AMERICAN UNIVERSITY OF BEIRUT

EXPRESSION OF CONNEXIN43 IN BREAST CANCER CELLS (MDA-MB-231): IMPLICATIONS IN CANCER METASTASIS

by
JALAL MAHMOUD KAZAN

A thesis
submitted in partial fulfillment of the requirements
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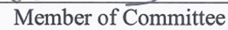

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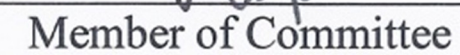

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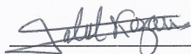

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AN ABSTRACT OF THE THESIS OF

Jalal Mahmoud Kazan for Master of Science
Major: Physiology

Title: Expression of Connexin43 in Breast Cancer Cells (MDA-MB-231): Implications in Cancer Metastasis.

Background: Connexins regulate cell proliferation, function and differentiation. Several human diseases are linked to mutations or dysfunction of connexin protein, and shown to be implicated in carcinogenic processes. For instance, a variety of breast cancer cell lines were shown to express low levels of connexin, or to lose the proper assembly of gap junctions. Several studies have reported channel-dependent and independent roles of connexin in tumorigenesis. A solid base was established proving that connexins are tumor suppressive proteins.

Hypothesis: Over-expression of connexin43 (Cx43) decreases the metastatic potential of a breast cancer cell line MDA-MB-231, while its knock down by shRNA enhances their invasive properties.

Materials and Methods: Upon over-expressing or down-regulating Cx43 in MDA-MB-231, metastatic abilities such as cell proliferation, cell aggregates' morphology in 3D culture system, invasive potential and localization of β-catenin were assessed *in vitro*. *In vivo*, tumor onset and volume, survival rate and cancer cell infiltration to secondary metastatic sites were investigated in immunocompromised mice injected subdermally with cells over-expressing or down-regulating Cx43.

Results: We have observed an epithelial phenotype with a suppressed potential to infiltrate lung and liver tissues upon Cx43 over-expression. On the other hand, Cx43 down-regulation induced a mesenchymal phenotype with higher expression levels of vascular endothelial growth factor (VEGF), invasive abilities and infiltration to secondary metastatic organ sites.

Conclusion: Cx43 over-expression in MDA-MB-231 breast cancer cell line suppresses its metastatic potential *in vivo* and induces an epithelial phenotype *in vitro*. On the other hand, knocking down Cx43 induces a mesenchymal phenotype with aggressive invasive abilities.

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ABBREVIATIONS

2D: two-Dimensional

3D: three-Dimensional

ATP: Adenosine Tri Phosphate

cDNA: complementary DNA

CMV: cytomegalovirus

c-Src: Proto-oncogene tyrosine-protein kinase Src

Cx26: Connexin26

Cx30: Connexin30

Cx32: Connexin 32

Cx43: Connexin43

Cx43D: Cx43-Dendra2

Cx45: Connexin 45

DNA: Deoxyribonucleic acid

ECM: Extra-Cellular Matrix

EMT: Epithelial to Mesenchymal Transition

ERK: extracellular-signal-regulated kinase

FACS: Fluorescence-Activated Cell Sorting

FRAP: Fluorescence Recovery After Photobleaching

GAPDH: Glyceraldehyde phosphate Dehydrogenase

GFP: Green Fluorescent Protein

GJIC: Gap Junctional Intercellular Communication

h: hour

HER2: Human Epidermal Growth Factor Receptor 2

HIF-1α: Hypoxia Inducible Factor-1 alpha

HIFs: Hypoxia-Inducible transcription Factors

kDa: kilo Dalton

M1: Mouse number 1

min: minute

miRNA: microRNA

MMP-2: Matrix MetalloProteases-2

MMP-9: Matrix MetalloProteases-9

MMPs: Matrix MetalloProteases

mRNA: messenger RNA

MW: Molecular Weight

^{o}C: Degree Celsius

PCR: Polymerase Chain Reaction

PET: polyethylene terephthalate

qRT-PCR: quantitative real time PCR

RNA: RiboNucleic Acid

RTCA: Real Time Cell Analysis

s.d: subdermally

shRNA: small hairpin RNA

ug: micro gram

ul: micro liter

um: micro meter

VEGF: Vascular Endothelial Growth Factor

CHAPTER I

INTRODUCTION

A. **Cancer**

1. Preface

Cancer is classified globally as a major cause of death. After surgery, current modalities of chemotherapy and radiation therapy have proven to be inefficient cancer treatment; scientists are searching for new therapeutic strategies. For instance, targeted therapy has become a major strategy to irradicate cancer cells. This study focuses on breast cancer as a model for solid malignant tumor in human beings with emphasis on the role of intercellular communication in the attenuation of invasion and metastasis of cancer cells.

2. Breast Cancer

According to U.S. Breast Cancer Statistics, about 1 in 8 U.S. women will develop invasive breast cancer over the course of her lifetime. In 2013, an estimated 230,000 new cases of invasive breast cancer were expected to be diagnosed (Cleator, Heller, and Coombes, 2007). There are two major different types of breast cancer. A hormone-dependent breast cancer and a triple-negative breast cancer variant. In the first case, cancer cells express one of the following receptors: estrogen, progesterone and/or Human Epidermal Growth Factor Receptor 2 (HER2). Binding of the hormone to its respective receptor stimulate genes regulating cell division,

leading to a rapidly growing tumor. Such type can be treated with hormone therapies (Sasa, Bando, Takahashi, Hirose, and Nagao, 2008). On the other hand, triple-negative breast cancer is an aggressive subtype which accounts for 10-15% of breast cancer cases (Dent et al., 2007). Triple-negative breast cancer lacks the above mentioned receptors, leading to a more complicated disease with fewer treatment options.

3. *Cancer Metastasis*

a. Growth at the Primary Site

Metastasis is a complex process requiring vascularization of the primary tumor through angiogenesis, tissue invasion to reach blood or lymphatic vessels, entering and exiting the blood stream respectively via intravasation and extravasation processes and finally the formation of secondary tumor foci (Figure 1).

At the primary growth site, tumor cells rest on an epithelial basement membrane, a dense meshwork of type IV collagen, glycoproteins, such as laminin and fibronectin, proteoglycans and embedded growth factors (Liotta and Kohn, 2000). Before tumor cell invasion, the basement membrane is continuous and separates the epithelium from the underlying connective tissue, thus from the underlying blood and lymphatic vessels. Tumor tissue integrity is stabilized by cell adhesion molecules, such as integrin and cadherin proteins. These proteins are cell surface receptors; that can be activated by external factors to transduce signals into the cell to direct cell behavior. The expression of such cell adhesion molecules is altered in tumor cells to enhance the metastatic potentials (Liotta and Kohn, 2000).

b. Angiogenesis

Angiogenesis, the process of formation of new blood vessels from pre-existing ones, is thought to be one of the rate-limiting steps for tumor growth and metastasis (Hanahan and Folkman, 1996). As the tumor mass increases in volume, more nutrients and oxygen are required for cellular needs, which can be provided by angiogenesis through the initiation of capillary sprouts in the direction of the tumor (Folkman, 1971). Upon insufficient supply of cellular needs, tumor cells become hypoxic and hypoxia-inducible transcription factors (HIFs) are activated, leading to the expression of various angiogenic factors, including Vascular Endothelial Growth Factor (VEGF). This causes a response in enzyme production related to invasion, migration, and proliferation in endothelial cells. Now tumor cells are nourished through blood vessels with the chance to metastasize to distant organ sites.

c. Tissue Invasion

Invasion does not occur in a passive manner due to excessive cell proliferation alone, but it is an active and dynamic process requiring Extra-Cellular Matrix (ECM) degradation (Kohn et al., 1995). Tissue invasion starts by the production of enzymes that degrade ECM for tumor cells to intravasate successfully. Matrix MetalloProteases (MMPs) is a family of these enzymes secreted as latent proenzymes which are activated by a proteolytic cleavage of their amino-terminal domain and by their binding to Zn^{2+} (Coussens and Werb, 1996). In breast cancer,

the invasive and metastatic potentials of tumor cells increase with the increase in MMP activity (Liotta, Saidel, and Kleinerman, 1976; Weiss, 1996). Cell surface integrins are essential in mediating cell-ECM interaction and in inducing cell motility and migration.

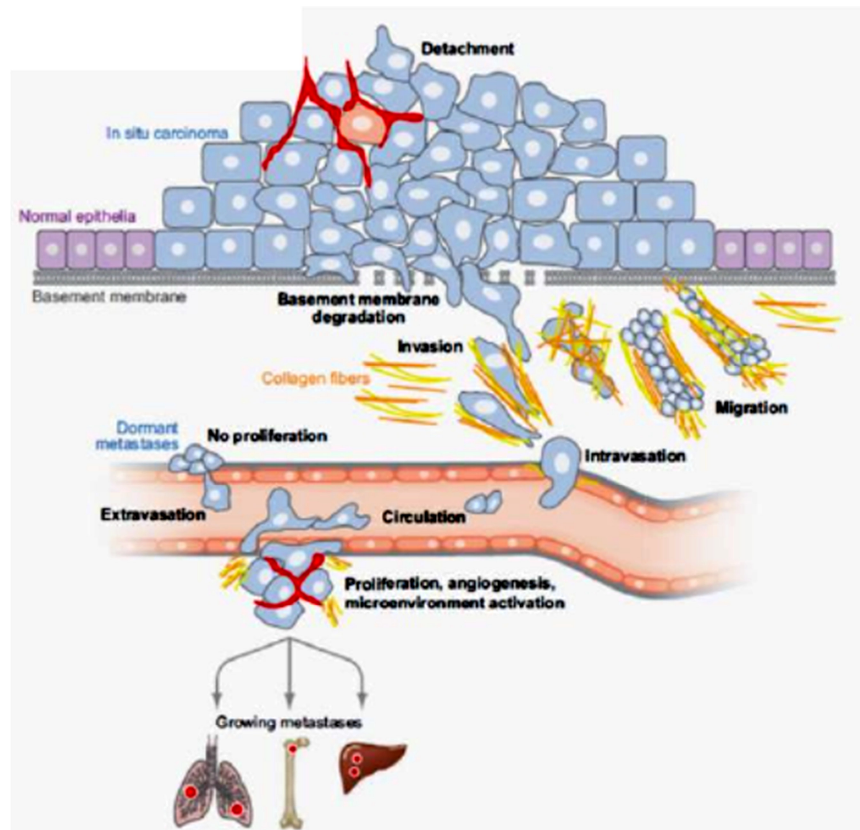


Figure 1: Schematic representation of the metastatic process. At the primary tumor growth site, tumor cells rapidly proliferate and as the tumor volume increases, tumor cells will have an insufficient supply of nutrients and oxygen. This requires the formation of new blood capillaries by the process of angiogenesis. Tumors cells may directly enter the blood stream through the newly formed blood capillaries or they start to degrade the surrounding ECM until they interact with endothelial cells of blood or lymphatic vessels where intravasation occurs. In the blood stream many tumor cells irradicate either due to physical forces or by the host defense mechanisms. The few remaining tumor cells interact with organ-specific adhesion molecules being expressed at the surface of endothelial cells to extravasate and to form a secondary tumor in another body organ.

d. Intravasation

Intravasation is the invasion of cancer cells through the endothelial basement membrane to enter the blood and/or lymphatic circulation. This process is facilitated by the secretion of proteolytic enzymes that degrade the endothelial basement membrane and retract endothelial cells. Once in the circulation, a huge number of cancer cells will be irradicated due to physical forces and host defense mechanism (Weiss, 1996). The few surviving tumor cells colonize selective organ sites by recognizing organ-specific adhesion molecules expressed on the surface of endothelial cells (Alby and Auerbach, 1984; Nicolson, 1988; Pauli and Lee, 1988; Pauli, Augustin-Voss, el-Sabban, Johnson, and Hammer, 1990).

e. Extravasation

Extravasation is a process by which cancer cells exit the bloodstream and infiltrate the surrounding tissue to establish a secondary site of metastasis. A model for cancer cell extravasation was proposed where it was shown that a cancer cell interact with endothelial cells of the target organ, through both cell-cell and paracrine interactions, inducing activation of endothelial MMPs and subsequent degradation of subendothelial basement membrane. This allows cancer cells to extravasate and to establish a secondary tumor (Figure 2). (Bazarbachi et al., 2004).

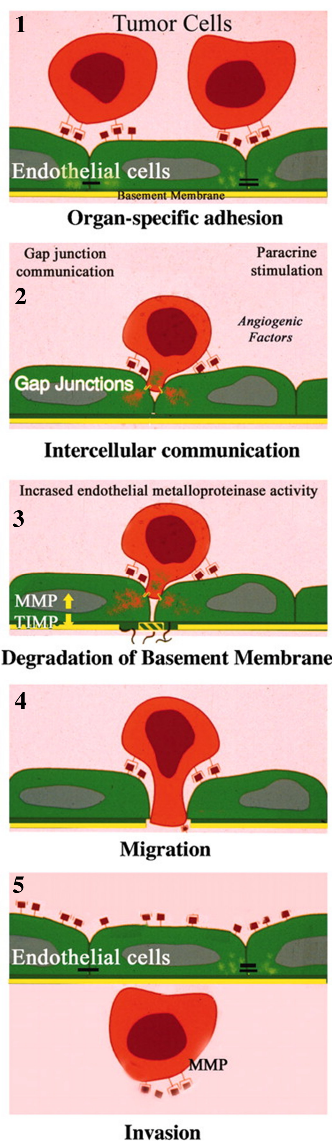


Figure 2: Proposed model for cancer cell extravasation. 1. Tumor cells in the blood stream interact with organ-specific adhesion molecules being expressed at the surface of endothelial cells. 2. Through direct cell-cell communication and paracrine stimulation between tumor and endothelial cells 3. MMP secretion levels increases and induces the degradation of the underlying basement membrane 4. facilitating the retraction of endothelial cells and the subsequent migration of tumor cells from the blood capillary

lumen into the tissue 5. where tumor cell invasion starts to form a secondary tumor foci. (Adopted and Modified) (Bazarbachi et al., 2004)

f. Secondary Tumor Formation

Tumor cells seed in particular organs as a final step of metastasis. This was first discussed as the "seed and soil" theory by Stephen Paget (Ribatti et al., 2006). It is difficult for cancer cells to survive outside their region of origin, thus the propensity for a metastatic cell is to survive in an organ with characteristics permissive with their needs. A second theory states that the selection of a secondary metastatic site depends on the recognition of organ-specific adhesion molecules, being expressed on the surface of endothelial cells (Pauli et al., 1990).

4. *Role of β-Catenin in Cancer Metastasis*

β-catenin is a dual function protein regulating the coordination of cell-cell adhesion and gene transcription. β-catenin is part of a protein complex that form adherens junctions (Bermbeck et al., 2006), which are essential for the creation and maintenance of epithelial layers and barriers. As a part of the complex, β-catenin can regulate cell growth and adhesion between cells. For instance, it is responsible for transmitting the contact inhibition signal causing to stop cell proliferation (Bermbeck et al., 2006). β-catenin is also a key player of the Wnt signaling pathway, once it dissociates from the adherens complex, it translocates to the nucleus where it induces the transcription of several genes related to cellular proliferation, angiogenesis, invasion, motility differentiation and stem cell renewal

(Talhouk et al., 2013). Malignant breast tumors are associated with mutations and over-expression of β-catenin. In other types of cancer, it is shown to have mutations in the genes coding for members inhibiting β-catenin activity. Several therapeutic strategies are focusing on how to sequester β-catenin away from the nucleus of tumor cells, so that their invasive and proliferative potential may decrease.

B. Epithelial to Mesenchymal Transition

Epithelial to Mesenchymal Transition (EMT) is the process by which an epithelial cell loses its cell polarity and cell-cell adhesion, and gain invasive and migratory properties with a mesenchymal phenotype. EMT is seen in normal physiological processes including embryonic development, wound healing and tissue regeneration. On the other hand, EMT also occurs in pathological disorders like organ fibrosis and cancer metastasis. As a result of EMT, cells become more motile and invade local tissue; hence facilitating the dissemination of cancer cells. Interestingly, EMT during embryogenesis occurs in an immunologically privileged setting, whereas under pathological disorders, inflammation and epigenetic factors induce EMT (Kalluri, 2009).

Some of the below listed EMT biomarkers are acquired and some are attenuated during transition. During EMT, the expression of the cell surface protein, E-cadherin, decreases (Hay et al., 1995). In fact, a cadherin switch from E-cadherin to N-cadherin, where the latter is expressed in mesenchymal cells and fibroblasts, occurs (Zeisberg et al., 2009). The process of EMT is also associated with a relocation of cells from the basement membrane microenvironment into a fibrillar

ECM (Li et al., 2003). As a result, an integrin switch occurs, where a change in the level of expression of different integrins is manifested (Raymond et al., 1989). For instance, some malignant breast cancer cell lines express β_1-integrin among other integrin families. It is worth to note that integrin signaling facilitates EMT (Boyer et al., 1989). Vimentin expression, an intermediate filament, is positively correlated with the increase in invasiveness and metastasis (Brabletz et al., 1998). Vimentin is used to identify cells undergoing EMT in cancers (Yook et al., 2006). Cancer cells undergoing EMT are also characterized by the dissociation of β-catenin away from the plasma membrane. β-catenin can be either in the cytoplasm, reflecting the down-regulation of E-cadherin, or in the nucleus, reflecting its role as a transcription factor (Yang et al., 2008). Various transcription factors are upregulated during EMT. For instance, β-catenin directly upregulates the expression of Snail1, a transcription factor associated with EMT. Twist transcription factor is also upregulated upon cancer cells transition. EMT biomarkers form a solid base to assess the phenotype of a cancer cell, so that a clear idea about its metastatic status can be built.

