Thus it can be said that the cancerous-CM obtained from MB231 has induced the iPSCs into differentiation and potentially converting them into cancer cell populations. However, the emphasis is not necessary on the type of differentiated cell acquired

but on the fact that the cancerous CM obtained were able to induce changes to these iPSCs in their surface markers to express similar or identical fluorescent readings observed throughout those tumor cell populations. The RT-PCR data in figure 27 shows an increase in alpha 6 integrin variant B, which also indicates an increase in cancer stem cells. Zooming into the hypoxic effect of a low oxygen concentration on stem cells, hypoxia has dual roles. It functions as a stemness factor by sustaining and preventing the differentiation of stem cells.

4.2 Identification of Proteins in iPSC conditioned media

For the second part of our project, we wanted to examine the factors secreted by induced pluripotent stem cells into the niche that promote optimistic changes in the cancer stem cell phenotype of MB231. For that purpose we used stem cell media mTeSR-1 as control and conditioned media from induced pluripotent stem cells for the analysis.

Tables 1-6 show us the descriptions of proteins identified by the Orbitrap at acetonitrile concentrations 30%, 60%, and 90%. We discovered that there were several proteins found that were exclusively present in the conditioned media. For the analysis we ignore the identification of BSA, serotransferrin, and trypsin due to the fact that these were defined by the composition of mTeSR-1 media (Figure 7) already. Keratin cannot be taken into consideration as well since its presence is due to skin contamination. When we take a deeper look into the proteins identified, we believe that some of the factors detected are from exosomes that were secreted by induced pluripotent stem cells. We conclude this from the mass spectrometric protein descriptions that show the presence of cytoskeletons and integral membrane proteins in the conditioned media. Tables 2, 4 and 6 shows us the presence of actin, profilin, cadherin, fibrilin-2, thymosin beta4, stathmin, and transgelin, which play vital roles in the cytoskeleton and structural function of the cells. These proteins are known to be part of vesicles such as exosomes or cell debris. However, we do not take the latter into consideration since this should have been identified at the control as well.

In table 2, we identify the cytokine tumor necrosis factor receptor superfamily member 12A (TNF), which is involved in systemic inflammation. TNF alpha regu-

lates immune cells and may induce fever, apoptotic cell death, inflammation, and can inhibit tumorigenesis and viral replication. A dysregulation of TNF production have been found to be responsible for various human diseases, including cancer. A study by Defilippi et al has shown that treatment with tumor necrosis factor alpha downregulates the expression of alpha 6 beta 1 integrin. After a 48hour treatment, the level of alpha 6 integrin expression was reduced to 20% of the control value (Defilippi, Silengo, & Tarone, 1992). From this we conclude that tumor necrosis factor receptor superfamily member 12A plays a role in the reduction of alpha 6 integrin in the cancer stem cell population of the our cancer cell lines MB231 and MCF-7.

List of References

Abdollahi, H., Harris, L. J., Zhang, P., Mcllhenny, S., Tulenko, T., & DiMuzio, P. J. (2012). The Role of Hypoxia in Stem Cell Differentiation and Therapeutics. *J Surg Res*, *165*(1), 112–117. http://doi.org/10.1016/j.jss.2009.09.057.The

Ailles, L., & Weissman, I. (2007). Cancer stem cells in solid tumors. *Current Opinion in Biotechnology*, *18*, 460–466. http://doi.org/10.1016/j.copbio.2007.10.007

Al-Hajj, M., & Clarke, M. F. (2004). Self-renewal and solid tumor stem cells. *Oncogene*, *23*(43), 7274–7282. http://doi.org/10.1038/sj.onc.1207947

Bauer, N., Wilsch-Br??uninger, M., Karbanov??, J., Fonseca, A. V., Strauss, D., Freund, D., … Corbeil, D. (2011). Haematopoietic stem cell differentiation promotes the release of prominin-1/CD133-containing membrane vesicles-a role of the endocytic-exocytic pathway. *EMBO Molecular Medicine*, *3*(7), 398–409. http://doi.org/10.1002/emmm.201100147

Bijnsdorp, I. V, Geldof, A. a, Lavaei, M., Piersma, S. R., van Moorselaar, R. J. a, & Jimenez, C. R. (2013). Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients. *Journal of Extracellular Vesicles*, *2*, 1–10. http://doi.org/10.3402/jev.v2i0.22097

