ROLE OF SEMA6D IN PROLIFERATION, EPITHELIAL-MESENCHYMAL TRANSITION AND MIGRATION OF BREAST CANCER CELL LINES

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

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December 2017
İZMİR

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ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and thanks to my supervisor Assoc. Prof. Özden YALÇIN ÖZUYSAL for her patience, encouragement, understanding, guidance, support and helps during my graduate studies. Furthermore, I would like to thank to Assist. Prof. Gülistan MEŞE ÖZÇİVİCİ and Assoc. Prof. Engin ÖZÇİVİCİ for their helps. Also, I would like to thank my lab mates for their help. I would like to thank my thesis committee members Assoc. Prof. Özden YALÇIN ÖZUYSAL, Assist. Prof. Gülistan MEŞE ÖZÇİVİCİ and Assist. Prof. Yavuz OKTAY for their help guidance.

I am so grateful to my family for their support all over my life and moral during my education. Also, I would like to thank my second family Zuhal & Hakan ARSEVEN for their support and hospitality. Lastly, I would like to thank all my close friends for their help, support and social environment, although I lived far away from them, they never forget to support me.

ABSTRACT

ROLE OF SEMA6D IN PROLIFERATION, EPITHELIAL-MESENCHYMAL TRANSITION AND MIGRATION OF BREAST CANCER CELL LINES

Breast cancer is one of the most common cancer types around world and the second leading cause of cancer related deaths among women. Not the primary tumor but distant metastases are mainly the reason of deaths. For metastasis, the cells may go through epithelial-mesenchymal transition (EMT), and acquire migration and invasion abilities.

SEMA6D is a transmembrane protein that belongs to a large semaphorin family. SEMA6D is involved in the migration of embryonic cardiac cells. Recently it was validated as an oncogene in osteosarcoma. Also, its oncogenic roles were investigated in gastric cancer and mesothelioma. According to in silico analysis of the Cancer Genome Atlas (TCGA), high SEMA6D expression level is associated with better survival of triple negative breast cancer patients. However, there is not any published study which investigates roles of SEMA6D in breast cancer yet, other than bioinformatic analysis. Therefore, we aimed to understand role of SEMA6D in proliferation, EMT and migration of breast cancer cells.

We observed that overexpression of SEMA6D reduces proliferation but enhances migration in non-invasive breast cancer cell line MCF7. Thereby, SEMA6D may increase metastatic ability of MCF7 cells. Its metastatic ability was also supported by changes in EMT markers. On the other hand, proliferation of metastatic breast cancer cell line MDAMB231 was not significantly changed by overexpression of SEMA6D and migration ability was slightly reduced but mesenchymal markers tended to increase in SEMA6D overexpressing MDAMB231 cells.

As a conclusion, SEMA6D tends to enhance proliferation, migration through EMT in MCF7 cell line whereas overexpression of SEMA6D did not demonstrate significant effect on metastatic MDA-MB-231 cell line. Therefore, we should separately evaluate role of SEMA6D in different breast cancer cell lines and further studies are required to understand role of SEMA6D in breast cancer.

ÖZET

SEMA6D'NİN MEME KANSERİ HÜCRE HATLARININ ÇOĞALMA, EPİTEL-MEZENKİMAL GEÇİŞ VE GÖÇ ETMESİNDEKİ ROLÜ

Meme kanseri dünyada yaygın görülen bir kanser çeşidi olmakla birlikte meme kanseriyle alakalı ölümler metastazdan kaynaklanmaktadır. Metastaz yapabilmek için kanser hücreleri göç etme ve invazyon özellikleri kazanırlar ve hücreler bu süreçte epitel-mezenkimal geçiş gösterebilir.

SEMA6D, semaforin ailesine bağlı transmembran bir proteindir. SEMA6D embriyonik kıkırdak hücrelerinin göç etmesinde görev alır. Son zamanlarda osteosarkoma'da onkogen olarak değerlendirilmektedir. Ayrıca onkogen fonksiyonları, mide kanseri ve mezotelyoma'da da keşfedilmiştir. Biyoinformatik analize göre, SEMA6D'nin gen ifadesi üçlü negatif meme kanseri olan hastaların yaşamlarıyla ilişkilendirilmiştir fakat SEMA6D'nin meme kanseri üzerindeki görevleri ve fonksiyonları ile ilgili yayınlanmış bir çalışma bulunmamaktadır. Bu sebeple, bu tez çalışmasında SEMA6D'nin meme kanseri hücrelerinde çoğalma, epitel-mezenkimal geçiş ve göç etme özelliklerini anlamak amaç edinilmiştir.

Bu çalışmada, SEMA6D'nin aşırı ekspresyonu, invazyon yeteneği olmayan meme kanseri hücre hattı MCF7'da çoğalmayı azaltmış fakat göç etmeyi arttırmıştır. SEMA6D'nin metastatik özelliği ayrıca epitel-mezenkimal geçiş ile de desteklenmiştir. Diğer yandan, SEMA6D'nin aşırı ekspresyonu metastatik ve agresif olan MDA-MB-231 hücre hattında çoğalmayı etkilememiş fakat göç etme yeteneğini azalttığı gözlemlenmiştir.

Sonuç olarak, SEMA6D, MCF7 hücre hattında çoğalmayı, epitel-mezenkimal geçiş aracılığıyla göç etme yeteneğini arttırırken, metastatik olan MDA-MB-231 hücre hattında önemli bir değişiklik yapmamıştır. Bu yüzden, SEMA6D'nin görevlerini değerlendirirken farklı hücre hatlarında farklı etkilere yol açabileceğini göz önünde bulundurmak ve görevlerini daha detaylı anlamak için daha fazla çalışma yapmak gerekmektedir.

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CHAPTER 1

INTRODUCTION

1.1 Human Breast

Human female breast mainly consists of adipose tissue (fatty tissue), lobules and milk ducts which provide production of milk (Figure 1.1) [1]. Adipose tissue of breast contains blood vessels, lymph nodes/vessels [2]. Human male breast is almost the same as female breast, except lacking of lobules for milk production [1].

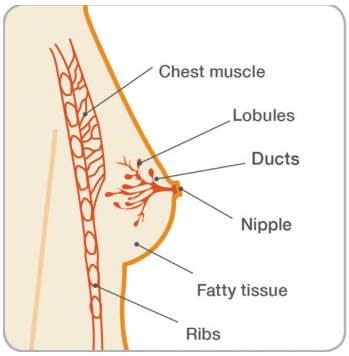

Figure 1.1 Structure of female breast including lobules, fatty tissue and milk ducts [37].

1.2 Breast Cancer

Breast cancer is highly prevalent worldwide, especially among women [3,4]. In United States, more than ten thousands of people are dying in every year due to breast cancer [3]. Breast cancers mainly originate from the lobules and ducts of breast and therefore, breast cancer ratio among men is less frequent than women [2].

Lobule Carcinoma in Situ (LCIS) is non-invasive breast cancer originated from lobules but it can gain invasive ability later and it is called invasive Lobule Carcinoma. Ductal Carcinoma in Situ (DCIS) is also type of non-invasive breast cancer originated from milk ducts. DCIS can gain invasive ability and turn into invasive Ductal Carcinoma[36]. Less frequently, stromal tissue of breast, which contains adipose tissue, blood vessels, lymph nodes, can also lead breast cancer[37]

Not primary tumors but metastasis causes most of deaths related with breast cancer [5,6]. Therefore, figuring out the mechanisms behind metastasis of breast cancer has a crucial importance.