C. Connexins and Cancer

Connexin protein is the building block of gap junctions. A gap junction is formed by the interaction of two connexons, where each connexon is located in the plasma membrane of a cell. A connexon is made up of a complex of six connexin proteins, stacked in a cylindrical form. Gap junctions allow intercellular exchange of molecules less than 1.5 kDa in molecular weight (Kumar & Gilula, 1996; Wei, Xu,

and Lo, 2004). Twenty-one different connexin genes have been identified in humans (Sohl and Willecke, 2004), each gene codes for a specific connexin isoform. In general, connexin protein is composed of nine domains which are: a cytoplasmic N-terminus, two extracellular loops, four transmembrane domains which are highly conserved among different isoforms and a cytoplasmic C-terminus domain that differs in length, sequence and proteins associated from one isoform to another (Dbouk, Mroue, El-Sabban, and Talhouk, 2009). Connexins can either assemble into a connexon hemichannel with identical subunits (homomeric) or different connexin isoforms (heteromeric) (Dbouk et al., 2009). Gap junctions can be formed by the assembly of two identical connexons (homotypic) or by the assembly of two dissimilar connexons (heterotypic) (Dbouk et al., 2009).

The C-terminal region of connexins interacts with a variety of proteins playing critical roles in regulating the assembly, function and life span of connexins (Herve, Bourmeyster, & Sarrouilhe, 2004; Herve, Bourmeyster, Sarrouilhe, & Duffy, 2007). For instance, zonula occludens-1 (ZO-1), a tight junction protein, is a type of connexin-associated protein. ZO-1 is involved in the organization and trafficking of gap junction, like in the delivery of connexin43 (Cx43) from a lipid raft domain to a gap junctional plaques, thus playing an essential step in gap junction formation. Another type of connexins-associated proteins, are the adherens junction proteins, where co-localization and co-immunoprecipitation experiments have shown this interaction. The upregulation of E-cadherin-dependent cell-cell contacts increases gap junctional intercellular communication (GJIC) in mouse epidermal cells (Jongen et al., 1991). Cx43 also interacts with β-catenin where they colocalize at the

cell membrane and the cytoplasm. This finding has raised the question whether Cx43 regulates β-catenin signaling by sequestering it away from the nucleus (Ai, Fischer, Spray, Brown, and Fishman, 2000). Connexins were also shown to interact with enzymes such as kinases and phosphatases which regulate the assembly, function and degradation of connexins (Figure 3).

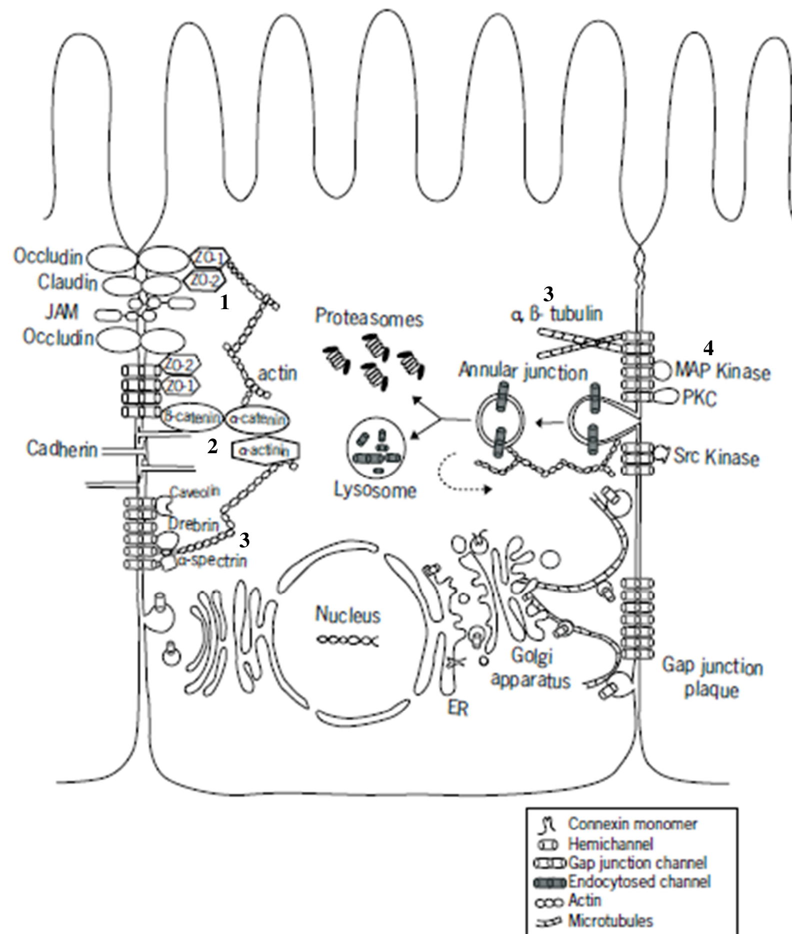


Figure 3: Schematic representation illustrating the life cycle and protein associations of connexins. Six connexins oligomerize to form a hemichannel or connexon. Connexin oligomerization occurs either in the rough endoplasmic reticulum (rER), ER/golgi intermediate compartment or the trans-golgi network. After that, connexon will be inserted in the plasma membrane where it docks with another connexon of an adjacent cell to form a gap junction. Connexins interact with a variety of proteins. For instance, 1. They interact with the members of tight junction proteins like cludins, occludins and ZO proteins. 2. Also

with members of adherens junction proteins like cadherins β or α-catenins. 3. They also interact with cytoskeletal members like actin, actin-binding proteins and microtubule filaments. 4. Connexins interact with enzymes such as kinases and phosphatases that have a role in the regulation of function and the life cycle of connexin proteins. (Adopted and Modified) (Dbouk et al., 2009).

Connexins have diverse functions whether in gap junctions, hemichannels or by their independent role from both gap junctions and hemichannels. Connexins are essential in many biological processes, since their loss or the existence of mutations in their corresponding genes affect their normal functions and are implicated in many diseases and disorders, including cancer (Laird, 2006; Mesnil, Crespin, Avanzo, and Zaidan-Dagli, 2005a).

1. ***Gap Junction Dependent Functions of Connexin***

Opening and closure of gap junctions are regulated by various cellular mechanisms. For instance, calcium dependent cellular processes might be regulating gap junction functions, since connexins interact with calcium-affected molecules such as calmodulin (Peracchia, Wang, and Peracchia, 2000; Peracchia, 2004). Regulatory sites that respond to intracellular pH levels were found in the intracellular loop and carboxy terminus domains of connexins. Thus connexins regulate the gating of gap junctions in response to changes in intracellular pH levels (Hirst-Jensen, Sahoo, Kieken, Delmar, and Sorgen, 2007; Spray and Burt, 1990). Connexin phosphorylation also affects gap junction function by controlling the trafficking and degradation of connexins, or by completely closing or opening the channel for passage of molecules (Anand and Hackam, 2005; Moreno and Lau, 2007).

Several studies have shown that gap junctions are essential for the proper tissue differentiation including the mammary gland, lens, bone marrow, sertoli cells, adipocytes and other cells and organs (El-Sabban et al., 2003; Gong, Cheng, and Xia, 2007; Yanagiya, Tanabe, and Hotta, 2007). Interestingly, the increased levels of Cx43 protein, correlates with the re-differentiation of colon cancer cells where gap junctional communication was restored (Nakamura et al., 2005).

Other studies have reported heterocellular gap junctions *in vitro* and *in vivo*, showing important regulatory roles for such junctions. Recent studies have shown that heterocellular gap junctions between myoepithelial-like cells and mammary epithelial cells can induce differentiation of the latter and can lead to the association of gap junction connexins with α-, β-catenin and ZO-2 proteins (Talhouk et al.).

Via the “bystander effect” gap junctions can spread a death signal among cells in a damaged tissue, probably by mediating Ca^{2+} influx between the cells. On the other hand, gap junctions can rescue dying cells by transporting metabolites such as ATP, glucose, ascorbic acid and miRNA etc. and by inhibiting the passage of cytotoxic agents (Contreras et al., 2004; Talhouk, Zeinieh, Mikati, and El-Sabban, 2008; Vinken et al., 2006).

2. *Hemichannel Dependent Functions of Connexins*

Hemichannels play roles in various physiological conditions. They are usually closed upon reaching the cell membrane, however their function is regulated by extracellular and intracellular factors, like changes in ionic concentration, membrane depolarization, metabolic inhibition and mechanical stimuli (Contreras,

Saez, Bukauskas, and Bennett, 2003; Gomez-Hernandez, de Miguel, Larrosa, Gonzalez, and Barrio, 2003; Saez, Retamal, Basilio, Bukauskas, and Bennett, 2005). Similar to gap junctions, hemichannels are also involved in cell death and survival. For instance, it was shown that Cx43 hemichannels accelerate cell death after staurosporin treatment (Hur et al., 2003). On the other hand, Cx43 hemichannels transduce survival signals by the activation of ERK and the attenuation of cell death after treating osteoblasts with biphosphonates (Plotkin, Manolagas, and Bellido, 2002).

3. *Gap Junction and Hemichannel-Independent Functions of Connexins*

Connexins by themselves also play roles independent of their channel-forming properties. The functions of an individual connexin protein are mediated by their various associating partners, resulting in wide changes in gene expression. It was shown that Cx26 inhibits cell invasion and migration in a gap-junction independent mechanism in MDA-MB-435 by regulating the expression levels of β_1-integrin and MMPs (Kalra et al., 2006). Thus Cx26 is acting as a tumor suppressive protein. Cx32 suppressed growth, invasion and metastasis of renal cell carcinoma cell lines (Sato et al., 2007). Through the association of Cx43 with c-Src and ZO-1 proteins, its expression has attenuated cell growth. Other studies have shown that changes in the expression of connexins directly affects gene expression resulting in cellular function alterations, including transcription, metabolism, cell-cell and cell-ECM

adhesion, cellular signaling, transport, cell cycle and division (Iacobas, Scemes, and Spray, 2004).

4. *Connexins and Metastasis in Breast Cancer*

Under normal physiological conditions three major connexins, Cx26, Cx32 and Cx43 are expressed in rodent mammary epithelium (Monaghan et al., 1994). For instance, Cx43 is expressed in myoepithelial cells throughout all the stages of mammary gland development (Monaghan et al., 1996). Cx26, expressed in luminal cells, its expression increases in pregnancy and peaks during lactation (Laird et al., 2002). Cx32 can only be detected in luminal cells of lactating gland (Monaghan et al., 1996). Several studies have shown that over-expression or down-regulation of connexins can lead to alterations in gene expression profile, thus affecting transcription, metabolism, cell-cell and cell-ECM interaction, cellular signaling, transport and cell cycle and division (Dbouk et al., 2009). Due to their wide range of function, many human diseases have been linked to connexin gene mutations leading to a malfunctional protein. Connexins are also implicated in the carcinogenic process (Iacobas et al., 2004). Connexin expression, assembly of gap junctions and GJIC are down-regulated in many neoplastic cell lines and tumor tissues (Naus et al., 1991). A variety of human breast tumor cell lines exhibit a down-regulation of connexin gene expression (Mesnil, Crespin, Avanzo, and Zaidan-Dagli, 2005b), and a deficiency of Cx43 gap junctions is considered as a marker of breast tumors (Lee, Tomasetto, Paul, Keyomarsi, and Sager, 1992). The restoration of connexin expression and GIJC has suppressed tumor cell proliferation

and induced cell differentiation (Yamasaki et al., 1999). Therefore, connexins are tumor suppressor proteins. An evidence for that came from Cx32 knockout mice where these animals were found to be more susceptible to liver tumor formation compared to wild type mice (Temme et al., 1997). It seems that the ability of gap junctions to suppress tumor development and metastasis is spatially regulated. There is a decrease in connexins' expression in primary tumors and an increase in heterocellular gap junctions with endothelial cells during intravasation and extravasation (Laird et al., 1999). Other studies have shown that the re-expression of Cx43 in breast cancer cell lines has decreased their metastatic potential by increasing homocellular GJIC. Various mammary tumor cell lines continue to express connexins but with the deficiency to assemble into functional gap junctions (el-Sabban and Pauli, 1991). It remains unclear whether the tumor suppressing activity of connexins is related to the formation of gap junction or to their roles which are independent of channel formation (el-Sabban and Pauli, 1994).

D. Aim of the Study

Connexins are well-documented tumor suppressors, regulating cell growth and differentiation. Most human breast tumors and cell lines tend to down-regulate connexins or to have defective gap junctions. In this study, we hypothesize that Cx43 over-expression in MDA-MB-231 cell line would reduce its aggressive metastatic abilities, and Cx43 down-regulation would induce a more aggressive phenotype. We investigated modifications in the expression levels of invasive, angiogenic and EMT markers at both transcriptomic and proteomic levels. Several

functional assays were conducted to assess the ability of tumor cells to adhere, to communicate with each other and to invade. Metastatic potentials upon Cx43 over-expression or down-regulation were also assessed *in vivo.* Tumor onset and volumes, survival rates and metastasis to secondary tissues were all investigated and recorded. This study will enhance our knowledge on the potential of Cx43 in suppressing the metastatic abilities of a malignant breast cancer cell line both *in vitro* and *in vivo.*

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

Human mammary cancer cell line: MDA-MB-231, a triple-negative highly metastatic human mammary adenocarcinoma cell line derived from a pleural effusion. Cells were maintained in RPMI-1640 medium (Lonza, Walkersville, USA) supplemented with 10% of heat-inactivated fetal bovine serum FBS (Sigma, St. Louis, USA) and 1% penicillin-streptomycin P/S (Sigma, St. Louis, USA), and incubated at 37° C in a humidified incubator (95% air, 5% CO_2). When 80% confluent, cells were washed with 1x Dulbecco's Phosphate Buffered Saline (PBS) then incubated with 1x trypsin (0.5% Trypsin-EDTA (10x), Gibco, UK) at 37° C for 1 minute.

1. Two-Dimensional Cell Culture

a. Transfection

pGFP-V-RS (Figure 4): Cx43 was silenced using the following constructs. The first is against Cx43 (shRNA-Cx43), and the other containing non-effective scrambled shRNACx43 nucleotide sequence (shRNA-scr). The above vector was purchased from OriGene Technologies, Inc.

MDA-MB-231 cells were plated on a 6 well tissue culture plates at a density of 125x10^3 cells per well in 2ml of media. After 48 h, media was removed, and cells were first washed with 1x PBS then washed with OptiMEM media (Gibco, UK).

Transfection was performed using Lipofectamine 2000 reagent (Invitrogen), according to manufacturer's instructions. Selection of MDA-MB-231 transfected cells was in RPMI with 10% FBS and 1% P/S supplemented with 0.2 ug/ml puromycin. Maintenance medium (RPMI with 10% FBS and 1% P/S supplemented with 0.1 ug/ml puromycin) was used throughout the study after cells have been selected and sorted. Lipofectamine transfection has provided our study with two different cell lines: MDA-MB-231 shRNA-Cx43 (down-regulating Cx43) and MDA-MB-231 shRNACx43-scr.

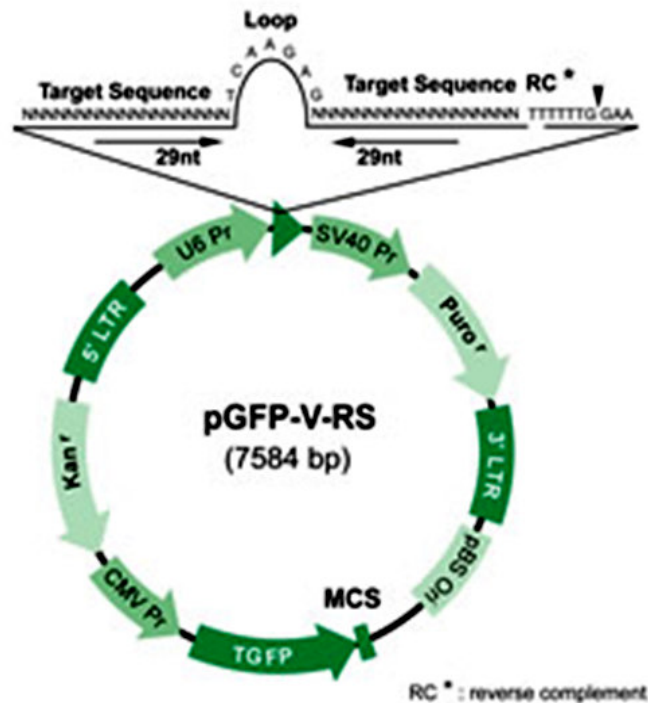


Figure 4: pGFP-V-RS vector used to transfect MDA-MB-231 by shRNA-Cx43.shRNA was inserted under the U6 promoter.

b. <u>Lentiviral Transduction</u>

pCSCW-Cx43-Dendra2: Cx43 was over-expressed using a lentiviral vector, into which Cx43 cDNA sequence was inserted downstream of a cytomegalovirus promoter (CMV) to the amino terminus of Dendra2 protein (Dendra2 is a green-to-red photoconvertable fluorescent protein used for trafficking studies).