Bjerkvig, R., Tysnes, B. B., Aboody, K. S., Najbauer, J., & Terzis, a J. a. (2005). Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nature Reviews. Cancer*, *5*(11), 899–904. http://doi.org/10.1038/nrc1740

Bonfanti, P., Barrandon, Y., & Cossu, G. (2012). “ Hearts and bones ”: the ups and downs of “ plasticity ” in stem cell biology, (5), 353–361. http://doi.org/10.1002/emmm.201200220

Camussi, G., Deregibus, M.-C., Bruno, S., Grange, C., Fonsato, V., & Tetta, C. (2011). Exosome/microvesicle-mediated epigenetic reprogramming of cells. *American Journal of Cancer Research*, *1*(1), 98–110.

Chen, X., Song, C.-H., Feng, B.-S., Li, T.-L., Li, P., Zheng, P.-Y., … Yang, P.-C. (2011). Intestinal epithelial cell-derived integrin alphabeta6 plays an important role in the induction of regulatory T cells and inhibits an antigen-specific Th2 response. *Journal of Leukocyte Biology*, *90*(4), 751–759. http://doi.org/10.1189/jlb.1210696

Chi, J.-T., Wang, Z., Nuyten, D. S. A., Rodriguez, E. H., Schaner, M. E., Salim, A., … Brown, P. O. (2006). Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. *PLoS Medicine*, *3*(3), e47. http://doi.org/10.1371/journal.pmed.0030047

Ciurea, M. E., Georgescu, A. M., Purcaru, S. O., Artene, S. A., Emami, G. H., Boldeanu, M. V., … Dricu, A. (2014). Cancer stem cells: Biological functions and therapeutically targeting. *International Journal of Molecular Sciences*. http://doi.org/10.3390/ijms15058169

Clayton, A., Turkes, A., Dewitt, S., Steadman, R., Mason, M. D., & Hallett, M. B. (2004). Adhesion and signaling by B cell-derived exosomes: the role of integrins. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *18*(9), 977–979. http://doi.org/10.1096/fj.03-1094fje

Cojoc, M., Mäbert, K., Muders, M. H., & Dubrovska, A. (2014). A role for cancer stem cells in therapy resistance: cellular and molecular mechanisms. *Seminars in Cancer Biology*, 1–12. http://doi.org/10.1016/j.semcancer.2014.06.004

Cozzio, A., Passegu??, E., Ayton, P. M., Karsunky, H., Cleary, M. L., & Weissman, I. L. (2003). Similar MLL-associated leukemias arising from self-renewing stem cells and short-lived myeloid progenitors. *Genes and Development*, *17*(24), 3029–3035. http://doi.org/10.1101/gad.1143403

Defilippi, P., Silengo, L., & Tarone, G. (1992). Alpha 6.beta 1 integrin (laminin receptor) is down-regulated by tumor necrosis factor alpha and interleukin-1 beta in human endothelial cells. *J Biol Chem*, *267*(26), 18303–18307. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1388151

Deregibus, M. C., Cantaluppi, V., Calogero, R., Lo Iacono, M., Tetta, C., Biancone,

L., … Camussi, G. (2007). Endothelial progenitor cell - Derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood*, *110*(7), 2440–2448. http://doi.org/10.1182/blood-2007-03-078709

Diaz, R., Nguewa, P. A., Redrado, M., Manrique, I., & Calvo, A. (2015). Sunitinib reduces tumor hypoxia and angiogenesis, and radiosensitizes prostate cancer stem-like cells. *Prostate*, *75*(11), 1137–1149. http://doi.org/10.1002/pros.22980

Goel, H. L., Gritsko, T., Pursell, B., Chang, C., Shultz, L. D., Greiner, D. L., … Mercurio, A. M. (2014). Regulated Splicing of the ??6 Integrin Cytoplasmic Domain Determines the Fate of Breast Cancer Stem Cells. *Cell Reports*, *7*(3), 747–761. http://doi.org/10.1016/j.celrep.2014.03.059

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*. http://doi.org/10.1016/j.cell.2011.02.013

Heijnen, H. F., Schiel, a E., Fijnheer, R., Geuze, H. J., & Sixma, J. J. (1999). Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood*, *94*, 3791–3799.

Honoki, K., Fujii, H., & Tsujiuchi, T. (2006). Cancer Stem Cell Niche : The Role of Mesenchymal Stem Cells in Tumor Microenvironment. *Canser Stem Cells*, *12*(1), 189–206.