Cancerous cells may start to spread from their primary location to other tissues or organs by gaining migratory and invasive features, this gain is called metastasis [6]. Breast cancer metastasize mainly to bone, lungs, liver and brain [7].

For metastasis cells may go through epithelial to mesenchymal transition (EMT)[8]. During EMT, immotile epithelial cells lose their polarity, cell to cell junctions and gain migratory features as in mesenchymal cells [8,9]. EMT involves in metastasis, tumor recurrence and drug resistance, therefore understanding mechanisms behind EMT is also crucial to find therapeutic solutions for cancer [9].

1.3 Molecular Subtypes and Cell Lines of Breast Cancer

There are five main molecular subtypes of breast cancer cells [10]. These are luminal A, luminal B, HER2-enriched, triple negative (basal-like) and normal-like breast cancers.

Estrogen receptor (ER) and progesterone receptor (PR) are found in Luminal A type of breast cancer, whereas HER2 receptor is not expressed in Luminal A type [10]. Luminal A type of breast cancer cells may grow slower than other subtypes thereby they can give better prognosis [10,11].

MCF7 (Michigan Cancer Foundation-7) breast cancer cell line was derived from invasive ductal carcinoma from Caucasian woman who was 69 years old. MCF7 is a luminal A type; ER and PR positive but HER2 negative cell line. MCF7 cell line has been commonly used by many breast cancer researchers [12].

Normal-like breast cancer type is also ER and PR positive and HER2 receptor negative [13]. Normal-like breast cancer have worse prognosis than luminal A type of breast cancer cells[37].

Luminal B type of breast cancer is ER and PR positive. However, absence of ERBB gene amplification, which encodes HER2 receptor, depends on high levels of Ki-67[37.] Luminal B type of cancer cells have faster growth rate than luminal A type therefore their prognosis is worse than luminal A type[37]. BT474 and ZR-75 are examples of luminal B type of breast cancer cell lines.

Triple-negative type of breast cancer does not have ER, PR and HER2 receptor [14]. Therefore, this type of breast cancer cannot be treated by neither hormone therapy nor targeting HER2 receptor [14]. MDA-MB-231, HS78T cell lines are examples of triple negative type of breast cancer cells. MDA-MB-231 cell line has highly aggressive tumor growth [15].

HER2-enriched breast cancer type is ER and PR negative but HER2 receptor is positive, oppositely to luminal A type of breast cancer[37]. Therefore, rather than hormone therapy, HER2 targeting therapy can give better results[37]. MDA-MB-453 and SKBR3 breast cancer cell lines, for instance, are HER2-enriched type. Unfortunately, this type of breast cancer usually grows faster than luminal types of breast cancers. For this reason, prognosis of HER2-enriched cancers is worse than other breast cancer types [37.]

1.4 Semaphorin Family

Semaphorin family is a part of membrane-associated and secreted proteins [16]. First semaphorin (Sema1A) was discovered in developing nervous system of grasshoppers [17]. Totally, eight classes and 28 members of semaphorins are discovered in distinct species. For instance, 20 members from class 3, 4, 5, 6 and 7 of semaphorins are found in human and mice, whereas 5 members from class 1, 2 and 5 are found in drosophila (Figure 1.4)[17].

Class 1 and 2 of semaphorins are located in invertebrates. Between Class 3-7 of semaphorin proteins are encoded in vertebrates, and Class V, which is last class, is included in virus [17]. Class 4-6 proteins of semaphorin family are transmembrane proteins and Class 3 proteins are secreted proteins [18]. According to domain arrangements, Class 6 proteins have resemblance to Class 1 whereas, Class 3 has resemblance to Class 2. However, phylogenetically Classes 6 and 3 are different from Class 1 and Class 2 [18].

Semaphorin structures are different from each other, however in their extracellular domain, about 500 amino acid sequences are conserved and this domain called sema domain [19]. Sema domain of semaphorins mediates its functions, for this reason, conservation of sema domain is crucial [20]. All semaphorins have Plexin-Semaphorin-Integrin (PSI) domain but some of them have immunoglobulin (Ig) domain, GPI-anchorage, trombospondin domains, according to functions and features (Figure 1.4) [17,18].

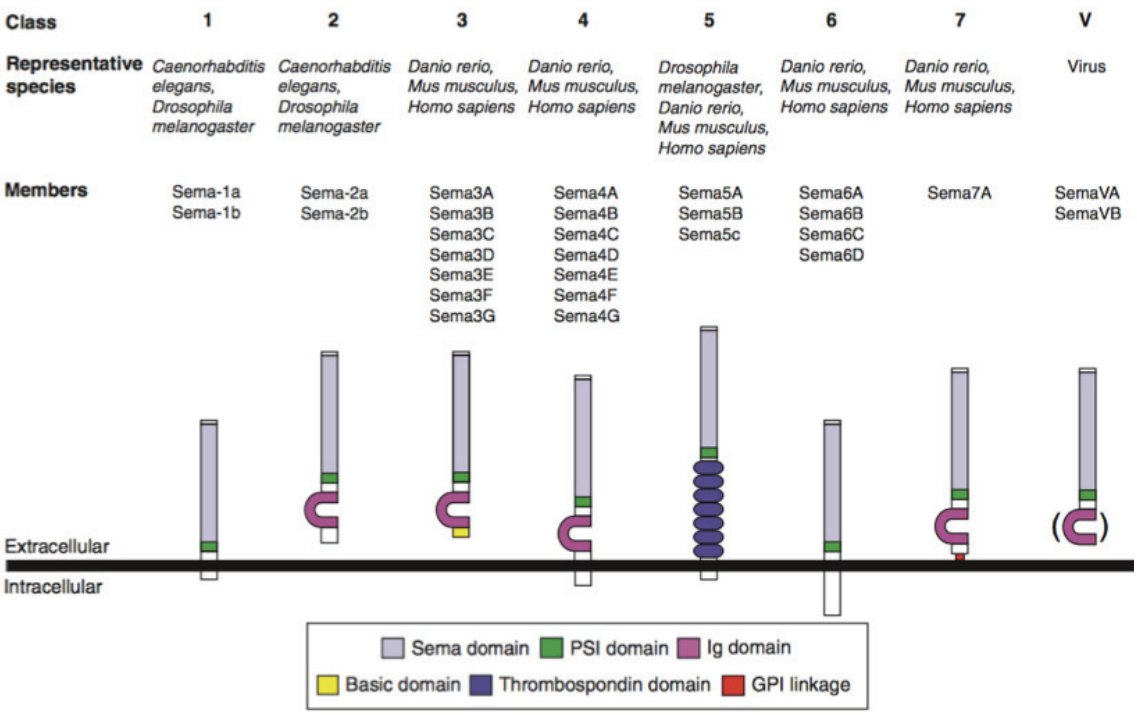

Figure 1.4 Representable figure of semaphorin family [17].

Semaphorins are expressed in many tissues and their expression levels may depend on age [17]. Most of semaphorin expressions are found in developing nervous system in both neuronal and non-neuronal cells. Besides, semaphorins are expressed in many systems, such as cardiovascular system, immune and respiratory systems [17]. Semaphorin function is known best in nervous system, especially semaphorins guide axons to find its target and semaphorins have repellent role in axon guidance [16,17].

Semaphorins have many roles in both development and adulthood in nervous system and so alterations of semaphorin expression were reported to cause serious diseases, such as Alzheimer's disease, motor neuron degeneration, epilepsy [17]. Besides, it is known that semaphorins have role in cancer via operating cell migration, angiogenesis, cell viability, vascularization, metastasis and other cancer related mechanisms [21].