Construction of Cx43-Dendra2 chimeras:

Total RNA from normal human keratinocyte cells were extracted using TRIZOL reagent (Invitrogen) and RNA extraction Kit (Invitrogen), respectively. cDNA were synthesized from 1 μg total cellular RNA, using reverse transcriptase (1st step PCR, Fermentas). cDNA covering the complete reading frame of Cx43 were then amplified by High fidelity PCR Kit (Rainbow). PCR products were subjected to electrophoresis on agarose gel, and visualized with ethidium bromide staining. Bands corresponding to Cx43-cDNA were cut, purified (Qiagen kit), and digested with the restriction enzymes (Fermentas) flanking its 5' and 3' sequences to be fused to the amino-terminus of pDendra2-N vector (Evrogen). The digested Cx43-cDNA and pDendra2-N were run on agarose gel and their corresponding bands were cut and purified. A ligation reaction containing three molar excess of the purified Cx43-cDNA to pDendra2-N was allowed to proceed for 20 min at room temperature using T4 DNA ligase (MBI Fermentas). Aliquots of the ligation reaction were transformed into DH5α competent bacteria by heat shock. Positive colonies were identified by midi plasmid purification and restriction enzymes analysis. The plasmids were then sequenced and used for transfections.

Production of lentiviruse: For efficient delivery of Cx43-Dendra2 chimeric protein into MDA-MB-231, lentiviral vectors were produced. Briefly Cx43, N-terminally tagged with Dendra2, were cloned into the transfer vector pCSCW under the control of the CMV promoter. Using the calcium phosphate transfection method, 293T cells were transfected with three lentiviral plasmids: pCMVDR8.91 (containing gag/pol), pVSVG2 (containing the envelope gene VSV-G) and pCSCW-Cx43-Dendra2 (Figure 5.A.B.C). The cell supernatant containing the recombinant lentivirus were collected 48-72 h post transfection, filtered, aliquoted, and frozen. To determine the viral titer, HeLa cells were transduced with the produced virus and the number of fluorescent cells was quantified by flow cytometry. The titer was extrapolated from the percentage of fluorescent cells, which correlates directly to the number of transducing viral units present in the supernatant used (tu/ml).

A.

B.

C.

Figure 5: A. pCMVDR8.91 vector B. pVSVG2 vector C. pCSCW-Cx43-Dendra2 vector (a part of the lentiviral vector shown in the figure) were used to transfect 293T cells for the production of viral particles.

Transduction: $125x10^3$ cells were cultured in a 6 well tissue culture plate and infected after 48 h with lentiviral particles. When culture reached confluence, cells were trypsinized and processed for FACS cell sorting.

2. *Three-Dimensional Cell Culture*

Growth Factor Reduced Matrigel obtained from BD Biosciences was used. $2x10^3$ cells were suspended in cold Matrigel/RPMI serum free medium (1:1) ratio (vol/vol) in a total volume of 50 ul. Cells were then plated uniformly in a circular manner around the bottom rim of a well in a 24 well tissue culture plate, and then incubated at 37^oC for one hour to form a bed of 100% solidified matrix before adding 0.5 ml of RPMI with 2% FBS and 1% P/S in the middle of each well. Cells were replenished with warm media as in the original plating every two days. Clusters start to form by day 3, and cells were kept in culture for 8 days.

B. Fluorescence-Activated Cell Sorting (FACS)

Fluorescent cells were isolated using a BD FACS Aria SORP cell sorter in the single cell mode at a low sort rate. A negative control of non-fluorescent cells was used to determine the background fluorescence. After sorting, cells were analyzed for their fluorescence. This analysis revealed about 20% non-fluorescent cells. Cells transfected by (shRNACx43 and shRNA-scr vectors) were sorted for GFP positive cells *versus* GFP negative cells (non-fluorescent cells). GFP negative cells were used in our study and were designated as sham cells.

Cells transduced by (Cx43-Dendra2 vector) were sorted according to their fluorescence intensity into low Cx43-Dendra2 and high Cx43-Dendra2 expressing cells, to study later on the effect of different expression levels of Cx43 on MDA-MB-231 cells' metastatic abilities. Thus, cell sorting has yielded two cell lines differing in the level of Cx43-Dendra2 expression. The first is MDA-MB-231 low Cx43-Dendra2 and the second is MDA-MB-231 high Cx43-Dendra2.

C. Cell Proliferation Assay

1. Cell Counting by Trypan Blue

In 2D cultures, cells were plated in 12-well tissue culture plates at a density of $25x10^3$ cells in each well. The cells were counted from duplicate wells after days 1, 2, 3 and 4. Cells were then diluted in Trypan Blue (1:1) ratio (vol/vol) and counted using a hematocytometer.

2. Real Time Cell Analysis (RTCA)

Cell proliferation was measured using xCELLigenec RTCA DP instrument (RTC; xCELLigenec Roche Penzberg, Germany). Cells were plated on a cellular proliferation plate (E-plate 16). The presence of cells on top of the E-Plate electrodes affects the local ionic environment at the electrode/solution interface, leading to an increase in electrode impedance. The more cells that are attached on the electrodes, the larger is the increase in electrode impedance. During proliferation, spreading of cells will lead to a larger change in electrode impedance. Thus, electrode impedance, which is displayed as Cell Index (CI) values, can be

used to monitor cell proliferation (Figure 6). For all proliferation assays, 150 µl of RPMI medium with 10% FBS and 1% P/S was added first in each well (used as a background measurement) and pre-incubated for 1 h at 37°C and 5% CO_2. MDA-MB-231 cells (down-regulating or over-expressing Cx43) were seeded at a density of $10x10^3$ cells per well in a volume of 150 µl of RPMI medium with 10% FBS and 1% P/S. Proliferation was monitored by recording cell impedance reading every 15 minutes for minimum of 48 h.

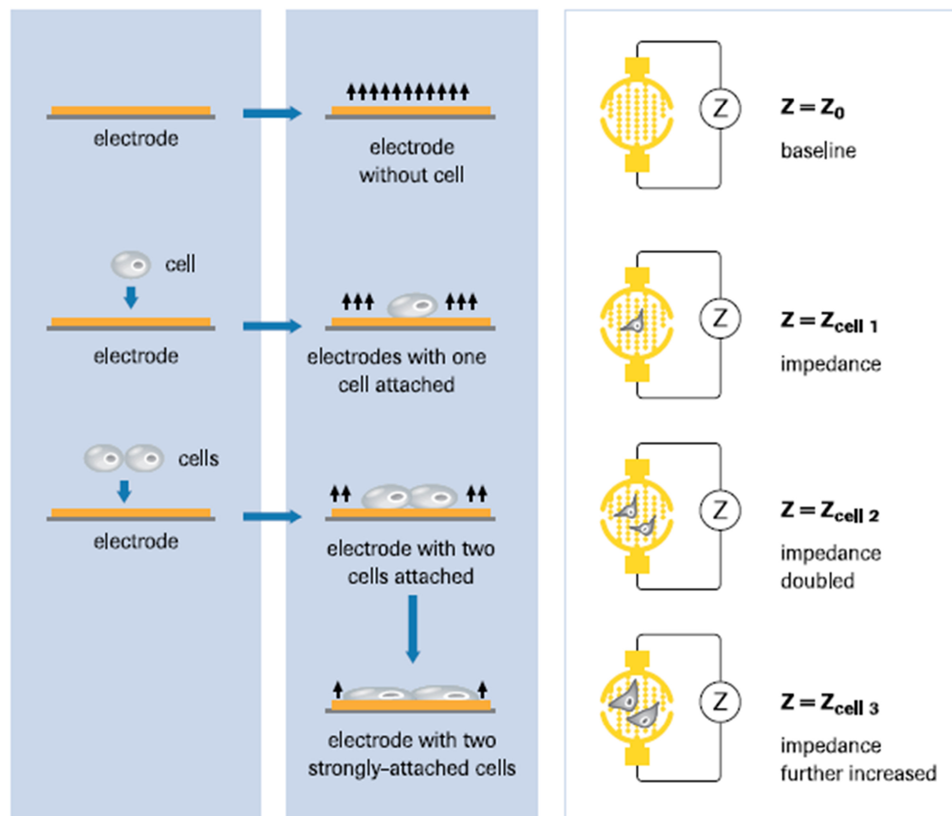


Figure 6: Schematic representation of the interdigitated microelectrodes on the well bottom of an E-Plate 16. Micro-electrode sensors on the bottom of the E-plate record changes in electric impedance upon cellular adhesion to the surface. Impedance increases as cells start to proliferate.

3. *Counting Spheres in Three-Dimensional Cell Culture*

MDA-MB-231 cells (down-regulating or over-expressing Cx43) were plated in matrigel and at least in triplicates in 24-well tissue culture plates at a density of $2x10^3$ in each well. The cells were maintained for 8 days in RPMI 1640 media supplied with 2% FBS before counting. Experiments were repeated at least two times. At day 8, both spherical and stellate colonies were counted in each well at 10 x magnification.

D. Fluorescence Recovery After Photobleaching (FRAP) Assay

Communication among MDA-MB-231 cells (down-regulating or over-expressing Cx43) through gap junctions was evaluated using FRAP assay. This consisted of measuring the transfer of the fluorescent dye calcein from cell to a cell that had been laser bleached to eliminate calcein fluorescence. Calcein-AM (39, 69-di [O-acetyl]-29, 79-bis [N, N-bis {carboxymethyl} amino methyl]-fluorescein, tetraacetoxy methyl ester, MW 994.87; Invitrogen) is a non-fluorescent, electrically neutral and highly lipophilic molecule that can permeate rapidly into the cytoplasm through cell membrane. Once inside the cell, nonspecific endogenous esterase hydrolyzes the acetoxymethyl groups to produce calcein (MW 622.54), a green fluorescent and negatively charged molecule that is unable to cross the plasma membrane but is able to cross from cell to cell via gap junctions only. Cells were plated on a confocal dish at a density of $20x10^3$ cells. After 72 h, 1ml of RPMI

medium with 10% FBS and 1% P/S containing 1 uM of calcein-AM was added to the cells and incubated at 37°C and 5% CO_2 for 1 h. Then, cells were washed with RPMI serum free media for 30 minutes to remove any calcein-AM. An LSM 710 confocal microscope (Carl Zeiss) with 488 nm laser was used at 60 x magnification to perform photobleaching on a specific cell surrounded by other cells. Fluorescence intensity was measured before bleaching, during and after bleaching and a picture was taken every 5 sec for 5 min post bleach period.

E. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from cells using NucleoSpin RNA II Kit according to manufacturer's instructions. 1 μg of total RNA was reversed transcribed to cDNA using RevertAid 1st strand cDNA synthesis kit (Fermentas). Real-time PCR was performed using iQ SYBR Green Supermix in a CFX96 system (Bio-Rad Laboratories), and its products were amplified using primers listed in table 1. Real-time PCR steps were as the following: A precycle of 95°C for 3 min followed by 40 cycles consisting of 95°C for 10 sec, X^{o}C for 30 sec and 72°C for 30 sec and a final extension step at 72°C for 5 min. To quantify changes in gene expression, the comparative C_t method was used to calculate the relative-fold changes normalized to GAPDH**.**

Genes	Primers' Sequences	Annealing Temp (^{o}C)
Cx43	F: CTTCACTACTTTTAAGCAAAAGAG R: TCCCTCCAGCAGTTGAG	52
Cx26	F: CCTCCCGACGCAGAGCAA R: CAGACAAAGTCGGCCTGCTCA	62
MMP-2	F: TTGACGGTAAGGACGGACTC R:ACTTGCAGTACTCCCCATCG	55
MMP-9	F: TTGACAGCGACAAGAAGTGG R: GCCATTCACGTCGTCCTTAT	55
HIF-1α	F: AGCCAGATCTCGGCGAAGT R: CAGAGGCCTTATCAAGATGCG	58
VEGF	F:AGGCCCACAGGGATTTTCTT R: ATCAAACCTCACCAAGGCCA	55
E-cadherin	F: CAGAAAGTTTTCCACCAAAG R: AAATGTGAGCAATTCTGCTT	58
N-cadherin	F: GGTGGAGGAGAAGAAGACCAG R: GGCATCAGGCTCCACAGT	58
Snail	F: CTTCCAGCAGCCCTACGAC R: CGGTGGGGTTGAGGATCT	58
Twist	F: AGCTACGCCTTCTCGGTCT R: CCTTCTCTGGAAACAATGACATC	58
ZO-1	F: CAGCCGGTCACGATCTCCT R: GTGATGGACGACACCAGCG	58
β-catenin	F: AGGGATTTTCTCAGTCCTTC R: CATGCCCTCATCTAATGTCT	52
GAPDH	F: TGGTGCTCAGTGTAGCCCAG R: GGACCTGACCTGCCGTCTAG	52-62

Table 1: Human real-time primers with their relative sequences and annealing temperatures (F= Forward primer R= Reverse primer).

F. Protein Extraction and Immunoblot

1. Cellular Protein Extraction

For two-dimensional cultures, cells for protein extraction were collected at 80% confluence. Cells were scraped into 300 µl of lysis buffer (0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) and 10% glycerol) to which 20 µl/ml protease inhibitors and 100 µl/ml phosphatase inhibitors were added. DC Protein Assay (Bio-Rad, Hercules, CA) was used to quantify proteins using bovine serum albumin (BSA, Sigma Chemical Co.) as standards.

2. Western Blot Analysis

From each sample 100 µg of proteins were resolved on SDS-PAGE gels of different concentrations (depending on the molecular weight of the protein being probed for). After electrophoresis, proteins were transferred overnight on PVDF membranes (Bio-Rad Laboratories), and then blocked by 5% skimmed milk before being incubated with specific primary antibodies. Finally, membranes were washed and protein bands were visualized using chemiluminescence after adding the corresponding horse reddish peroxidase conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Equal loading was determined by probing for GAPDH. For quantitation of the results, protein bands were taken at different intensity levels and their densities were analyzed by densitometry using Image J software, where the expression levels of each protein sample were normalized to GAPDH, and results were plotted accordingly. All primary antibodies were used in a concentration of 1 µg/ml and are listed in table 2.

Primary anti-bodies	Source
Cx43	Invitrogen
Cx26	Invitrogen
Cx45	Chemicon
HIF-1α	Novus Biologicals
VEGF	Santa Cruz
E-cadherin	Cell Signaling
N-cadherin	Invitrogen
ZO-1	Invitrogen

Table 2: Primary antibodies recognizing human antigens used in the *in vitro* immunoblotting experiments.

G. RTCA Invasion and Migration Assays

Invasion or migration assays were performed using xCELLigenec RTCA DP instrument (RTC; xCELLigenec Roche Penzberg, Germany). Cells were plated on a cellular invasion/migration plate (CIM-plate 16) containing micro-electronic sensors on the underside of an 8 um microporous polyethylene terephthalate (PET) membrane of a Boyden-like upper chamber. As cells invade or migrate from the upper chamber through the membrane into the lower chamber in response to a chemoattractant (FBS in our case), they tend to interact and adhere to the micro-electronic sensors, thus causing an increase in electrical impedance. Changes in electrical impedance reflect the number of cells on the underside of the membrane

that have invaded or migrated, therefore allowing a continuous and automatic measurement of invasion or migration (Figure 7). For invasion assays, the upper surface of the membrane were coated with 30 ul of growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) diluted in RPMI serum free medium at a ratio of 1:20 (vol/vol) and incubated at 37°C and 5% CO_2 for 4 h. For all invasion and migration assays, 160 ul of RPMI medium with 10% FBS and 1% P/S was added to the lower chamber of each well (used as a chemoattractant) and 20 ul to the upper chamber, then the plate was pre-incubated for 1 h at 37°C and 5% CO_2. MDA-MB-231 cells (down-regulating or over-expressing Cx43) were plated at a density of $20x10^3$ cells per well into the upper chambers in a volume of 150 ul of RPMI serum free medium. Invasion and migration assays were monitored by recording the electrical impedance every 15 min for minimum 48 h.

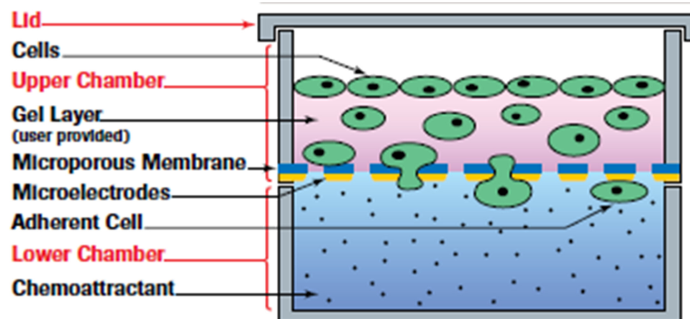


Figure 7: Schematic representation of a well of the CIM-Plate 16. Cell invasion will be assessed by recording the number of cells degrading the underlying ECM and penetrating a microporous membrane in response to a chemoattractant. (Adopted and Modified).

H. MMP Enzymatic Activity

1. Serum Starvation and Supernatant Preparation

MDA-MB-231 cells (down-regulating or over-expressing Cx43) were plated in 6 well tissue culture plate at a density of $0.25x10^6$ cells per well. After 48 h, cells have reached 100% confluence and were starved by 1ml of RPMI serum free media for 72 h. After starvation, the supernatant was collected from each well, spun down at 160 g for 5 min to get rid of any cell, and finally it was stored at -80°C.

2. Gelatin Zymography

Supernatants from MDA-MB-231 cells (down-regulating or over-expressing Cx43) were analyzed by zymography to detect MMP-2 and MMP-9 enzymatic activities. 50 µg of protein from supernatant were electrophoresed on 10% polyacrylamide gel copolymerized with 1mg/ml of gelatin. Gels were then washed twice for 30 min with 2.5% Triton X-100, and incubated overnight in substrate buffer (50 mM Tris-HCl (pH 8), 5 mM $CaCl_2$ and 0.02% sodium azide) at 37°C with gentle shaking. Next day, gels were stained with Coomasie Brilliant Blue R-250 (0.5% in 10% acetic acid, 30% isopropanol and 60% water) for 1 h at 37°C with gentle shaking and then destained in destaining buffer (30% ethanol, 10% acetic acid and 60% water). Enzymatic activity was visualized as white band on a blue background. A positive control of 2.5% FBS was used to detect MMP-2 and MMP-9 enzymatic activities at 68 and 92 kDa, respectively.