Hu, M., & Polyak, K. (2008). Microenvironmental regulation of cancer development. *Current Opinion in Genetics & Development*, *18*(1), 27–34. http://doi.org/10.1016/j.gde.2007.12.006

Huang, E. H., Heidt, D. G., Li, C.-W., & Simeone, D. M. (2007). Cancer stem cells: a new paradigm for understanding tumor progression and therapeutic resistance. *Surgery*, *141*(4), 415–419. http://doi.org/10.1016/j.surg.2006.12.015

Huntly, B. J. P., Shigematsu, H., Deguchi, K., Lee, B. H., Mizuno, S., Duclos, N., … Gilliland, D. G. (2004). MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. *Cancer*

Cell, *6*(6), 587–596. http://doi.org/10.1016/j.ccr.2004.10.015

Iero, M., Valenti, R., Huber, V., Filipazzi, P., Parmiani, G., Fais, S., & Rivoltini, L. (2008). Tumour-released exosomes and their implications in cancer immunity. *Cell Death and Differentiation*, *15*(1), 80–88. http://doi.org/10.1038/sj.cdd.4402237

Jamieson, C. H. M., Ailles, L. E., Dylla, S. J., Muijtjens, M., Jones, C., Zehnder, J. L., … Weissman, I. L. (2004). Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *The New England Journal of Medicine*, *351*(7), 657–667. http://doi.org/10.1056/NEJMoa040258

Jordan, C. T., Guzman, M. L., & Noble, M. (2006). Cancer stem cells. *The New England Journal of Medicine*, *355*(12), 1253–1261. http://doi.org/10.1056/NEJMra061808

Juliano, R. (1994). Signal transduction by integrins and its role in the regulation of tumor growth. *Cancer Metastasis Reviews*, *13*(1), 25–30. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8143343

Kasai, T., Chen, L., Mizutani, A., Kudoh, T., Murakami, H., Fu, L., & Seno, M. (2014). Cancer stem cells converted from pluripotent stem cells and the cancerous niche. *Journal of Stem Cells and Regenerative Medicine*.

Klonisch, T., Wiechec, E., Hombach-Klonisch, S., Ande, S. R., Wesselborg, S., Schulze-Osthoff, K., & Los, M. (2008). Cancer stem cell markers in common cancers - therapeutic implications. *Trends in Molecular Medicine*. http://doi.org/10.1016/j.molmed.2008.08.003

Krivtsov, A. V, Twomey, D., Feng, Z., Stubbs, M. C., Wang, Y., Faber, J., … Armstrong, S. a. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature*, *442*(7104), 818–822. http://doi.org/10.1038/nature04980

Lee, H. D., Kim, Y. H., & Kim, D. S. (2014). Exosomes derived from human macrophages suppress endothelial cell migration by controlling integrin trafficking. *European Journal of Immunology*, *44*(4), 1156–1169. http://doi.org/10.1002/eji.201343660

Liu, H.-G., Chen, C., Yang, H., Pan, Y.-F., & Zhang, X.-H. (2011). Cancer stem cell subsets and their relationships. *Journal of Translational Medicine*, *9*, 50. http://doi.org/10.1186/1479-5876-9-50

Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., & Wang, Y. (2014). Immunobiology of mesenchymal stem cells. *Cell Death and Differentiation*, *21*(2), 216–25. http://doi.org/10.1038/cdd.2013.158

Mathieu, J., Zhang, Z., Nelson, A., Lamba, D. A., Reh, T. A., Ware, C., & Ruohola-Baker, H. (2013). Hypoxia induces re-entry of committed cells into pluripotency. *Stem Cells*, *31*(9), 1737–1748. http://doi.org/10.1002/stem.1446

Mathieu, J., Zhang, Z., Zhou, W., Wang, A. J., Heddleston, J. M., Pinna, C. M. A., … Ruohola-Baker, H. (2011). HIF induces human embryonic stem cell markers in cancer cells. *Cancer Research*, *71*(13), 4640–4652. http://doi.org/10.1158/0008-5472.CAN-10-3320

Medema, J. P. (2013). Cancer stem cells: the challenges ahead. *Nature Cell Biology*, *15*(4), 338–44. http://doi.org/10.1038/ncb2717

Mimeault, M., & Batra, S. K. (2013). Hypoxia-inducing factors as master regulators of stemness properties and altered metabolism of cancer- and metastasis-initiating cells. *Journal of Cellular and Molecular Medicine*, *17*(1), 30–54. http://doi.org/10.1111/jcmm.12004