Some of semaphorin proteins are immune regulatory molecules which are expressed on immune cells[36]. Especially, Sema4D protein was known as immune regulatory protein but also other semaphorins, which are Sema7A, 6D, 6B, 6A, 4F, 4G, 4B, 4A and Sema3A, are discovered as functional molecules on immune cells, they are called immune semaphorins[34].

1.5 SEMA6D

SEMA6D is a member of semaphorin family in class 6. Gene of SEMA6D encodes functional Sema6D transmembrane protein. Sema6D protein has five functional isoforms by alternative splicing in humans [18].

SEMA6D provides growth cone collapse in rat neurons. Also, their inhibition on axonal extension has been demonstrated in PC12 cells (derived from rat brain) of nerve growth factor [18]. Plexin and neuropilin are only recognized receptors of semaphorin and they bind semaphorins directly[22]. Plexin A1 (PlexA1) is a receptor of both SEMA6D and SEMA3A [16]. PlexA1 directly binds Sema6D protein and their interaction was reported to initiate osteoclast differentiation and also cardiac morphogenesis [23]. Nevertheless, role of SEMA6D has not been understood completely.

1.6 SEMA6D in Cancer

SEMA6D has important functions in physiological process and as axonal guidance molecule [23]. On the other hand, roles of SEMA6D had started to be discovered in many type of cancers.

SEMA6D and PlexA1 expressions were found high level in malignant mesothelioma cells and it was demonstrated that SEMA6D/PlexA1 complex mediates tumor cells to grow without anchorage by binding to Vascular Endothelial Growth Factor Receptor type (VEGFR-2) [24]. Similarly, according to another study, expressions of SEMA6D and PlexA1 were found high level in vascular endothelial cells. Therefore, it is speculated that SEMA6D-PlexA1complex is related with tumor angiogenesis correlatively with VEGFR-2 [25]. It is also shown that SEMA6D expression increased in gastric cancer cells compared to normal gastric cells and it has a crucial role in promotion of tumor [25].

Figure 1.6 demonstrates mRNA expressions of SEMA6D in different cancer types. According to TCGA (The Cancer Genome Atlas), glioma has the highest expression levels of SEMA6D calculated by RNA-seq data set in 17 cancer types (Figure 1.6)[38].

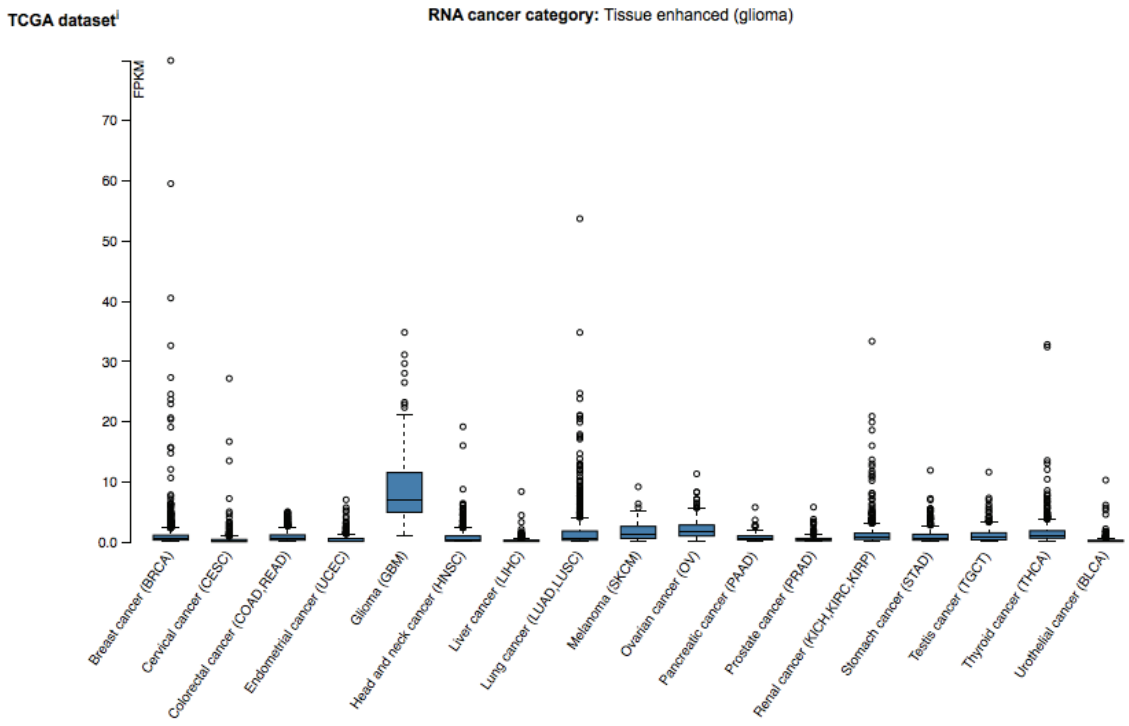

Figure 1.6 The Cancer Genome Atlas (TCGA) RNA-seq data among 17 different cancer types. FPKM is a median calculated from Fragment number Per Kilobase of exon in Million reads [38].

Expression of SEMA6D was also found significantly increased in osteosarcoma in mice and SEMA6D is functionally validated as oncogene in human osteosarcoma[26]. According to in silico analysis by TCGA dataset, SEMA6D expression in breast cancer tissue was found lower in African-American women than Caucasian-American women[27]. Chen et al. concluded that high expression of SEMA6D level linked with better survival of breast cancer patients, especially in patients who have triple negative breast cancer type[27]. On the other hand, in the same study, they divided patients' data according to expression level of SEMA6D. Then, they compared expression level of SEMA6D with some of EMT markers and other genes which have a role in tumor metastasis. According to their results, tumor promoters such as ZEB1,ZEB2 was coregulated in SEMA6D-high patient group suggesting that balance between tumor promoters and suppressors is also important to figure out role of SEMA6D during EMT [27]. Besides, the same group performed NCBI GEO data analysis and according to their results, SEMA6D expression was decreased in breast cancer tissues compared to normal tissues of patients [27].

1.7 Aim of The Project

There is not any published study which investigates SEMA6D function in breast cancer yet. Therefore, we aimed to figure out role of SEMA6D in proliferation, epithelial to mesenchymal transition (EMT) and migration of breast cancer cells.

CHAPTER 2

MATERIALS & METHODS

2.1 Cell lines & Cell culture

For this project, four different cell lines were cultured. In order to understand role of SEMA6D in breast cancer, MCF7 and MDA-MB-231, which are human breast cancer cell lines were chosen. 293T, human embryonic kidney cell line for virus production and NIH3T3, mouse embryonic fibroblast cell line for virus titration, were cultured.

All cell lines were cultured at 37^0C, 5% CO_2 in incubator with containing particular mediums for each of them. High glucose DMEM medium (Gibco, Cat# 41966-029) containing L-Glutamine, 10% Fetal Bovine Serum (FBS) (Biological Industries (BI), Cat# 04-007-1A), 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B) was used for MDA-MB-231, MCF7 and 293T cell lines. Besides, high glucose DMEM medium (Gibco, Cat# 41966-029) containing L-Glutamine, 10% New Born Calf Serum (NBCS) (BI, Cat# 04-102-1A), 1% Penicillin/Streptomycin (BI, Cat# 03-031-1B) was used for NIH3T3 cell line.

After infection (explained in Section 2.4), MCF7 and MDA-MB-231 cell lines were cultured with 5µg/ml Blasticidin antibiotic (Santa Cruz, Cat#G3115) to obtain stable cell lines.