I. Immunocytochemistry

Cells were plated at a density of $25x10^3$ cells per well, on the top of glass coverslips. After reaching 60%-80% confluency, cells were fixed with 4% paraformaldehyde, washed by PBS and stored at 4^oC. Cells were then permeablized for 10 min with 0.2% Triton X-100 at room temperature, and then blocked for 1 hour at room temperature by a buffer consisting of 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20 and 10% normal goat serum. Cells were then incubated overnight at 4^oC in a humidified chamber with the specific primary antibody. This was followed with PBS washes and 1 h incubation with an appropriate secondary antibody. Nuclei were counterstained with Hoechst stain, and mounted with prolong anti-fade, for analysis using LSM 710 confocal microscope (Carl Zeiss).

J. *In Vivo* Study

1. Cell Injections and Sacrifice

6-7 weeks postnatal immunocompromised mice were subdermally injected by MDA-MB231 cells (down-regulating or over-expressing Cx43). Two experiments were conducted in this *in vivo* study. The first is a survival rate experiment, and the second is a gene profiling one, where mice were sacrificed at specific time points. In each experiment, three cell lines were used. The first cell line is MDA-MB-231 cells used as a control; the second is MDA-MB-231 shRNA-Cx43 cells (down-regulating Cx43) and the third is MDA-MB-231 high Cx43-Dendra2 cells (over-expressing Cx43). In both experiments, 20 immunocompromised mice were injected with the same cell line (60 mice per experiment). For the gene profiling

experiment, 5 mice from each condition were sacrificed at week 3, 5, 7 and 9. From each sacrificed mouse, primary tumor, lung and liver tissues, directly snap frozen by liquid nitrogen, were taken to extract protein and RNA for further molecular studies. Primary tumor, lung, liver, heart, spleen, kidney, bone and pancreas tissues were collected and directly fixed with 10% formalin to be stained with Hematoxylin and Eosin stain (H and E stain).

2. *Tumor Volume Measurement*

Starting from week 3 post-injection, tumor volume of each mouse was measured by a caliper at a weekly basis. Three-dimensional measurements were recorded, and the volume was calculated using the sphere volume formula to be plotted *versus* time in weeks.

3. *Monitoring Survival Rates*

NSG injected-mice were checked 3 days a week. Mice health status was recorded, in addition to the date at which a mouse has died or being sacrificed after making sure it will no longer survive for even a few hours. Data was plotted as percentage of survived mice *versus* time in weeks.

4. *Protein Extraction and Immunoblot*

Primary tumor, lung and liver tissues were homogenized in 1ml of lysis buffer (0.125 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS) and 10% glycerol) to which 20 ul/ml protease inhibitors were added, using a tissue tearor. DC Protein

Assay (Bio-Rad, Hercules, CA) was used to quantify proteins using bovine serum albumin (BSA, Sigma Chemical Co.) as standards.

Immunoblotting was performed using the same method as described previously.

Chapter III

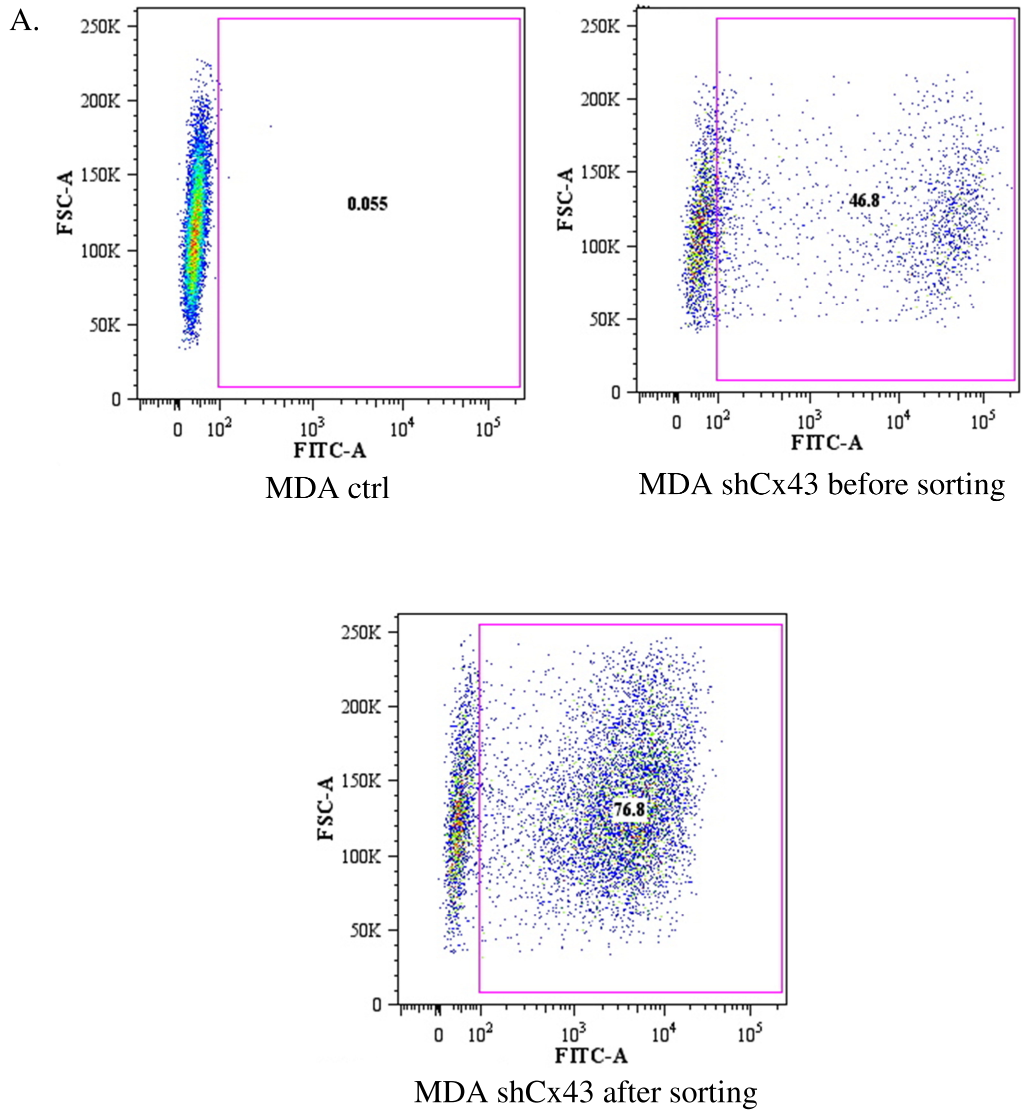
Results

Upon Cx43 over-expression *in vitro*, MDA-MB-231 cells demonstrated an epithelial phenotype with a decrease in their metastatic potential. On the other hand, a mesenchymal phenotype was acquired with more invasive potential upon Cx43 down-regulation. These results were also manifested in an *in vivo* experiment with variations in survival rates upon Cx43 over-expression or down-regulation.

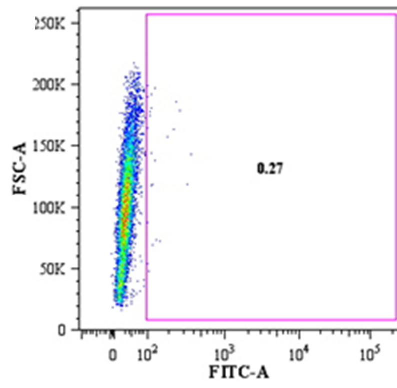
A. Cx43 Down-regulation and Over-Expression in MDA-MB-231

After transfection or transduction, cell sorting was performed to select for fluorescent cells. This yielded five different cell lines: MDA-MB-231 shRNA-Cx43 (down-regulating Cx43), MDA-MB-231 shRNACx43-scr (transfected with a scrambled nucleotide sequence of the shRNA-Cx43), MDA-MB-231 sham (transfection protocol minus plasmid), MDA-MB-231 low Cx43-Dendra2 (expressing Cx43-Dendra2 (Cx43D) at low levels) and MDA-MB-231 high Cx43-Dendra2 (over-expressing Cx43D). All cell lines contained about 70% fluorescent cells (Figure 8). To ensure that down-regulation or over-expression of Cx43 has been established, immunoblots were performed detecting Cx43 (43 kDa) and Cx43D (70 kDa) levels in each cell line. Densitometric analysis revealed no effect of shRNA-scr vector on the expression of Cx43, whereas a 30% decrease was manifested in MDA-MB-231 shRNA-Cx43 cell line. On the other hand, Cx43 expression level has

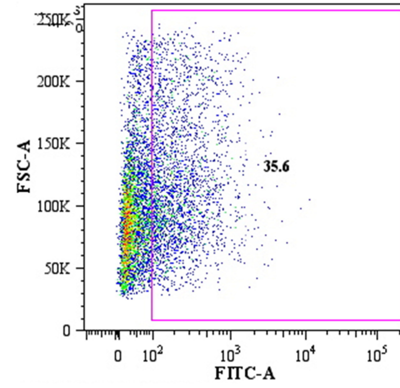
increased by 25% and 75% in cells expressing Cx43D at low and high levels, respectively. A clear difference in the level of Cx43D was demonstrated between MDA-MB-231 low Cx43-Dendra2 and MDA-MB-231 high Cx43-Dendra2 cell lines (Figure 9).



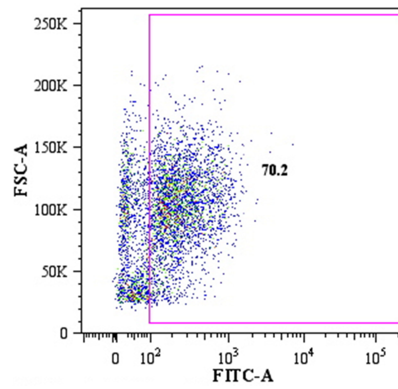
B.



MDA ctrl

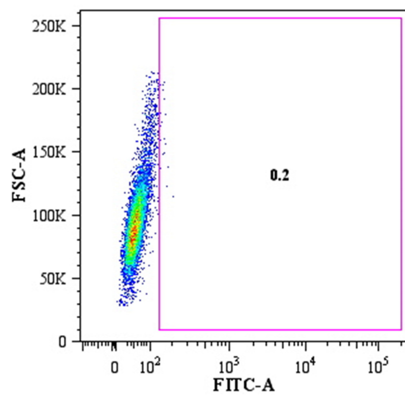


MDA low Cx43D before sorting

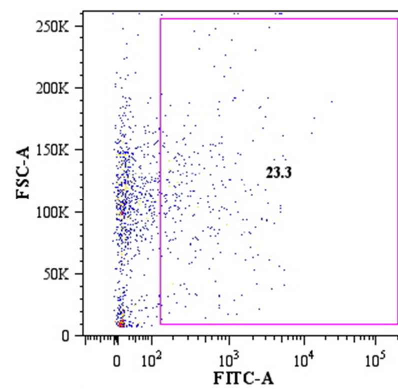


MDA low Cx43D after sorting

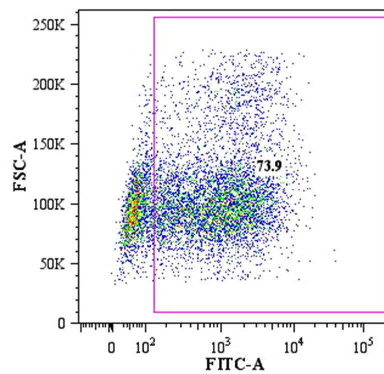
C.



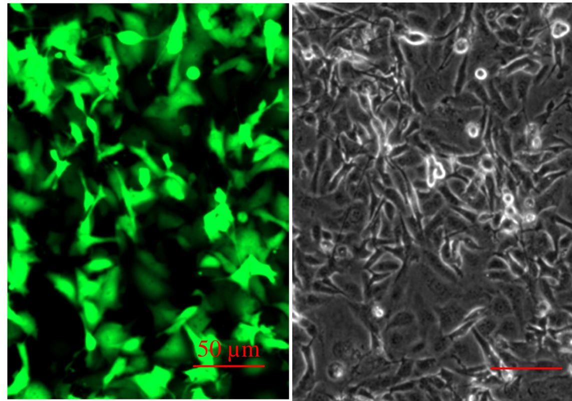
MDA ctrl



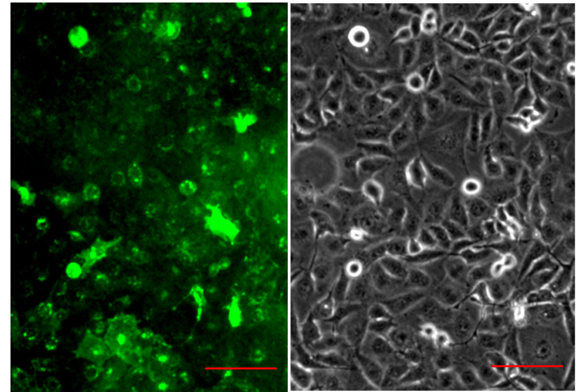
MDA high Cx43D before sorting



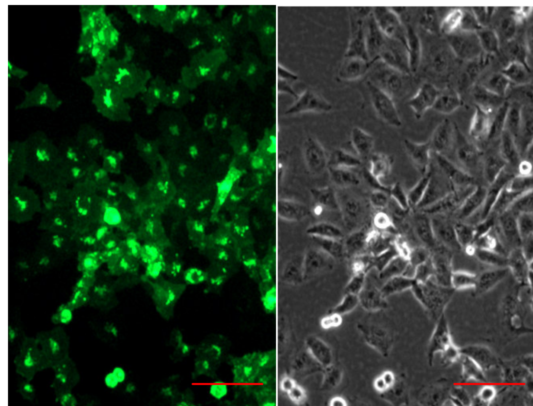
MDA high Cx43D after sorting



MDA-MB-231 shRNA-Cx43 after sorting



MDA-MB-231 low Cx43-Dendra2 after sorting



MDA-MB-231 high Cx43-Dendra2 after sorting

Figure 8: A. MDA-MB-231 shRNA-Cx43 cell sorting B. MDA-MB-231 low Cx43-Dendra2 cell sorting. C. MDA-MB-231 high Cx43-Dendra2 cell sorting with their respective fluorescent and light microscopic images.