Moore, K. a, & Lemischka, I. R. (2006). Stem cells and their niches. *Science (New York, N.Y.)*, *311*(5769), 1880–1885. http://doi.org/10.1126/science.1110542

Mukhopadhyay, R., Theriault, R. L., & Price, J. E. (1999). Increased levels of alpha6 integrins are associated with the metastatic phenotype of human breast cancer cells. *Clinical & Experimental Metastasis*, *17*(4), 325–332.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., & D'Souza-Schorey, C. (2009). ARF6-Regulated Shedding of Tumor Cell-Derived Plasma Membrane Microvesicles. *Current Biology*, *19*(22), 1875–1885. http://doi.org/10.1016/j.cub.2009.09.059

O'Brien, C. A., Kreso, A., & Dick, J. E. (2009). Cancer Stem Cells in Solid Tumors: An Overview. *Seminars in Radiation Oncology*.

http://doi.org/10.1016/j.semradonc.2008.11.001

Pardal, R., Clarke, M. F., & Morrison, S. J. (2003). Applying the principles of stem-cell biology to cancer. *Nature Reviews. Cancer*, *3*(12), 895–902. http://doi.org/10.1038/nrc1232

Plaks, V., Kong, N., & Werb, Z. (2015). The cancer stem cell niche: How essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell*. http://doi.org/10.1016/j.stem.2015.02.015

Raposo, G., & Stoorvogel, W. (2013). Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology*. http://doi.org/10.1083/jcb.201211138

Regenbrecht, C. R. A., Lehrach, H., & Adjaye, J. (2008). Stemming cancer: Functional genomics of cancer stem cells in solid tumors. *Stem Cell Reviews*. http://doi.org/10.1007/s12015-008-9034-0

Rich, J. N., & Eyler, C. E. (2008). Cancer stem cells in brain tumor biology. In *Cold Spring Harbor Symposia on Quantitative Biology* (Vol. 73, pp. 411–420). http://doi.org/10.1101/sqb.2008.73.060

Seguin, L., Weis, S. M., & Cheresh, D. A. (2014). Variety in the tumor microenvironment: Integrin splicing regulates stemness. *Cell Stem Cell*. http://doi.org/10.1016/j.stem.2014.04.011

Singh, S., Hawkins, C., Clarke, I., & Squire, J. (2004). Identification of human brain tumour initiating cells. *Nature*, *432*, 396–401. http://doi.org/10.1038/nature03031.1.

Strauss, R., Hamerlik, P., Lieber, A., & Bartek, J. (2012). Regulation of Stem Cell Plasticity: Mechanisms and Relevance to Tissue Biology and Cancer. *Molecular Therapy*, *20*(5), 887–897. http://doi.org/10.1038/mt.2012.2

Tannishtha, R., Morrison, S. J., Clarke, M. F., & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature*, *414*(November), 105–111. http://doi.org/10.1007/978-1-60327-933-8

Tirino, V., Desiderio, V., Paino, F., De Rosa, A., Papaccio, F., La Noce, M., … Papaccio, G. (2013). Cancer stem cells in solid tumors: an overview and new

approaches for their isolation and characterization. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *27*(1), 13–24. http://doi.org/10.1096/fj.12-218222

Visvader, J. E., & Lindeman, G. J. (2012). Cancer stem cells: Current status and evolving complexities. *Cell Stem Cell*. http://doi.org/10.1016/j.stem.2012.05.007

Webber, J., Yeung, V., & Clayton, A. (2015). Extracellular vesicles as modulators of the cancer microenvironment. *Seminars in Cell and Developmental Biology*. http://doi.org/10.1016/j.semcdb.2015.01.013

Yan, T., Mizutani, A., Chen, L., Takaki, M., Hiramoto, Y., Matsuda, S., … Seno, M. (2014). Characterization of cancer stem-like cells derived from mouse induced pluripotent stem cells transformed by tumor-derived extracellular vesicles. *Journal of Cancer*, *5*(7), 572–584. http://doi.org/10.7150/jca.8865

Ye, J., Wu, D., Wu, P., Chen, Z., & Huang, J. (2014). The cancer stem cell niche: cross talk between cancer stem cells and their microenvironment. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *35*(5), 3945–51. http://doi.org/10.1007/s13277-013-1561-x