2.2 Plasmids & Virus Production

For transfection of cells and virus production, pLX304-LacZ (Addgene, plasmid #42560) as control or pLX304-SEMA6D (Dharmacon, plasmid # OHS6085-213576758) lenti-viral expression vectors with packaging vectors psPAX2 (Addgene, plasmid # 12260) and pMD2.G (Addgene, plasmid # 12259) were used. Both packaging plasmids were a gift from Didier Trono. psPAX2 is a package vector containing pol and gag viral structural genes, which express reverse transcriptase and capsid protein, respectively. pMD2.G vector contains viral coating protein and so it has a role as an envelope vector in packaging.

pLX304 backbone for LacZ and SEMA6D genes has CMV promoter, HIV-1 5' LTR and 3' LTR and resistant gene for Blasticidin. pLX304-SEMA6D plasmid has C-terminal V5 epitope of SEMA6D (Figure 2.2).

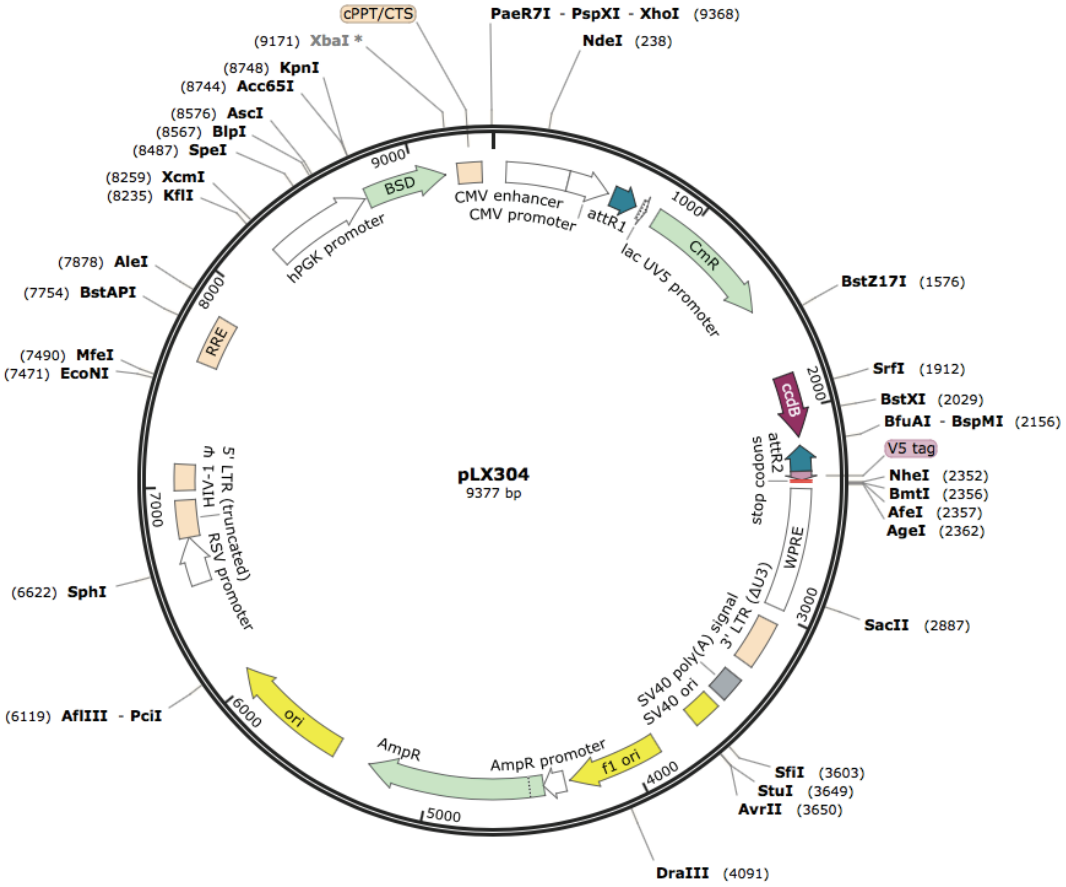

Figure 2.2 Plx304 plasmid map

For transfection, $3.5x10^6$ 293T cells were cultured onto 10cm cell culture plates, 24 hours before transfection. On transfection day, 0.7 µg from pMD2.G, 1.3 µg from psPAX2 and 2 µg from lenti-viral expression vector pLX304-LacZ or pLX304-SEMA6D were gently mixed with 12 µL of transfection reagent Fugene HD (Promega, Cat# E2311) in 500 µL serum-free DMEM medium (Gibco, Cat# 41966-029) to provide 1:3 plasmids and transfection reagent ratios. Mixture was left for 30 min at room temperature and was added drop by drop to 293T cell plates. After 24-hour incubation of 293T cells, medium of plates was replenished with fresh one. Since 48 hours and 72 hours after transfection are suitable for viral RNA to be packaged into virus, medium including viruses was collected from plates in 48 and 72 hours, then they were aliquoted and stored at -80^0C to keep ready for titration or infection.

2.3 Virus Titration

In order to measure efficiency of viruses, titration was performed with NIH3T3 cell line. 150.000 NIH3T3 cells were cultured into 6-well plate. 24 hours later, 10^{-3}, 10^{-4}, 10^{-5} serial dilutions were obtained from virus medium and Polybrene (Sigma, Cat# 107689) in 8 µg/ml final concentration was added to serial diluted medium. Then, serial diluted medium was replaced with old medium of NIH3T3 6-well plate and centrifuged at 2500 rpm, 32^0C for 2 hours. After centrifugation, virus medium was replaced with fresh medium and 6-well plate was incubated in incubator. 48 hours after infection, NIH3T3 cells in 6-well plate were transferred to 10 cm plates and 5 µg/ml Blasticidin antibiotics Santa Cruz, Cat#G3115) were added into their medium. Changing medium including antibiotic was continued until non-transfected NIH3T3 cells (Mock) were all dead. Then, plates were washed with 1X PBS and 0.5% Crystal Violet staining were performed for 10 minutes. After staining, washing with 1X PBS were maintained until mock plate was seen as unstained. Lastly, stained colonies were counted and compared to see efficiency of viruses. Almost equal efficiencies of viruses were used for infection of breast cancer cell lines.

2.4 Infection of Breast Cancer Cell Lines

250.000 cells of MCF7 cells and 300.000 MDA-MB-231 cells were cultured into 6-well plates, separately. LacZ (control) expressing viral medium and SEMA6D expressing viral medium were replaced with old medium and centrifuged at 2500 rpm, 32^0C for 2 hours. After centrifugation, virus medium was replaced with fresh medium and 6-well plate was incubated in incubator. 48 hours after infection, MCF7 or MDA-MB-231 cells from 6-well plates were transferred to 6 cm plates. One day later, to obtain stable cell lines by antibiotic selection, medium was replenished with fresh medium containing 5 µg/ml Blasticidin antibiotics Santa Cruz, Cat#G3115) for infected and non-infected (Mock) MCF7 cells or 8 µg/ml Blasticidin antibiotics Santa Cruz, Cat#G3115) for infected and non-infected (Mock) MDA-MB-231 cells. Passaging cells and changing with fresh medium including antibiotic was continued until non-infected cells (Mock) died. After obtaining stable cell lines, flash freezing of plates was performed for RNA and protein isolations from each cell lines by putting plates on liquid nitrogen.

Four different stable clones after four different infections of MCF7 cells and three different stable clones after three different infections of MDA-MB-231 cells were generated to obtain biological repeats.