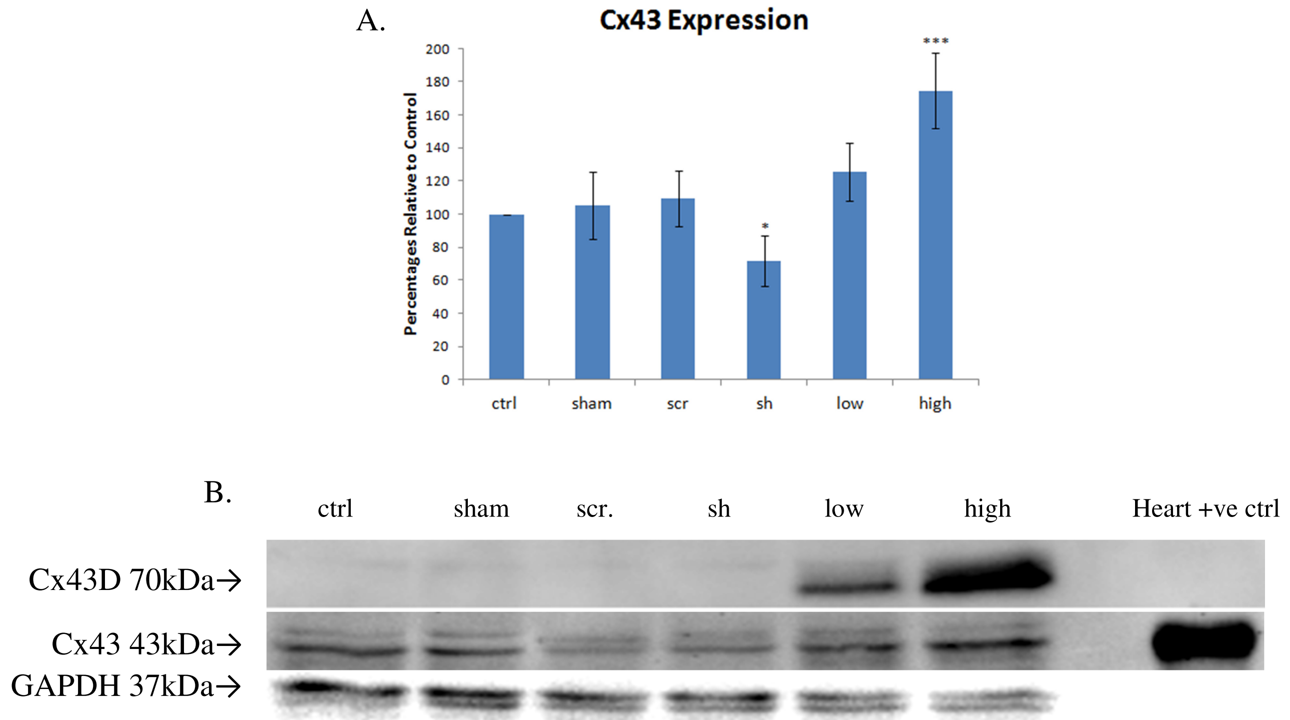
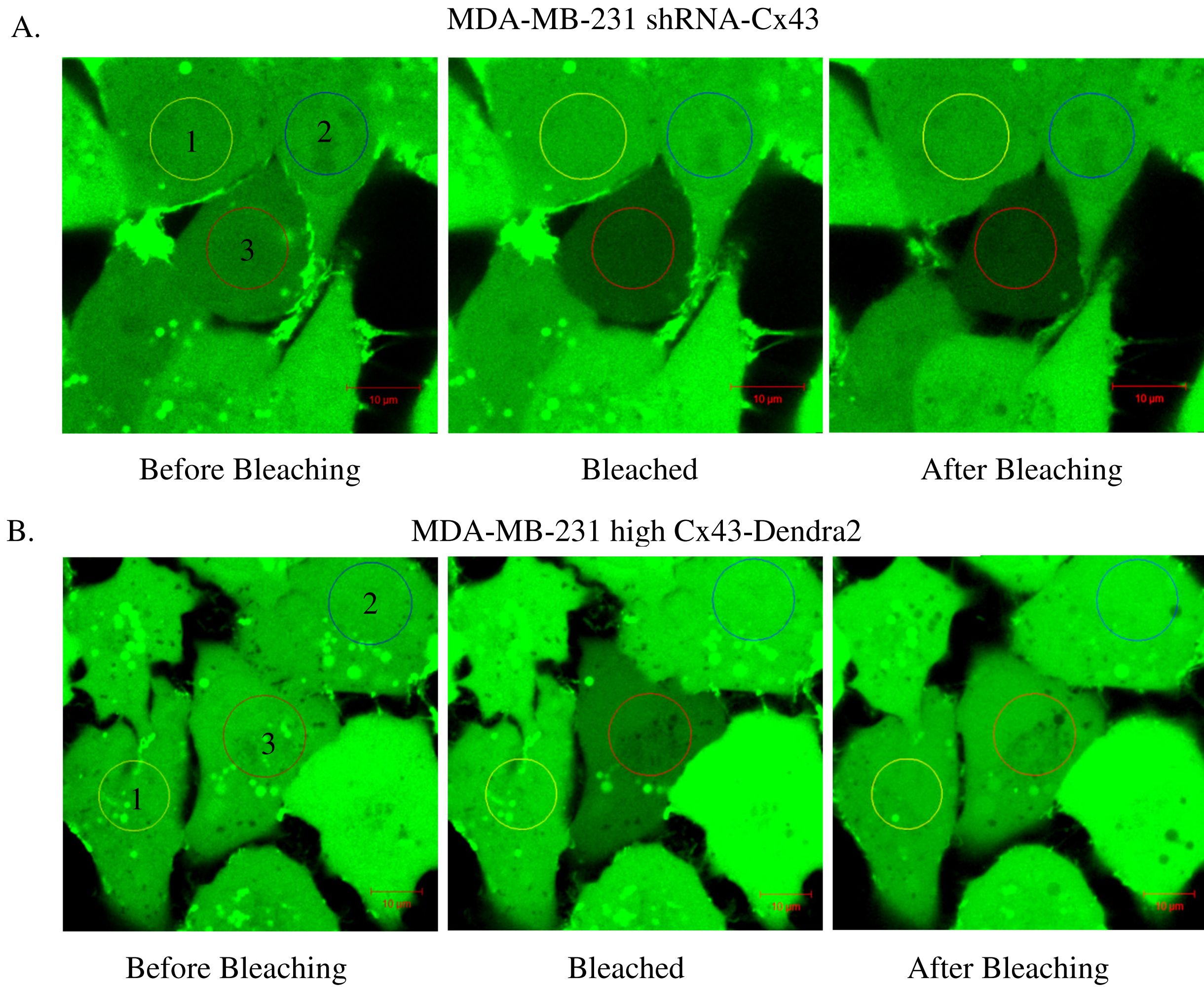


Figure 9: A.B. Immunoblots and densitometric analysis detecting Cx43/Cx43D expression levels in different MDA-MB-231 cell lines. Ctrl: MDA-MB-231. Sham: MDA-MB-231 sham. Scr: MDA-MB-231 shRNACx43-scr. Sh: MDA-MB-231 shRNA-Cx43. Low: MDA-MB-231 low Cx43-Dendra2. High: MDA-MB-231 high Cx43-Dendra2. Heart +ve ctrl: A murine heart tissue lysate was used as a positive control for Cx43. P value for high ($p<0.005$). P value for sh ($p<0.05$)

B. Functionality of Over-Expressed Cx43

To further confirm the over-expression or down-regulation of Cx43, and to assess the functionality of over-expressed Cx43 are functional *via* the formation of intercellular gap junctions, FRAP assay was conducted on four cell lines: MDA-MB-231, MDA-MB-231 shRNA-Cx43, MDA-MB-231 low Cx43-Dendra2 and MDA-MB-231 high Cx43-Dendra2. After photobleaching, fluorescence recovery was only recorded in cell lines over-expressing Cx43D at low or high levels, and

not in the other two cell lines (Figure 10). This has confirmed that Cx43 is over-expressed and performing its normal functions in the cell.



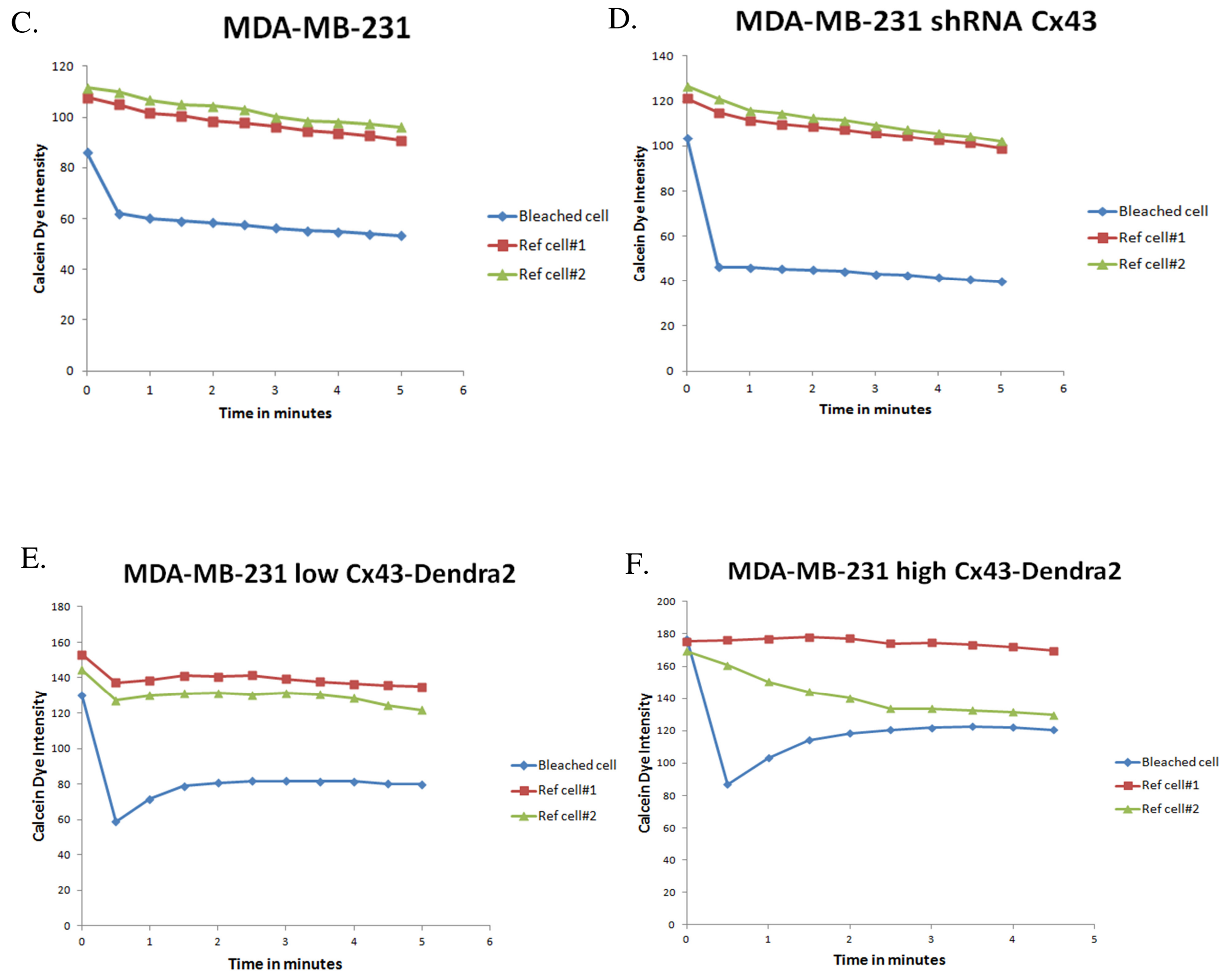


Figure 10: FRAP analysis showing intensities of calcein dye in photobleached cells in reference to two adjacent cells. A. B. Live imaging of cells, over-expressing or down-regulating Cx43, being photobleached. C.D. No fluorescence recovery in MDA-MB-231 control and cells down-regulating Cx43. E.F. Fluorescence recovery in cells over-expressing Cx43 at low and high levels.

C. **Effect of Cx43 Over-Expression and Down-Regulation on Cell Proliferation in 2D and 3D Culture Systems.**

Due to its tumor suppressive abilities, Cx43 is expected to reduce tumor growth. For that reason, we assessed the effect of Cx43 over-expression and down-regulation on cellular growth and proliferation in both 2D and 3D culture systems. Trypan blue dye exclusion assay and RTCA were performed on cells down-regulating or over-expressing Cx43 grown in 2D culture system over a period of 4 days. For 3D culture system, cell aggregates were counted after 8 days in culture, differentiating between stellar and spherical morphology. According to both trypan and RTCA assays, cells down-regulating or over-expressing Cx43 did not show difference in growth rate in 2D culture system (Figure 11). In 3D culture system, Cx43 down-regulation has favored cell aggregates with stellar morphology (invasive morphology) and large diameters. However, the over-expression of Cx43 favored a spherical cell aggregate morphology resembling the growth morphology of normal mammary epithelial tissue. Compared to other cell lines, small diameter cell aggregates were formed with cells over-expressing Cx43D at high levels (Fig. 12).

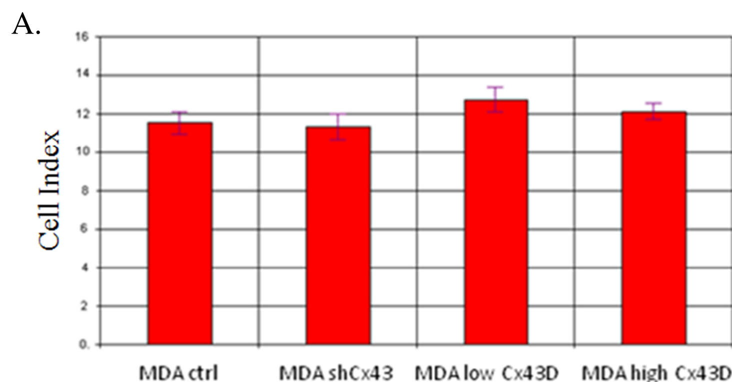
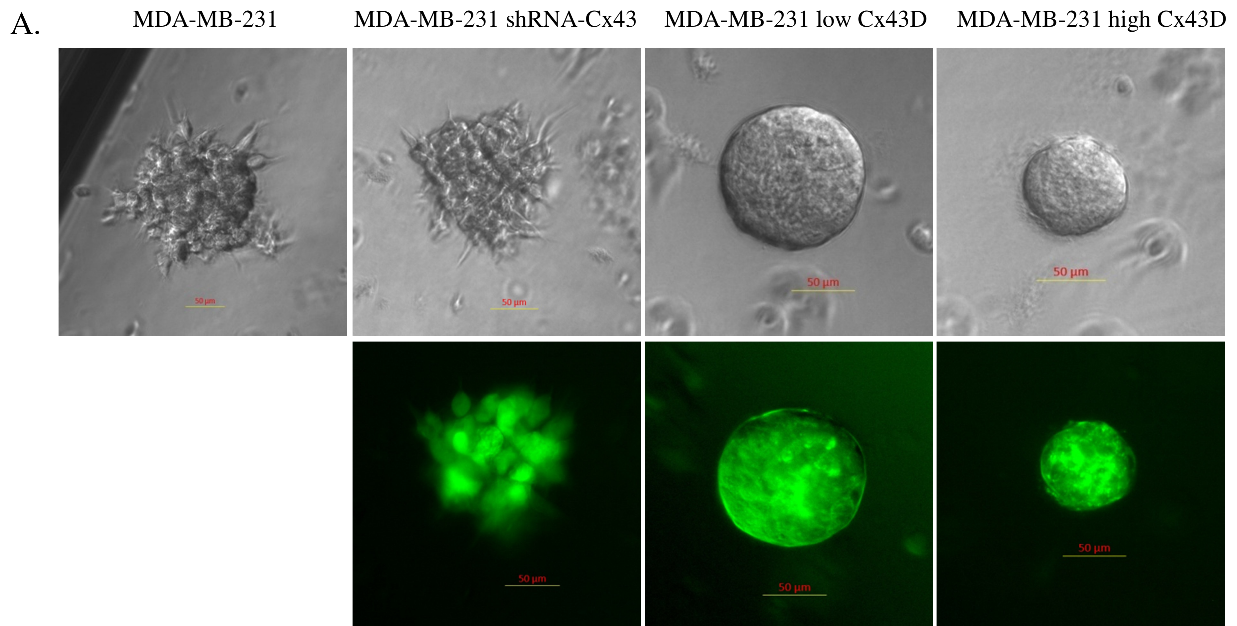


Figure 11: A. RTCA graphical representation showing cell proliferation in 2D culture system



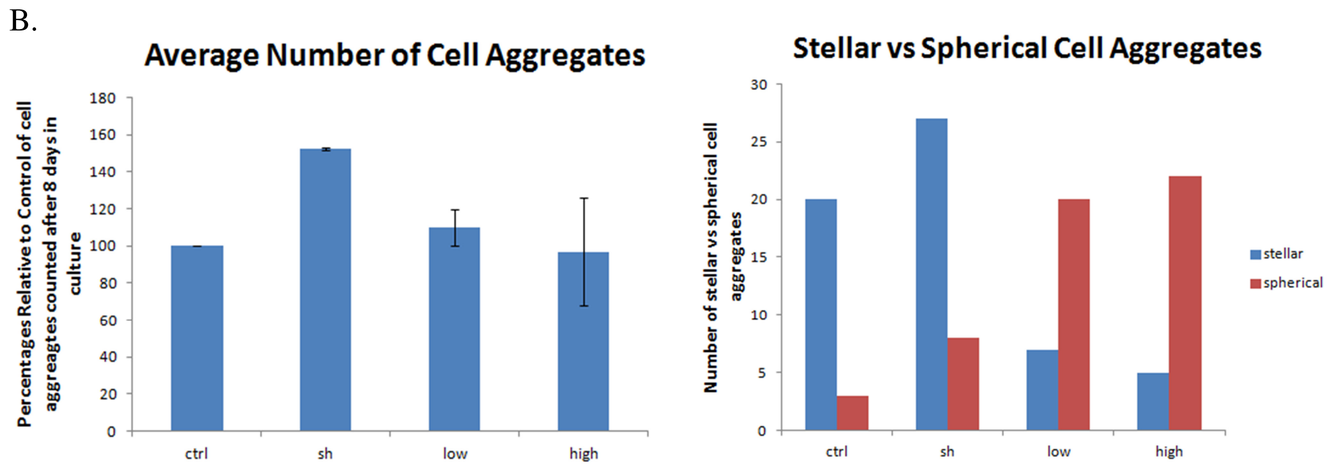


Figure 12: A. Light and fluorescent images for cell aggregates from each cell line. B. Graphical representations showing the average number of cell aggregates with the differentiation between stellar versus spherical morphologies.

D. Gene Expression Profile at the Transcriptional Level in Cells Down-Regulating and Over-Expressing Cx43

As a first step in our gene expression profile, qRT-PCR was performed to detect changes in the expression level of connexins (Cx26, Cx30, Cx32, Cx43 and Cx45), EMT markers (E-cadherin, N-cadherin, Snail, Twist and ZO-1), angiogenic markers (VEGF and HIF-1α), MMPs (MMP-2 and MMP-9) and the transcription factor β-catenin. Results have shown no significant changes in the expression level of Cx26 (data not shown), but a 0.5 fold decrease in the expression of Cx43 and approximately a 1 fold increase in the expression of Cx45 in cells down-regulating Cx43 (Figure 13 A). Cx30 and Cx32 were not expressed in any cell line (data not shown). E-cadherin expression increased 6 folds and decreased approximately by 0.8 fold in MDA-MB-231 high Cx43-Dendra2 and MDA-MB-231 shRNA-Cx43, respectively (Figure 13 B). The epithelial marker ZO-1 has increased with Cx43 over-expression and decreased upon Cx43 down-regulation by approximately 0.5 fold (Figure 13 C). N-cadherin, Snail, Twist, HIF-1α and VEGF did not show significant changes in their mRNA expression levels (data not shown). MMP-9 expression level increased by approximately 1.3 folds in MDA-MB-231 shRNA-Cx43 (Figure 13 D). β-catenin showed no changes in its transcriptional expression level among the different cell lines (Figure 13 E).