2.5 BrdU Cell Proliferation Assay of MCF7 Cell Line

In order to understand proliferation capacity of breast cancer cell lines, Bromodeoxyuridine (BrdU) assay was performed. 100.000 cells were cultured in 8-well culture chamber (8-well on PCA detachable) (Sarstedt, Cat# 94.6140.802). After 24 hours, MCF7 or MDAMB231 cells were washed with 1X PBS (from 10X stock, BI, Cat#02-024-5A) and mixture of 20 µM BrdU (BD Pharmingen, Cat# 51-9000019BK) in serum-free medium was added to 8-well culture chamber and incubated for 2 hours at 37^0C. After incubation with BrdU, plate was washed twice with 1X PBS. 4% Paraformaldehyde (PFA) was added and incubated for 45 minutes at room temperature to fix cells. After fixation and washing step, cells were treated with 1.5M HCl solution for 30 minutes and washed again. In order to prevent unspecific binding, 5% Normal Horse Serum (NHS) solution including 0.2% Triton X-100 (Sigma-Aldrich, Cat# T8787), NHS (BI, Cat# 04-004-1A) and 1X PBS, was added in 8-well culture chamber and incubated for 1 hour. After blocking, APC anti-BrdU monoclonal antibody (BU20A) (eBioscience, Cat# 17-5071-41) was prepared in 2% NHS solution including NHS, 1% Triton X-100 (Sigma-Aldrich, Cat# T8787) and 1X PBS with 1:50 dilution. Anti-BrdU antibody was incubated overnight at room temperature. After antibody treatment, plate was washed with 1X PBS and DAPI (Sigma Aldrich, Cat# D95242) solution was prepared in 1:1000 dilution with same solution as anti-BrdU antibody and incubated for 30 minutes. After DAPI staining, underside of 8-well culture chamber was separated from its upper part and underside part was coated with mounting oil and cover glass. Then, slides were visualized by fluorescence microscope (Leica SP8) in 20X magnification and analysed with ImageJ software by particle analysis. Each analysis was performed by counting cells with DAPI staining and with anti-BrdU antibody staining for same image position. At least three images and at least 750 cells were counted and analysed for every single cell line by image J particle analysis.

In order to test Mitomycin C efficiency for inhibiting proliferation, BrdU assay was also performed at 6, 24 and 48 hours' time periods. First of all, $2x10^6$ MCF7 cells were cultured on cover glass in 6-well plate. After one day 6-wells were washed gently with 1X PBS. Then, 10 µg/ml Mitomycin C (SCBT, Cat# sc-3514) in serum-free medium was added into all 6-well plate and incubated for 2 hours at 37^0C. After incubation, Mitomycin C medium was removed and starvation medium was added to all plate. 4, 22 and 46 hours after Mitomycin C addition, cells were incubated with 20 µM of BrdU for two hours to complete 6, 24 and 48 hours' time periods. At the end of the BrdU incubation, immunostaining (anti-BrdU and DAPI staining) was performed and glass slides were mounted. After completing BrdU assay for all time periods, slides were visualized in 20X magnification by fluorescence microscope (Leica SP8). Each image was taken from same position with DAPI staining and anti-BrdU antibody staining, respectively.

2.6 MTT Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to measure cell viability and indirectly proliferation of cells.

2000 cells of MCF7 or MDA-MB-231 cell lines were cultured into 12-well plates. One day later, MTT (Amnesco, Cat# 0793) was diluted 1:10 with normal DMEM medium (Gibco, Cat# 41966-029). Old medium of cells was discarded only for wells of specific day and MTT-medium mixture was added to those wells and incubated at 37^0C for 3-4 hours. After 3-4 hours, MTT-medium mixture was removed and DMSO (Sigma Aldrich, Cat# CAS 67-68-5) was added to solubilize formazan crystals. Wells were turned to purple-like colour in presence of formazan and optical density (OD) of wells was measured in 570nm and 630nm by spectrophotometer. DMSO was used for a blank. On each 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 7^{th}, 8^{th} day, three wells were treated with MTT and measured for each infection and each cell line.

2.7 Semi-quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

In order to perform qRT-PCR, RNA isolations by PureLink RNA mini kit (ambion, Cat# 12183-018A) and PureLink DNase (Thermo Scientific, Cat# 12185-010) to remove any contamination of DNA were used. Also, cDNAs were obtained from 2µg of isolated RNA by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Cat# K1621) with following manufacturer's instructions.

cDNAs were amplified by qRT-PCR machine (Roche, Light Cycler® 96) with particular forward and reverse primers (shown in Table 1) and SYBR Green master mix (Thermo Scientific, Cat# KO252). SYBR Green master mix contains Taq Polymerase, SYBR Green I dye, buffer and dNTPs. ZO-1, E-Cadherin were analysed as epithelial markers; and Zeb-1, Zeb-2, Vimentin, Twist, Snai1, Snai2 (Slug), N-Cadherin were analysed as mesenchymal markers. TBP is used as housekeeping gene control. By using delta-delta Ct method, mRNA levels were analysed. Two tailed unpaired student's t-test were used to calculate statistical significance.

Table 2.7 qRT-PCR primers and sequences

TBP_Forward	TAGAAGGCCTTGTGCTCACC
TBP_Reverse	TCTGCTCTGACTTTAGCACCTG
SEMA6D_For	TTTCCCAGTTGAGGGCAGTC
SEMA6D_Rev	AGGGCGTCCTCTAAAAACCG
ZEB1_For	CCCAGGTGTAAGCGCAGAAA
ZEB1_Rev	GTCTGGTCTGTTGGCAGGTC
ZEB2_For	ATAAGGGAGGGTGGAGTGGAA
ZEB2_Rev	GTTAATTGCGGTCTGGATCGTG
Vimentin_For	GCTAACCAACGACAAAGCCC
Vimentin_Rev	CGTTCAAGGTCAAGACGTGC
SNAI1_For	CTAGGCCCTGGCTGCTACAA
SNAI1_Rev	TGTGGAGCAGGGACATTCG
SNAI2_For	CTCCTCATCTTTGGGGCGAG
SNAI2_Rev	TTCAATGGCATGGGGGTCTG
TWIST1_For	CTGTCCATTTTCTCCTTCTCTGG
TWIST1_Rev	TTCTCGGTCTGGAGGATGGA
E-cadherin_For	CAGCACGTACACAGCCCTAA
E-cadherin_Rev	GGTATGGGGGCGTTGTCATT
ZO1_For	ATGGAGGAAACAGCTATATGGGA
ZO1_Rev	CCAAATCCAAATCCAGGAGCC
N-Cadherin_For	GGCTTCTGGTGAAATCGCAT
N-Cadherin_Rev	GCAGGCTCACTGCTCTCATA

2.8 Western Blotting

After flash freezing of cells, proteins were isolated and concentrations were measured by Bradford assay. 60 µg of proteins were used for each experiments. Proteins with 5X Loading dye (5% β-mercaptoethanol, 10% SDS, 250 mM Tris-HCI, 30% Glycerol and 0,02% Bromophenol Blue) were incubated at 95^0C for 10 minutes and loaded 20 µl into SDS-polyacrylamide gel (5% Stacking Gel, 10% Resolving Gel). Loaded proteins were run between 18-23 mA for 2-3 hours until protein marker (New England Biolabs, Cat# P7712S) opened clearly. After running of gels, polyvinyldene difluoride (PVDF) membranes were used for transferring proteins from gel to membrane. To do this, PVDF membranes were activated by methanol then transfer of proteins were performed at 60V, 4^0C for 3 hours. After transferring of proteins to membrane, membranes were blocked with 5% milk powder in Tris-Buffered Saline with Tween 20 (TBS-T) for 2 hours at room temperature. After blocking, primary antibody treatments were performed in different conditions (Table 2.8). After primary antibody treatments, membranes were washed with TBS-T in 3 x 15 minutes. 1:2000 dilution of goat conjugated anti-rabbit monoclonal antibody (Dako, Cat# P0449) was used for 1 hour at room temperature for all primary antibodies. Luminata Forte Western Blot HRP substrate (Merck Millipore, Cat# WBLUF0100) was used for visualizing protein expression. Beta-actin primary antibody was used for housekeeping protein expression and binding images of proteins were analysed according to beta-actin expressions.