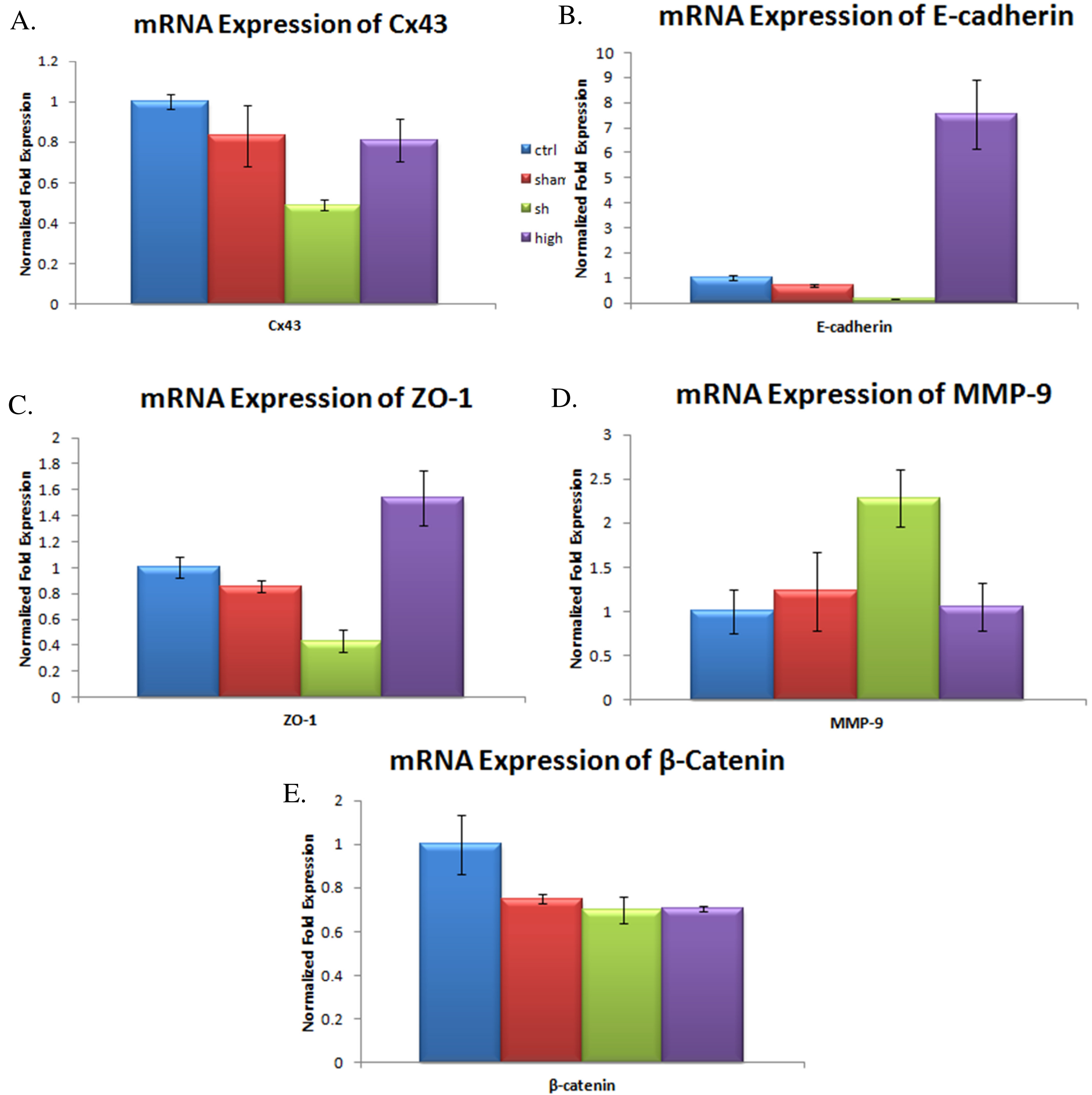
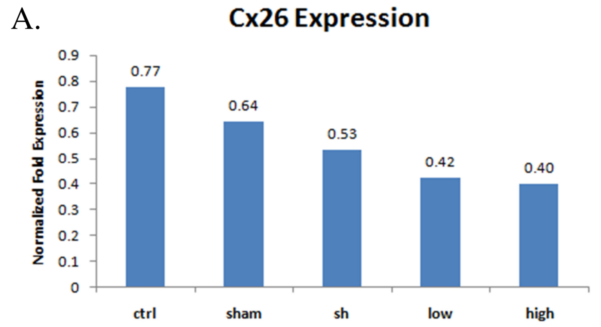
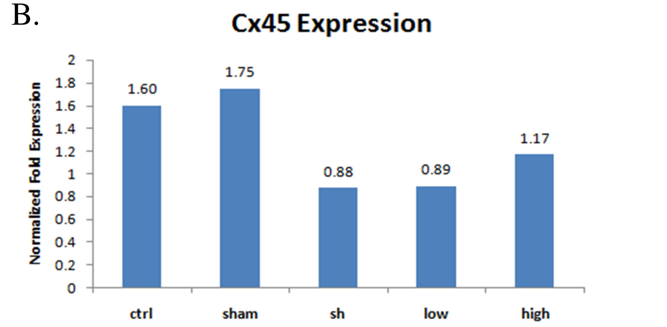


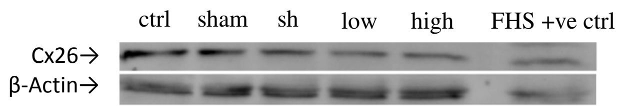
Figure 13: Graphical representation of changes in mRNA expression level normalized to GAPDH housekeeping gene. A. 50% decrease in mRNA level of Cx43 upon knocking down by an shRNA. B.C. Increase in the mRNA expression level of E-cadherin and ZO-1 upon Over-expressing Cx43D at high levels. D. Increase in the mRNA expression level of MMP-9 upon knocking down Cx43. E. No changes in the mRNA expression level of β-catenin upon over-expressing or knocking down Cx43.

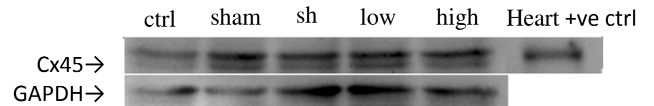
E. Gene Expression at the Translational Level in Cells Down-Regulating and Over-Expressing Cx43

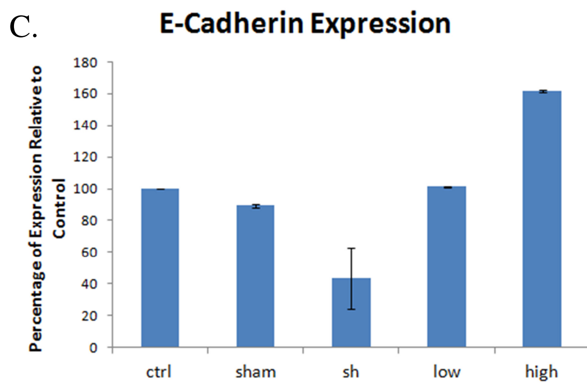
Immunoblots were performed as a second step in our gene expression profile. Connexins (Cx26 and Cx45), EMT markers (E-cadherin, N-cadherin, Vimentin and ZO-1) and angiogenic markers (HIF-1α and VEGF) expression levels were investigated. Results have revealed that Cx26 and Cx45 were down-regulated upon knocking down Cx43 via shRNA (Figure 14 A, B). The epithelial marker, E-cadherin was up-regulated by approximately 60% with the over-expression of Cx43D at high level and down-regulated by approximately 70% in cells down-regulating Cx43. E-cadherin expression was not affected in MDA-MB-231 low Cx43-Dendra2 cell line (Figure 14 C). In addition, ZO-1 was also up-regulated by approximately 35% in MDA-MB-231 high Cx43-Dendra2 (Figure 14 D). N-cadherin, a mesenchymal marker, was only expressed in cells down-regulating Cx43 (Figure 14 E), but the expression of vimentin was not affected in any cell line (Figure 14 F). Investigating angiogenic markers, results have shown no effect on the expression levels of HIF-1α transcription factor (Figure 14 G), but VEGF levels were up-regulated by 44% and down-regulated by 22% in cells down-regulating and over-expressing Cx43, respectively (Figure 14 H).

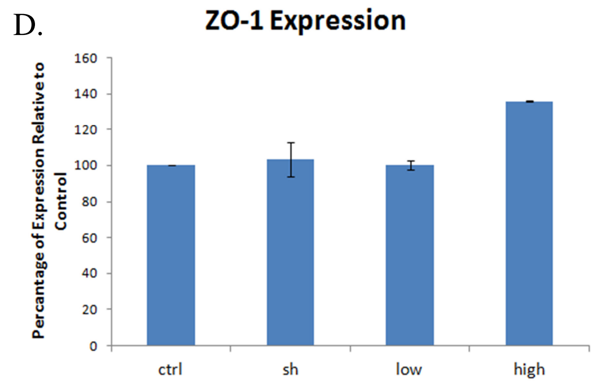

A.
Cx26 Expression
Normalized Fold Expression
0.77
0.64
0.53
0.42
0.40
ctrl
sham
sh
low
high

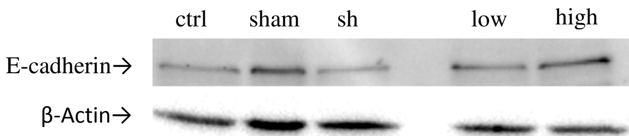

B.
Cx45 Expression
Normalized Fold Expression
1.60
1.75
0.88
0.89
1.17
ctrl
sham
sh
low
high


ctrl sham sh low high FHS +ve ctrl
Cx26→
β-Actin→

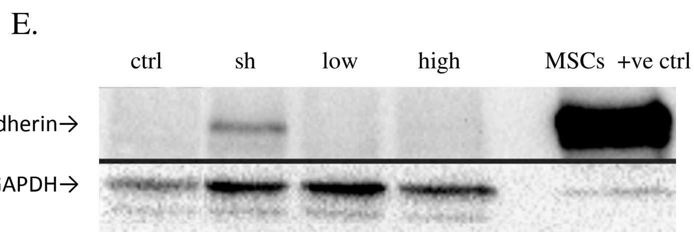

ctrl sham sh low high Heart +ve ctrl
Cx45→
GAPDH→


C.
E-Cadherin Expression
Percentage of Expression Relative to Control
ctrl
sham
sh
low
high


D.
ZO-1 Expression
Percantage of Expression Relative to Control
ctrl
sh
low
high


ctrl sham sh low high
E-cadherin→
β-Actin→

ctrl sh low high
ZO-1→
β-Actin→


E.
ctrl sh low high MSCs +ve ctrl
N-cadherin→
GAPDH→

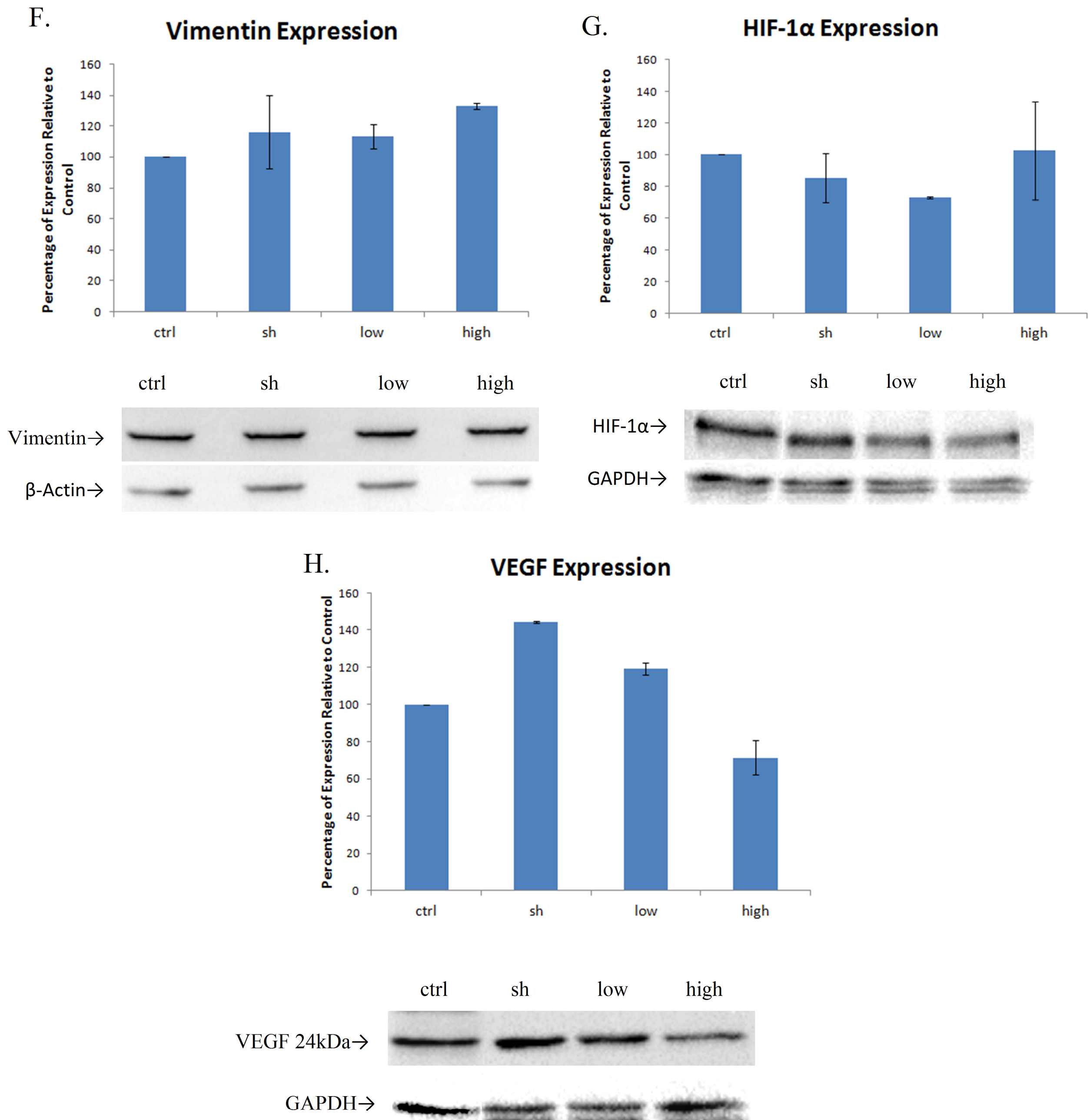


Figure 14: Normalized fold expression and immunoblots of each of the following proteins A. B. Cx26 and Cx45 are down-regulated upon knocking down Cx43. C. D. E-cadherin and Zo-1 are up-regulated upon over-expressing Cx43D. E. N-cadherin is only expressed in cells knocking down Cx43. F.G. No changes in vimentin and HIF-1α expression. H. Upregulation and down-regulation of VEGF expression upon down-regulating and over-expressing Cx43, respectively.

F. Effect of Cx43 Down-Regulation and Over-Expression on MMP-9 Enzymatic Activity, Cell Invasion and Migration

In order to assess the ability of cancer cells to migrate and to invade the surrounding ECM, three separate functional assays were conducted. Using RTCA machine, invasion and migration assays were performed, where cells down-regulating or over-expressing Cx43, were plated above matrigel (no ECM layer in migration assays), where cells invading this layer in response to FBS, acting as a chemoattractant, will be recorded by microelectrode sensors. Results have shown no effect of Cx43 over-expression on cells' invasive potential, whereas Cx43 down-regulation has enhanced cells' invasive ability in comparison to control cells (Figure 15 A, B). Both Cx43 down-regulating and over-expression had no effect on cell migration (Figure 15 C). To detect activity and levels of the secreted MMP-2 and MMP-9, gelatin zymography assay was performed. After cells were starved by serum free media for 72 h, the supernatant were loaded on a gelatin-acrylamide page and results have shown a high level of MMP-9 level in MDA-MB-231 shRNA-Cx43 cell line compared to other cell lines (Figure 16 A, B), but no differences in the levels of MMP-2 were detected (data not shown).