Table 2.8 Antibodies with dilution ratios and treatment conditions (Overnight; ON, Room temperature; RT, Hours; h)

Antibodies	Catalog #	Dilution	Condition
SEMA6D	Abcam, ab191169	1:250	ON, 4^0C
V5	Cell Signalling, 13202	1:1000	2h, RT
Beta-actin	Abcam, ab5694	1:6000	2h, RT
Slug	Cell Signalling, 9585P	1:1000	ON, 4^0C
Sma	Cell Signalling, 14968	1:250	ON, 4^0C
Twist	Calbiochem, DR108	1:1000	ON, 4^0C
E-Cadherin	Cell Signalling, 3195P	1:1000	ON, 4^0C
N-Cadherin	Cell Signalling, 13116P	1:1000	ON, 4^0C
ZO-1	Cell Signalling, 8193P	1:1000	ON, 4^0C
Zeb-1	Cell Signalling, 3396P	1:1000	ON, 4^0C
Zeb-2	Merck, ABT332	1:1000	ON, 4^0C
Vimentin	Cell Signalling, 5741P	1:1000	ON, 4^0C

3.9 Wound-Healing Assay

Migration ability of MCF7 and MDA-MB-231 cells were tested by Wound-Healing assay. $2x10^6$ MCF7 cells (LacZ and SEMA6D infected) were cultured into 6-well plate or 500.000 MCF7 cells were cultured into 12-well plate. MDA-MB-231 cell line were only performed in 12-well plates with culturing 400.000 cells of each well. After one day from culturing, cells were covering surface of plate. Plates were washed gently with 1X PBS once then, 10ug/ml Mitomycin C (SCBT, Cat# sc-3514) in serum-free medium was added into 6-well plate or 12-well plate and incubated at 37^0C for 2 hours. After 2-hour incubation, well plates were scratched with sterile 10ul pipette tips from the middle of wells and washed with 1X PBS gently. Then, starvation medium (DMEM medium with 1% FBS, 1% P/S in presence of 5% CO_2 or L15 medium (Gibco, Cat# 21083-027) with 1% FBS, 1% P/S in absence of 5% CO_2) was added and time-lapse microscopy had started to take photos in every 1 hour. Closing areas in particular time periods, which are zero, 6^{th}, 12^{th}, 18^{th}, 24^{th}, 36^{th} and 48^{th} hours, were calculated by ImageJ-Macro MRI plugin software. Area normalizations were calculated according to area of time zero from each image. Then, relative proportions were computed and bar graph were drawn with paired t-test analysis. Calculations of every single well had at least three different positions. Single MCF7 cells were counted in closing area on ImageJ software and dot plot was drawn to show migrating cells.

CHAPTER 3

RESULTS

3.1 Generation of SEMA6D Overexpressing Cell Lines

To understand role of SEMA6D on breast cancer cells, tumorigenic but non-invasive cell line MCF7 and highly tumorigenic and invasive cell line MDA-MB-231 were used. SEMA6D was overexpressed by viral infection and LacZ is used as control on all MCF7 and MDA-MB-231 cell lines. After infection, stable cell lines were generated by antibiotic selection. Then, expression of SEMA6D mRNA and protein was confirmed by qRT-PCR (Figure 3.1.1 A and Figure 3.1.2 A) and Western Blotting (Figure 3.1.1 B and Figure 3.1.2 B), respectively. According to qRT-PCR result, SEMA6D expression is significantly increased by 1450 folds on SEMA6D overexpressed MCF7 cells compared to control group (Figure 3.1.1 A). Besides, Western Blot image confirms that SEMA6D expression level is higher in SEMA6D overexpressed MCF7 cells than a control group (Figure 3.1.1 B).

A

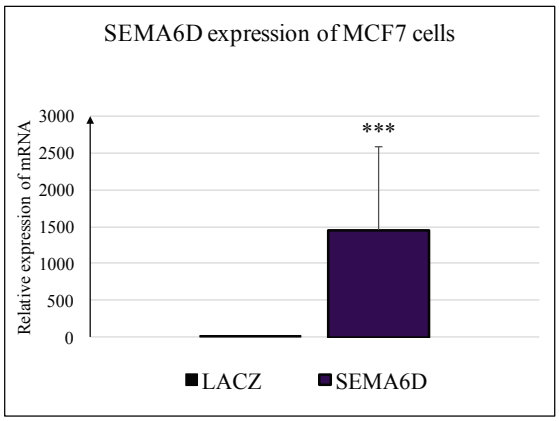

Figure 3.1.1 A) qRT-PCR quantification of MCF7 cells. Six independent experiments and four biological repeats were performed. (***$p<0.0005$) B) Western Blotting images of MCF7 cells with SEMA6D antibody. SEMA6D protein is expected in 120kDa and red arrow indicates SEMA6D protein. Labels represent short description of LACZ as L and SEMA6D as S; Numbers after labels represents biological repeats of cell lines by infection; e.g. LACZ infection #1 as labelled L1. C) Quantification of Figure 3.1.1 B.

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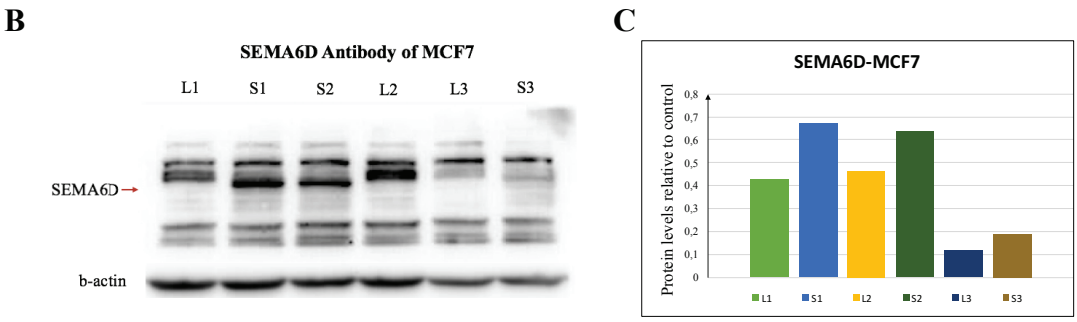

Figure 3.1.1 (Cont.)

According to qRT-PCR data, expression of SEMA6D is significantly 702 folds higher in SEMA6D overexpressed MDA-MB-231 cells (Figure 3.1.2 A). Also, Western blot image demonstrates that SEMA6D expression on protein level of SEMA6D-overexpressed MDA-MB-231 cells is higher than LacZ control group. (Figure 3.1.2 B).

Overall, LacZ expressing and SEMA6D expressing stable cell lines were successfully generated from MCF7 and MDA-MB-231 cells.