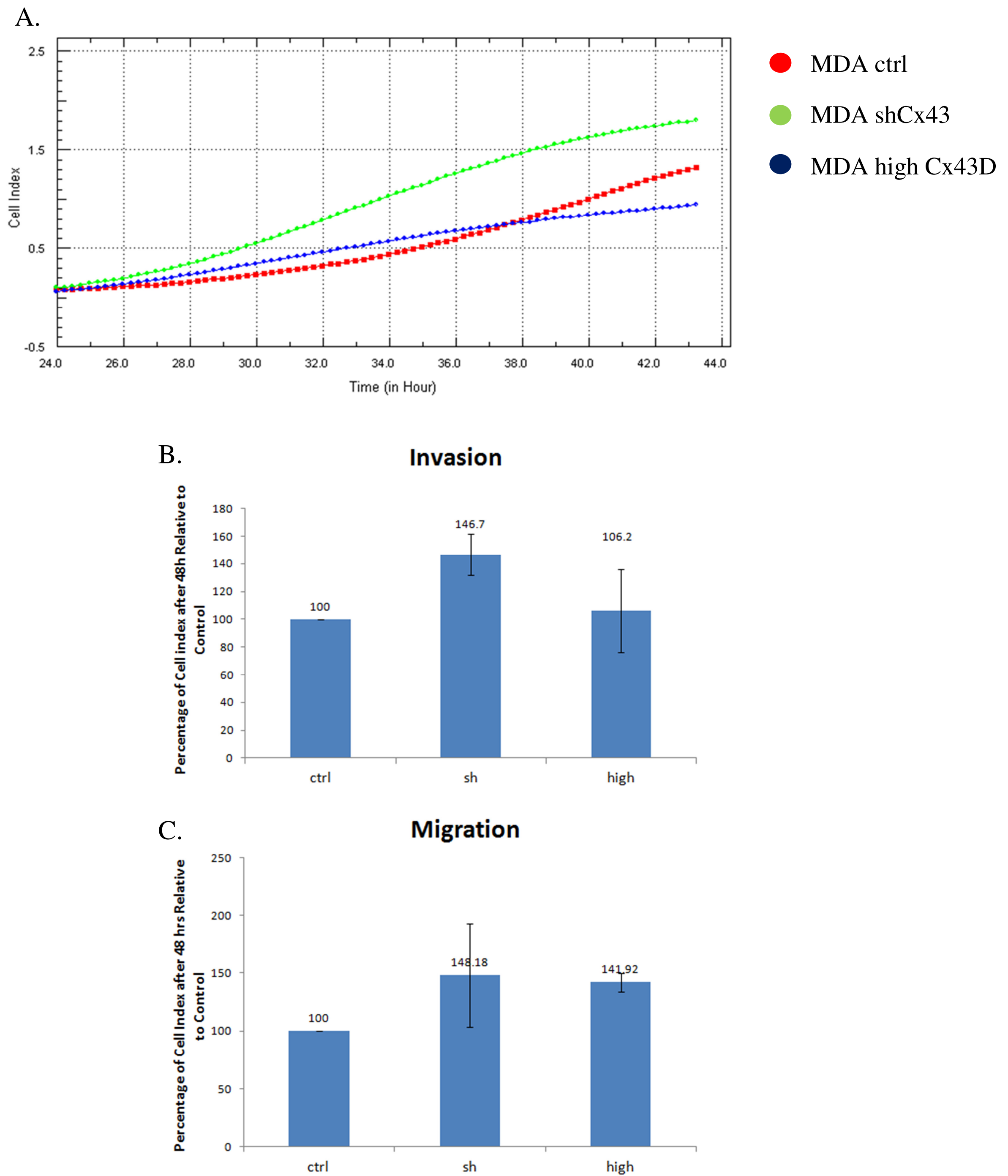
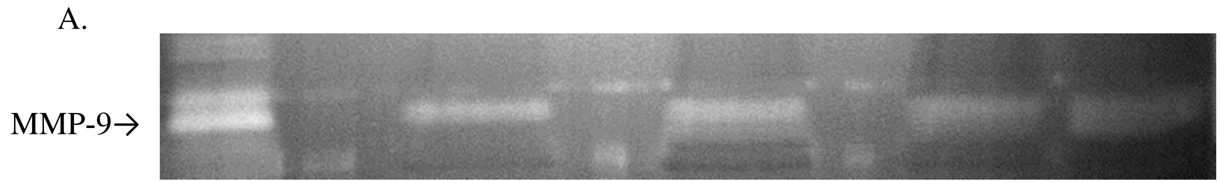


Figure 15: A. Graphical representation showing the invasive potential of cells down-regulating or over-expressing Cx43. Histogram showing the cell index of each cell line after 48 hr, which illustrates the percentages of cells that invaded (B) the ECM, or migrated (C).

A.

MMP-9→



B.

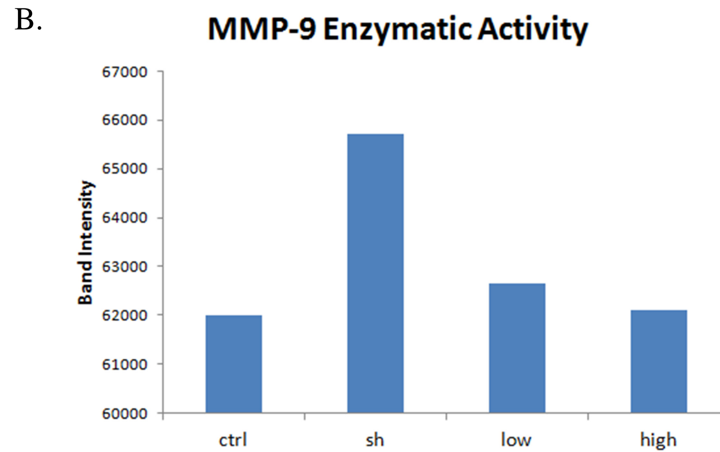


Figure 16: MMP-9 enzymatic activity in cells down-regulating or over-expression Cx43. A. Gelatin zymography gels where bands show the activated MMP-9 enzyme. B. Densitometric analysis where band intensities were measured.

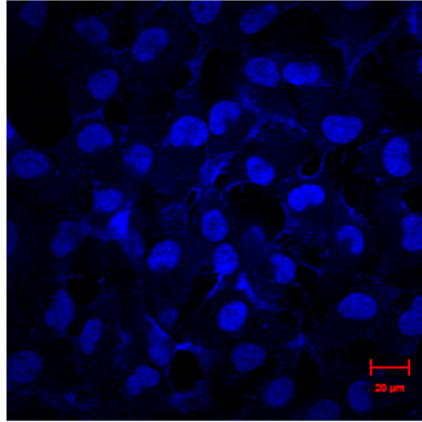
G. High Expression of Cx43 in MDA-MB-231 Cells Sequesters β-Catenin at the Cell Membrane

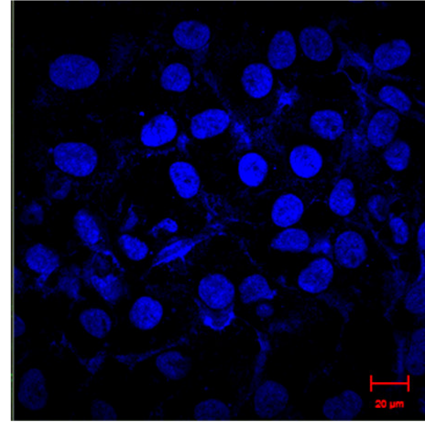
β-catenin is a dual function protein, it could be a part of the adherens junction complex or a transcription factor regulating the expression of genes related to metastasis in tumor cells. To localize whether β-catenin is in the nucleus or on the cell membrane, immunofluorescent staining was performed on cells over-expressing and down-regulation Cx43. Results have demonstrated that with Cx43 over-expression more β-catenin was found on the cell membrane and less in the nucleus compared to cells down-regulating Cx43 (Figure 17).

A.

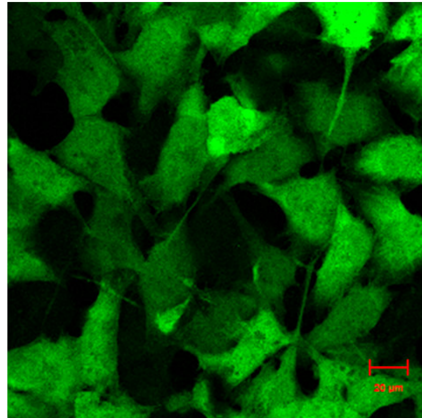
MDA-MB-231 shRNA-Cx43 | MDA-MB-231 high Cx43-Dendra2

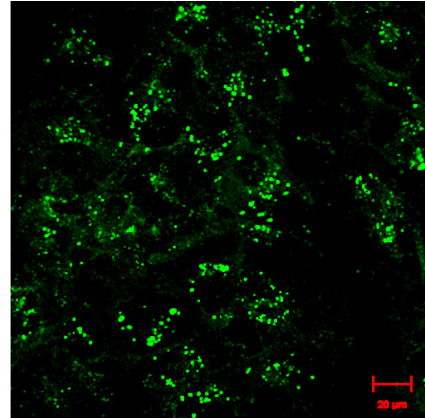
DAPI



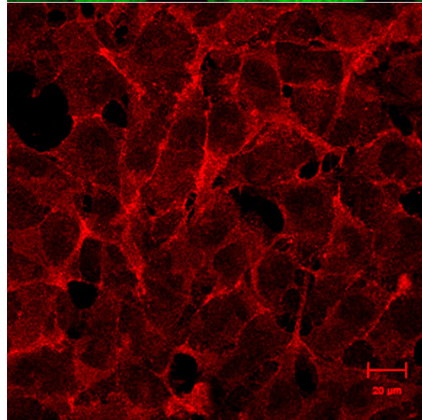


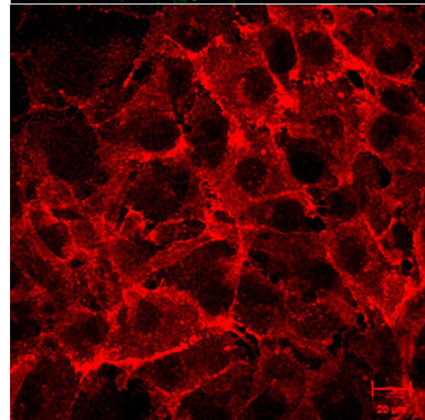
GFP/Cx43-Dendra2



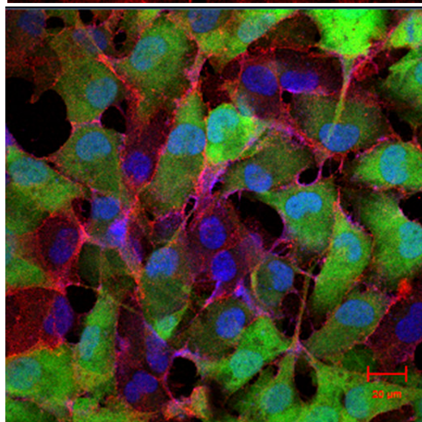


β-Catenin





Merged



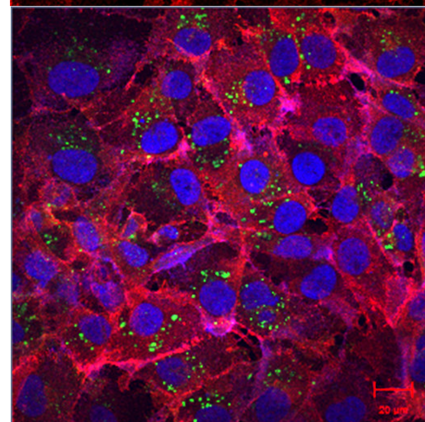
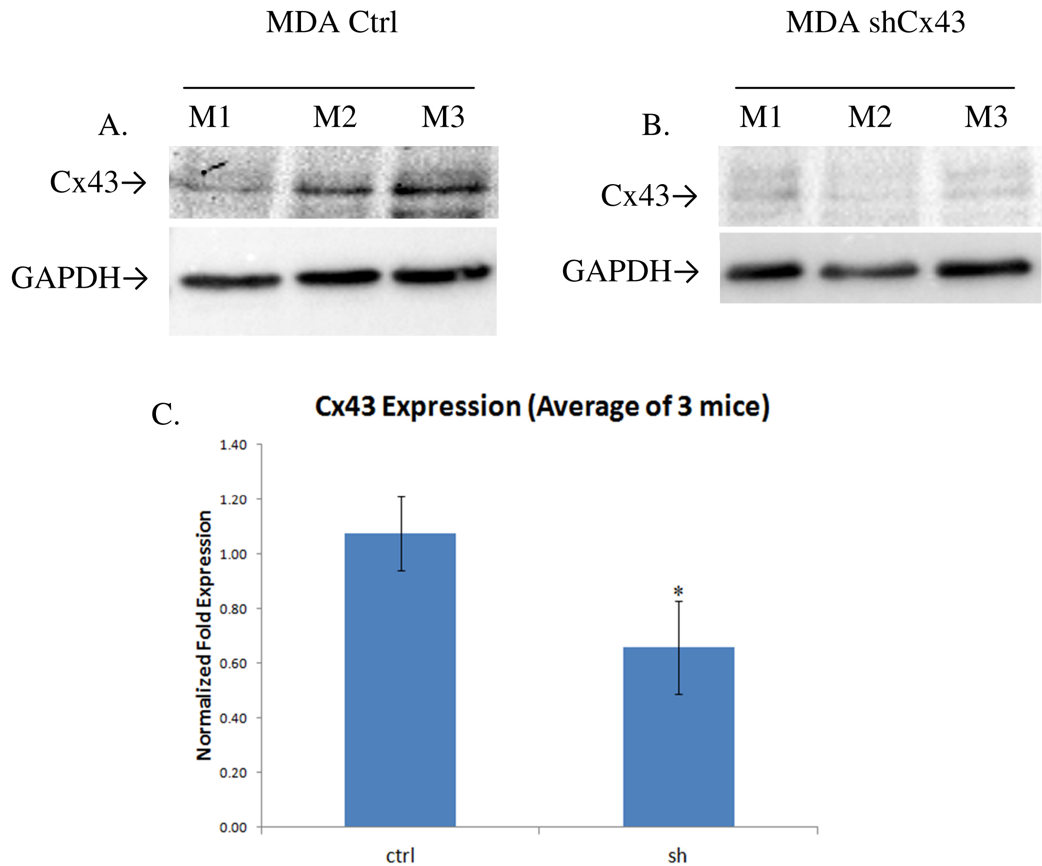


Figure 17: Immunocytochemistry staining β-catenin in cells over-expressing or down-regulating Cx43. By probing for β-catenin, it was shown that more β-catenin are sequestered on the plasma membrane away from the nucleus upon over-expressing Cx43D in comparison to cells knocking down Cx43.

H. Cx43 Over-Expression or Down-Regulation in Primary Tumor Tissues

To ensure that the results of tumor onset, tumor volume, survival rate and H&E stains are due to Cx43 over-expression or down-regulation, immunoblots were performed on primary tumor tissues. Results have revealed expression of Cx43D and down-regulation of Cx43 in primary tumor tissues taken after nine weeks post-injection (Figure 18).



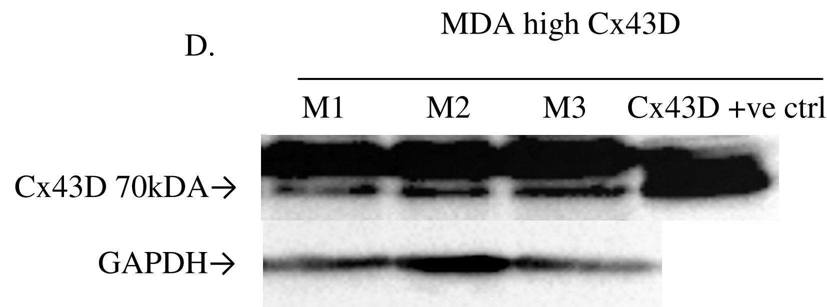


Figure 18: A.B. Immunoblots probing for Cx43 in primary tumor tissues taken after nine weeks post-injection. C. Densitometric analysis showing down-regulation of Cx43 in primary tumors of mice injected by cells down-regulating Cx43, compared to primary tumors taken from mice injected by control cells. D. Immunoblot probing for Cx43D in primary tumors tissues taken after nine weeks post-injection. Cx43 +ve ctrl is a cell lysate from cells highly expressing Cx43D, used as our positive control.

H. Mice Injected with Cancer Cells Over-Expressing Cx43 have a Delayed Onset of Palpable Tumors

To study the effect of Cx43 over-expression or down-regulation on tumorigenesis, injected mice were checked for tumors by palpation. After no tumors were detected by week one, 50% of mice injected with control cancer cells have developed palpable tumors by week two, compared to 30% and 12% of mice injected with MDA-MB-231 shRNA-Cx43 and MDA-MB-231 high Cx43-Dendra2 cells, respectively. By week three, tumor onset was delayed in the group injected with cancer cells over-expressing Cx43D with 24% of the mice having palpable tumors. However, 92% and 65% for those injected with control cancer cells and cancer cells down-regulating Cx43, respectively. By week four, all mice injected with control cells had a palpable tumor. Ninety five percent of mice injected with cancer cells

over-expressing or down-regulating Cx43 had palpable tumors, until week 5 when all mice developed visible tumors at the site of injection (Figure 19).

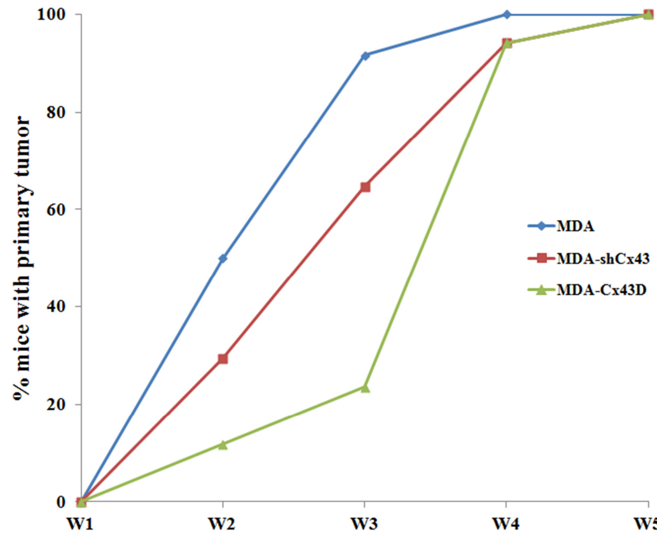


Figure 19: The rate of tumor development has decreased in mice injected with cancer cells over-expressing Cx43D.

I. Mice Injected with Control Cells and Cells Down-Regulating Cx43 have Larger Tumors Compared to those injected with Cells Over-expressing Cx43D.

On a weekly basis, the tumor dimensions were recorded in all the groups to assess the effect of Cx43 over-expression or down-regulation on the primary tumor volume. Starting from week one till week three, all groups showed small tumors with a comparable slow increase in size. At week four, there was no difference in tumor volume between groups injected with cancer cells down-regulating or over-expressing Cx43 (≈0.5 cm^3), but it has increased up to ≈ 0.15 cm^3 in those injected with control cancer cells. From week five, the tumor volume of groups injected by

control and Cx43 down-regulating cancer cells started to increase sharply reaching ≈0.6 cm^3 by week seven, whereas a shy increase was recorded in groups injected by Cx43D over-expressing cancer cells, reaching ≈0.25 cm^3 by week seven (Figure 20).