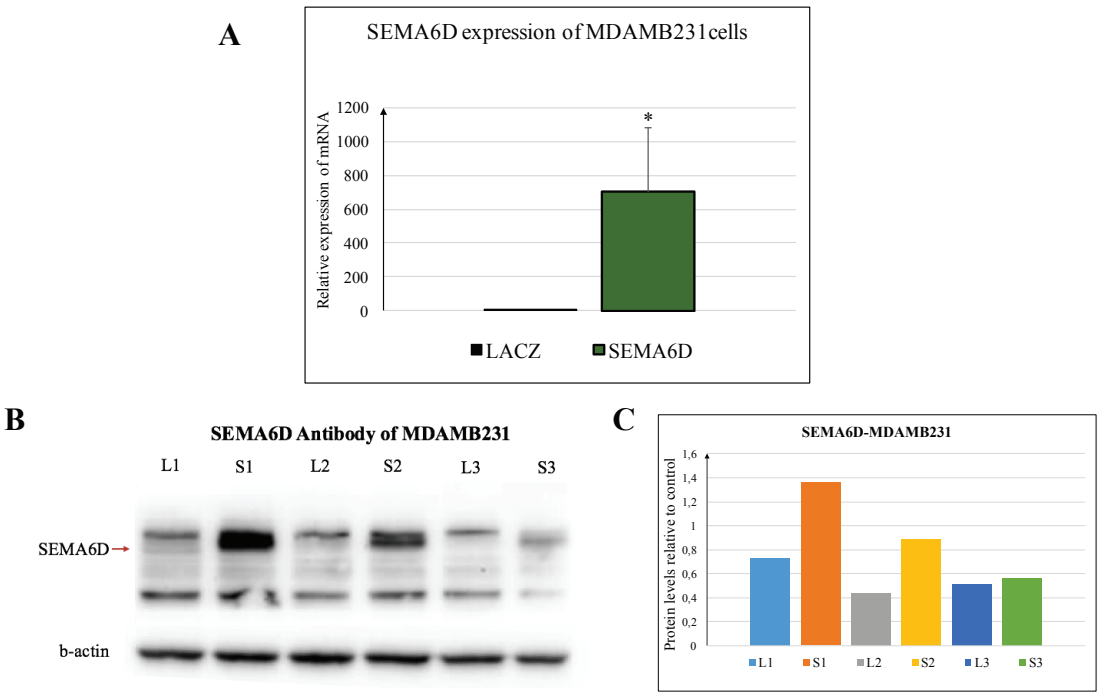

Figure 3.1.2 A) qRT-PCR quantification of MDAMB231 cells. two independent experiments and three biological repeats were performed. (***p<0.0005) B) Western Blotting images of MDAMB231 cells with SEMA6D antibody. SEMA6D protein is expected in 120kDa and red arrow indicates SEMA6D protein. Labels represent short description of LACZ as L and SEMA6D as S; Numbers after labels represents biological repeats of cell lines by infection; e.g. LACZ infection #1 as labelled L1. C) Quantification of Figure 3.1.2 B.

3.2 Effects of SEMA6D on Proliferation of Breast Cancer Cell Lines

In order to figure out the effects of SEMA6D overexpression on proliferation capacity of MCF7 and MDA-MB-231 cells, MTT Assay and BrdU Assay were performed (Figure 3.2.1 and Figure 3.2.2).

3.2.1 Proliferation Analysis by MTT Assay

MTT assay demonstrates cell viability and indirect proliferation via reducing metabolic dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals[28].

Overexpression of SEMA6D on MCF7 cell line shows a significant decrease in proliferation by MTT assay compared to control group (Figure 3.2.1 A). However, overexpression of SEMA6D on MDA-MB-231 cells does not demonstrate a significant change in MTT assay (Figure 3.2.1 B).

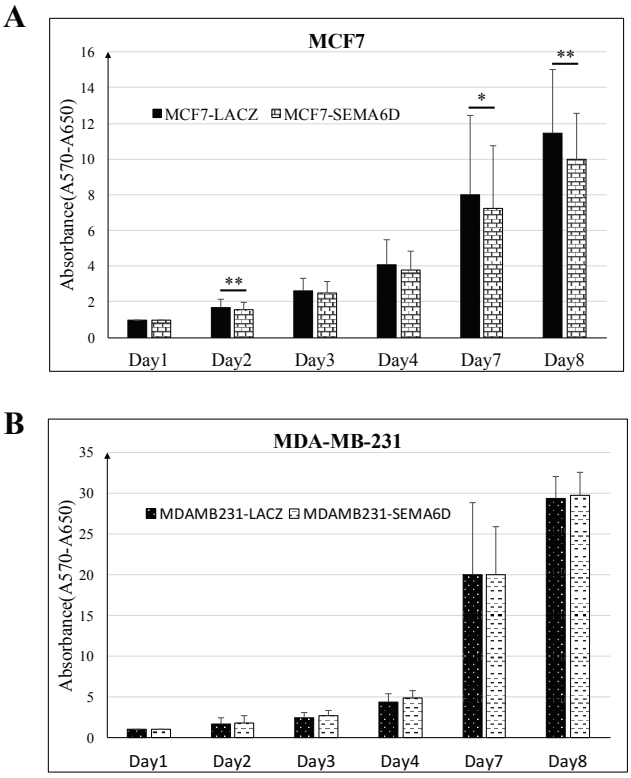

Figure 3. 2.1 MTT Assay, normalized cell viability values for 8 days A) with MCF7 stable cell line, eight independent experiments, four biological repeats, B) with MDA-MB-231 stable cell line, three independent experiments, three biological repeats. (* $p<0.05$, ** $p<0.005$)

3.2.2 Proliferation Analysis by BrdU Assay

Bromodeoxyuridine (BrdU) Assay demonstrates direct cell proliferation via BrdU, which acting as a thymidine analogue and incorporating into new DNA during cell replication[29].

Figure 3.2.2 A and B show examples of microscope images of respectively MCF7 and MDAMB31 cells stained with anti-BrdU monoclonal antibody (red) and DAPI (blue). There is a significant decrease in proliferation on SEMA6D-overexpressed MCF7 cells, consistent with MTT assay results (Figure 3.2.2 C). However, although there is a trend to decrease in proliferation of SEMA6D overexpressed MDAMB232 cells, statistical analysis does not demonstrate any significances (Figure 3.2.2 D).