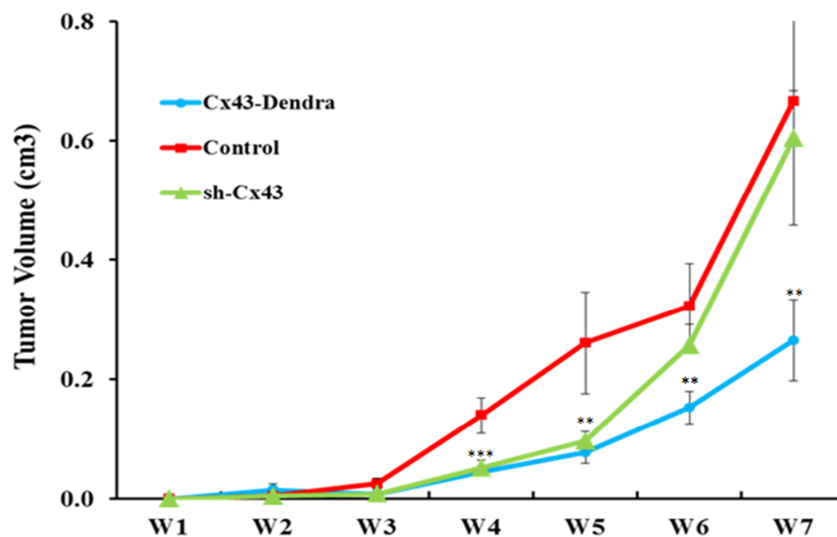


Figure 20: Tumor volume suppression in mice injected with Cx43 over-expressing cancer cells (mean ± s.e.m). At week 4, p value<0.005. At week 5, 6 and 7 p value<0.01

J. Over-Expression of Cx43D in Breast Tumor Cells Increases Mice Survival Rate Compared to those Injected with Control or Cx43 Down-Regulating Tumor Cells

Following injections, mice were left for survival experiments where overall survival of the mice was defined from the injection of the cell lines to the death of mice from any cause. Cx43 down-regulation caused a lower survival rate compared to control and Cx43D over-expressing groups, where all mice have died at day 92. Cx43 over-expression delayed the death by two weeks and by ten days compared to Cx43 down-regulating and to control groups, respectively (Figure 21).

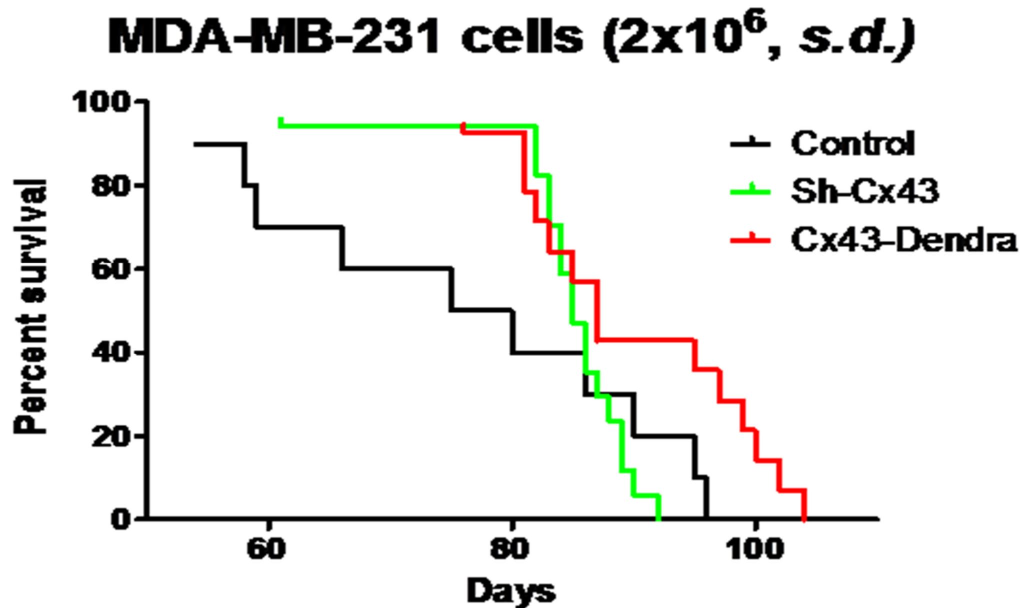
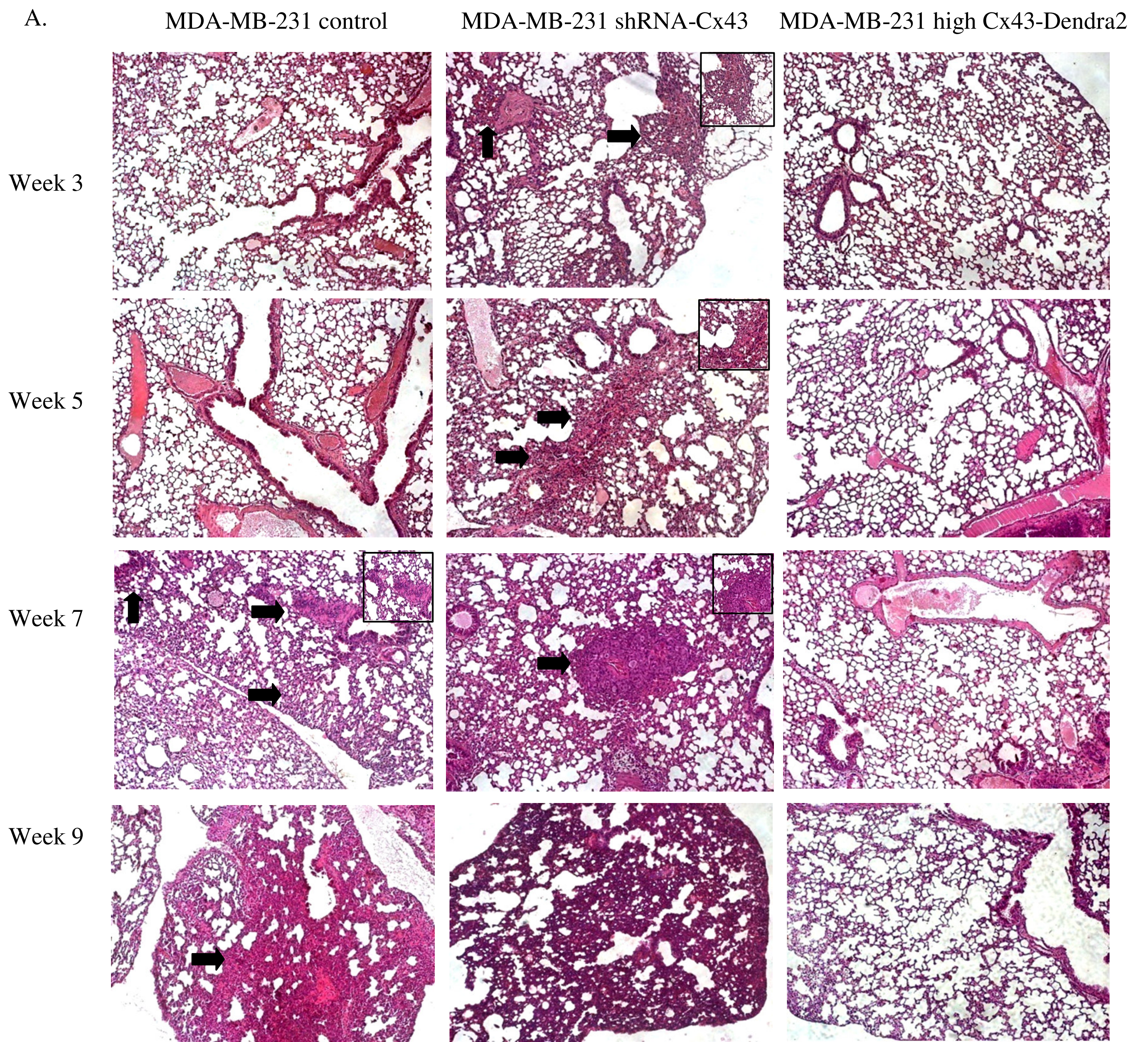


Figure 21: Survival curves of mice injected subdermally by $2x10^6$ cells from each cell line.(Control vs Cx43D $p<0.05$, shCx43 vs Cx43D $p<0.05$)

K. Cx43 Over-Expression Decreases Tumor Cell Infiltration to the Lung and Liver Tissues

Histological examination of lung and liver tissues taken at weeks three, five, seven and nine, showed that Cx43D over-expression suppressed the potential of tumor cells to infiltrate the lung and liver compared to the two other cell lines. On the other hand, Cx43 down-regulation lead to more aggressive infiltrations compared to the control cell line (Figure 22 A, B).


A.
MDA-MB-231 control
MDA-MB-231 shRNA-Cx43
MDA-MB-231 high Cx43-Dendra2
Week 3
Week 5
Week 7
Week 9

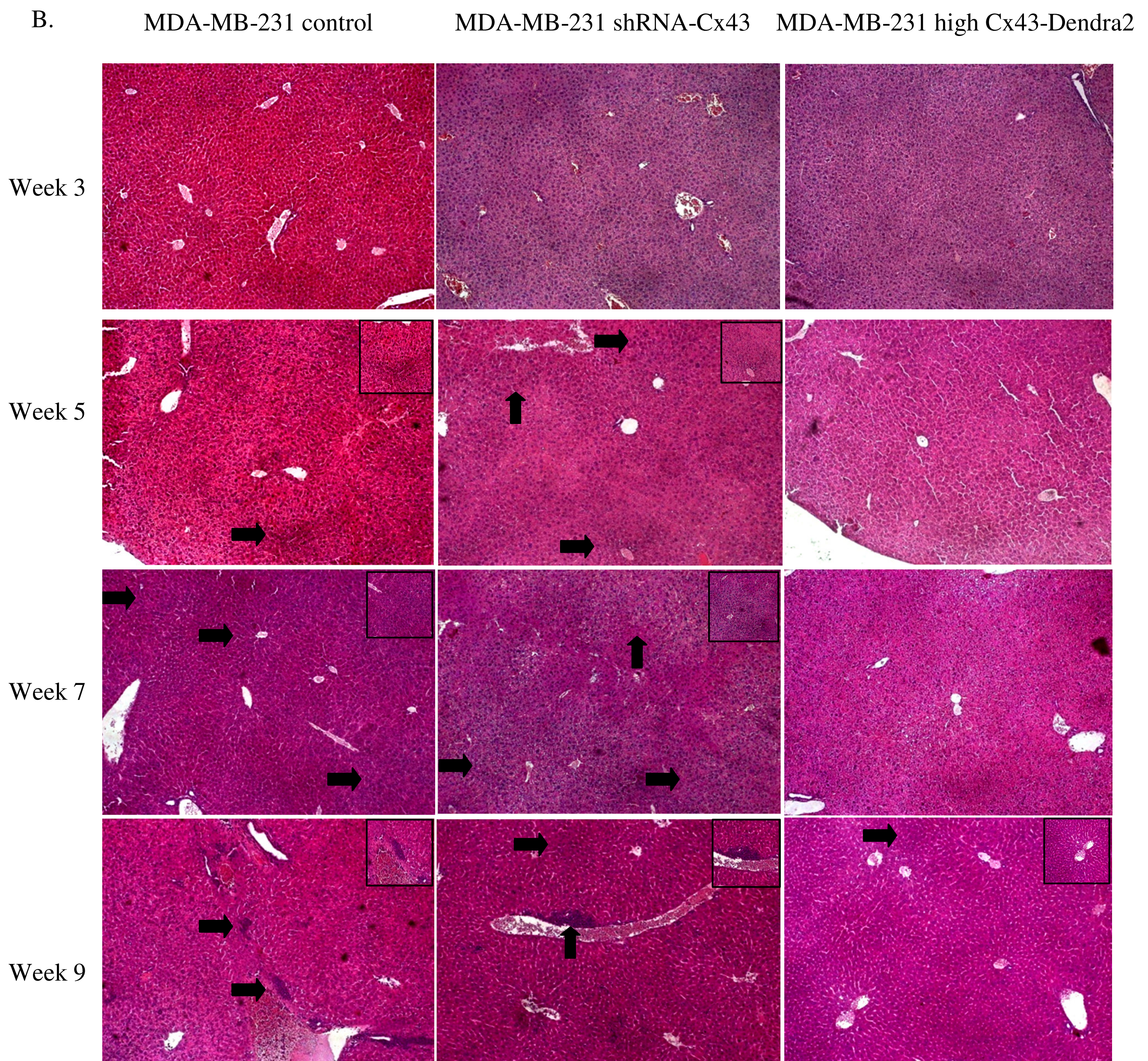


Figure 22: H&E staining of lung and liver tissues taken at week three, five, seven and nine post-injection from mice injected with MDA-MB-231 control, MDA-MB-231 shRNA-Cx43 and MDA-MB-231 high Cx43-Dendra2 cells. Arrows showing tumor tisuues.

CHAPTER IV

DISCUSSION

Intercellular junctions are essential for maintaining proper tissue physiology. Junctional molecules act as signaling units integrating signals from external stimuli or from within the cell (Lee et al., 2008). Gap junctions have the unique ability to directly transfer molecules between adjacent cells. Moreover, connexins have channel-independent functions which control other cellular processes such as cellular growth, apoptosis and differentiation (Dbouk, Mroue, El-Sabban, and Talhouk, 2009).

In cancer, junctional molecules are found to be down-regulated, mutated or mis-localized (Hanahan et al., 2000). Gap junction assembly and connexin expression are differentially regulated throughout the metastatic process of cancer (El-Saghir et al., 2011). For instance, gap junction down-regulation may occur during tumor cell proliferation and invasion, and it will be re-expressed and assembled to facilitate extravasation and secondary tumor formation (Laird et al., 2002).

Many studies have confirmed a tumor suppressive role for connexins after showing a decrease in cancer cell proliferation and invasive abilities upon connexin over-expression, and a more aggressive phenotype upon down-regulation (Talhouk et al., 2013). The mechanism of this effect remains unclear, whether it is channel-dependent or independent. In this study, we have aimed to assess the effect of Cx43 over-expression or down-regulation on metastatic abilities of MDA-MB-231, a triple-negative highly invasive breast cancer cell line. Since several studies have proposed that connexins' tumor suppressive

abilities are channel-independent, we investigated *in vitro* the expression of several metastatic markers related to invasion, angiogenesis and EMT in cells over-expressing or down-regulating Cx43. In an *in vivo* study, tumor onset and volume, survival rates and secondary tissue infiltration by cancer cells were also assessed.

As an initial step, levels of Cx43 protein were assessed in both over-expressing and down-regulating Cx43 cell lines. We chose to include the cell line over-expressing Cx43D at low levels to investigate whether there is a gradual suppression of metastasis as the expression of Cx43 increases from low to high Cx43 levels. Immunoblot showed that Cx43 was down-regulated and over-expressed in their respective cell lines. In addition, FRAP assay has confirmed our immunoblot results and added an evidence for an increase in GJIC upon Cx43D over-expression. Laird et al. have also shown that upon over-expression of Cx43 in MDA-MB-231, the number of cells transferring Lucifer yellow dye to adjacent cells has increased compared to control cells (Laird et al., 2002). In 3D culture system, Cx43 down-regulation induced a higher number of cell aggregates with dominant stellar morphology, whereas the over-expression of Cx43D decreased the number of cell aggregates favoring spherical structures. This 3D morphogenesis assay matches with other studies where the same effects were shown to occur upon Cx43 down-regulation and over-expression in MDA-MB-231 (Talhouk et al., 2013). Gene expression profile showed that an epithelial phenotype was favored in cells expressing Cx43D at high levels by up-regulating certain epithelial markers (E-cadherin and ZO-1), in contrast to Cx43 down-regulating cells which favored a mesenchymal phenotype through the up-regulation of mesenchymal markers and down-regulating epithelial ones. Furthermore, VEGF biosynthesis increased with Cx43 down-regulation and decreased upon Cx43D over-expression. These findings are in line

with studies reporting the domination of an epithelial phenotype and an attenuation of angiogenesis upon Cx43 up-regulation (Laird et al., 2002). Functional assays have also proven that the invasive properties of tumor cells have decreased upon Cx43D over-expression in comparison to control and cells down-regulating Cx43. Results from our functional assays match with a study showing that tumor cell extravasation decreased upon Cx43D over-exprerssion in MDA-MB-231 (Talhouk et al., 2013). Our immunocytochemical results are in line with a study showing that upon Cx43D over-expression, β-catenin is sequestered away from the nucleus (Talhouk et al., 2013).

In vivo experiment showed that a delay in tumor onset and volume and death time occurred upon injecting nude mice with MDA-MB-231 cells over-expressing Cx43D, compared to mice injected with control and Cx43 down-regulating cells where the above parameters occupied more aggressive settings. Lung and liver tissues have showed less cancer cell infiltration upon Cx43D over-expression. All these stated results match with a study showing that upon mutations and down-regulation of Cx43 in a breast cancer mouse model, an earlier palpable tumors and a more aggressive cancer cell infiltration to the lungs were detected (Plante et al., 2011)

When Cx43 was expressed in human glioblastomas, cell proliferation was attenuated both *in vitro* and *in vivo* (Huang et al., 1998). Moreover, it was shown that the expression of the carboxyl terminal end of Cx43 alone in Neuro2a cells, has suppressed cell growth (Moorby et al., 2001). This suggests that connexins can regulate gene transcription *via* interactions with transcription factors.

All in all, our results demonstrate that the over-expression of Cx43D decreases the metastatic potentials of a mammary adenocarcinoma tumor cell line both *in vitro* and *in vivo*. We further report, for the first time to our knowledge that the expression of Cx43D at low levels does not have a significant effect on the malignant phenotype of MDA-MB-231 cell line *in vitro.* These results would suggest that gene therapy by delivering Cx43 gene to tumor cells may have future value as part of a treatment regimen for human breast cancer.

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