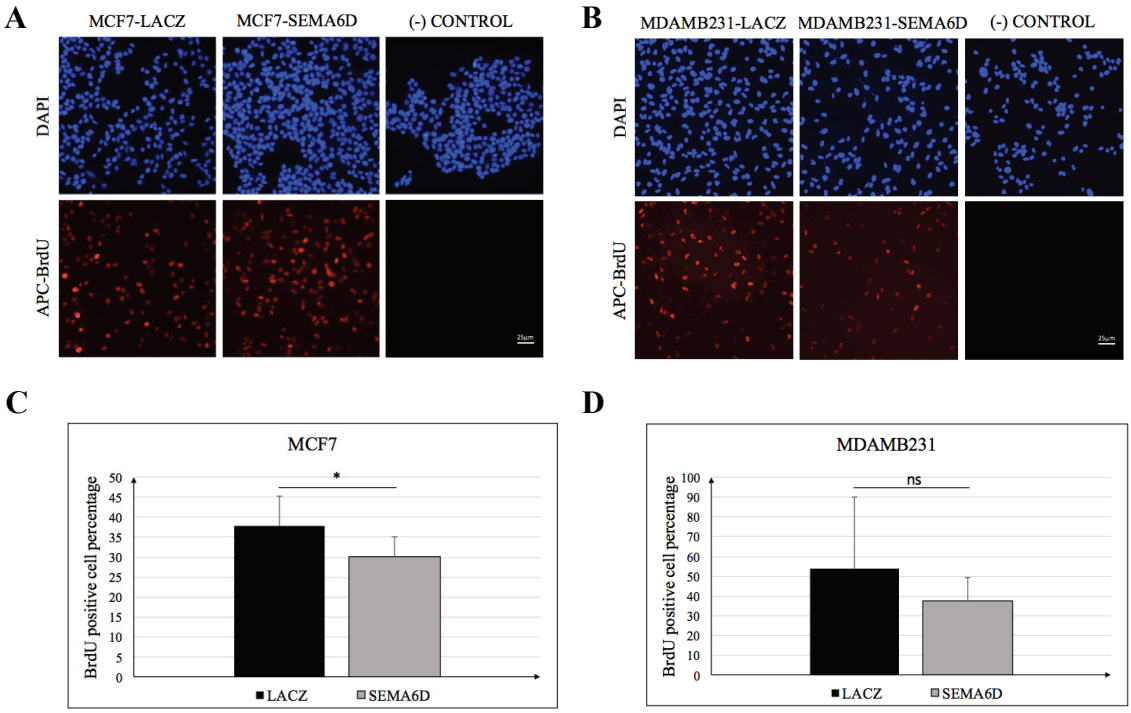

Figure 3.2.2. Cell nuclei counter-DAPI staining (Blue) and Anti-BrdU monoclonal antibody staining (Red) A) of MCF7 cells, B) MDAMB231 cells. Quantification of BrdU assay of C) MCF7 cells, D) MDAMB231 cells. All images show same region with 20X magnification, scale bar: 25µm under Fluorescence Microscope. Negative Control was performed without BrdU treatment LACZ shows control group and SEMA6D shows SEMA6D-overexpressed MCF7 cells. (ns: not significant, * $p<0.05$)

3.3 Effect of SEMA6D Overexpression on EMT of Breast Cancer Cell Lines

To test overexpression of SEMA6D in both mRNA and protein level of MCF7 and MDA-MB-231 cells, EMT markers were used. ZO-1 and E-Cadherin were used for epithelial markers in both qRT-PCR and Western Blotting. N-Cadherin, Twist, Vimentin, Snai1, Snai2 (Slug), Zeb-1 and Zeb-2 was used as mesenchymal markers in qRT-PCR and Western Blotting.

Additionally, Sma (Smooth Muscle Actin) protein and V5 tag were used in Western Blotting. Sma is mainly used for a marker of myofibroblast formation which shows mesenchymal characterization. Plx304-LacZ and plx304-SEMA6D plasmids have C-terminal V5 epitope in their sequences and V5 antibody shows exogenous expression of plasmids to confirm that plasmids produce SEMA6D instead of basal levels (Figure 3.2.1).

3.3.1 Effects of SEMA6D Overexpression on EMT of MCF7 Cells

EMT markers were tested for MCF7 cells. ZO-1 and E-Cadherin primers were used as epithelial marker whereas Snai1, Snai2 (Slug), Vimentin, N-Cadherin, Zeb-1, Zeb-2 and Twist were used as mesenchymal markers in both qRT-PCR and Western Blotting experiments. Additionally, V5 tag antibody was used for Western Blotting and it is confirmed MCF7 cells have SEMA6D protein from viral infection (Figure 3.3.1.1).

Figure 3.3.1.1 and Figure 3.1.1.2 represents all Western Blot results of MCF7 cells. According to Western Blot analysis, C-terminal epitope V5 tag antibody had more intense band in control group (LacZ) and this intensity had 40% significant decrease in SEMA6D overexpressed cells (Figure 3.3.1.1). Also, even though there were a decreasing trend in both Twist and ZEB2 proteins, they do not have significant change in protein levels (Figure 3.3.1.1). Vimentin and ZEB1 antibodies did not give substantial results for MCF7 cell line.

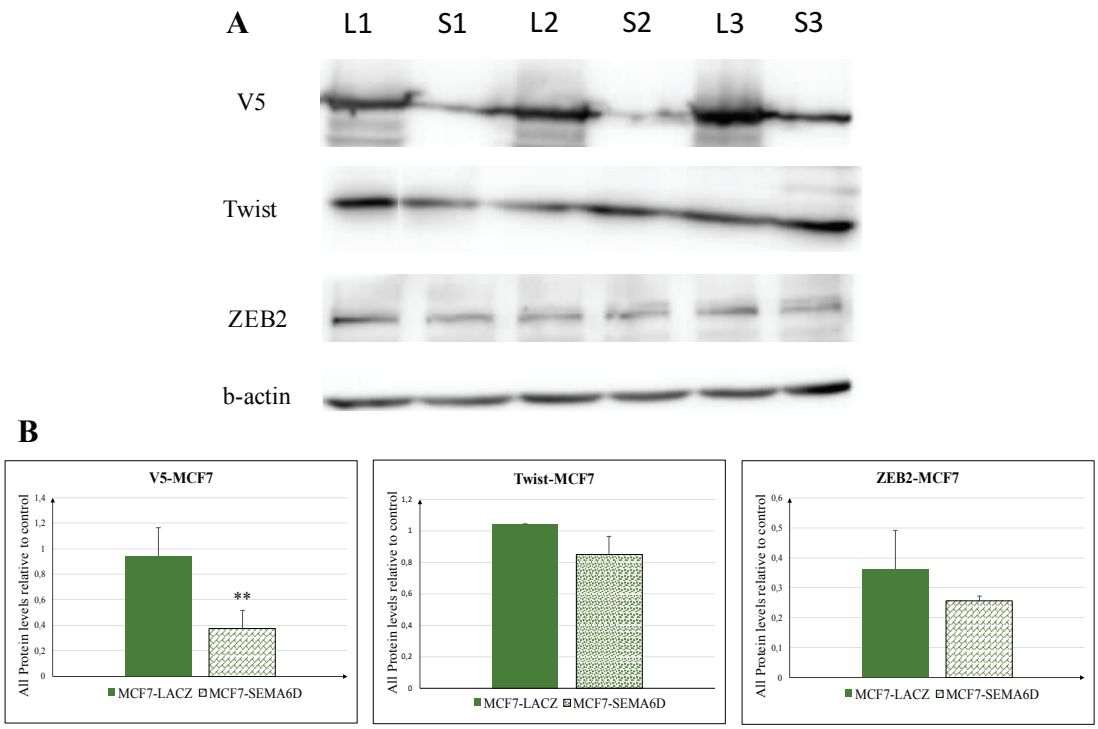

Figure 3.3.1.1 A)Western Blot images of MCF7 cells with three different stable clones B) Quantification of three stable clones: V5 tag antibody, 120 kDa; ZEB2, 220kDa; Twist, 25kDa. Labels represent short description of LACZ as L and SEMA6D as S; Numerical letter after labels represents biological repeats of cell lines by infection; e.g. LACZ infection #1 as labelled L1, SEMA6D infection #3 as labelled S3.

According to four different stable clones; Sma (Smoot Muscle Action) and also E-Cadherin that is epithelial marker, did not have significant change in SEMA6D overexpressed MCF7 cells whereas, mesenchymal markers Slug, and N-Cadherin had significantly increased by 1.8 fold and 2 fold, respectively (Figure 3.3.1.2). According to one Western Blot experiments with four stable clones, epithelial marker ZO-1 had tended to decrease in SEMA6D-overexpressed cells, especially in the clone 3 but still its statistical analysis did not reflect significant results between control group and SEMA6D-overexpressed MCF7 cells (Figure 3.3.1.2).

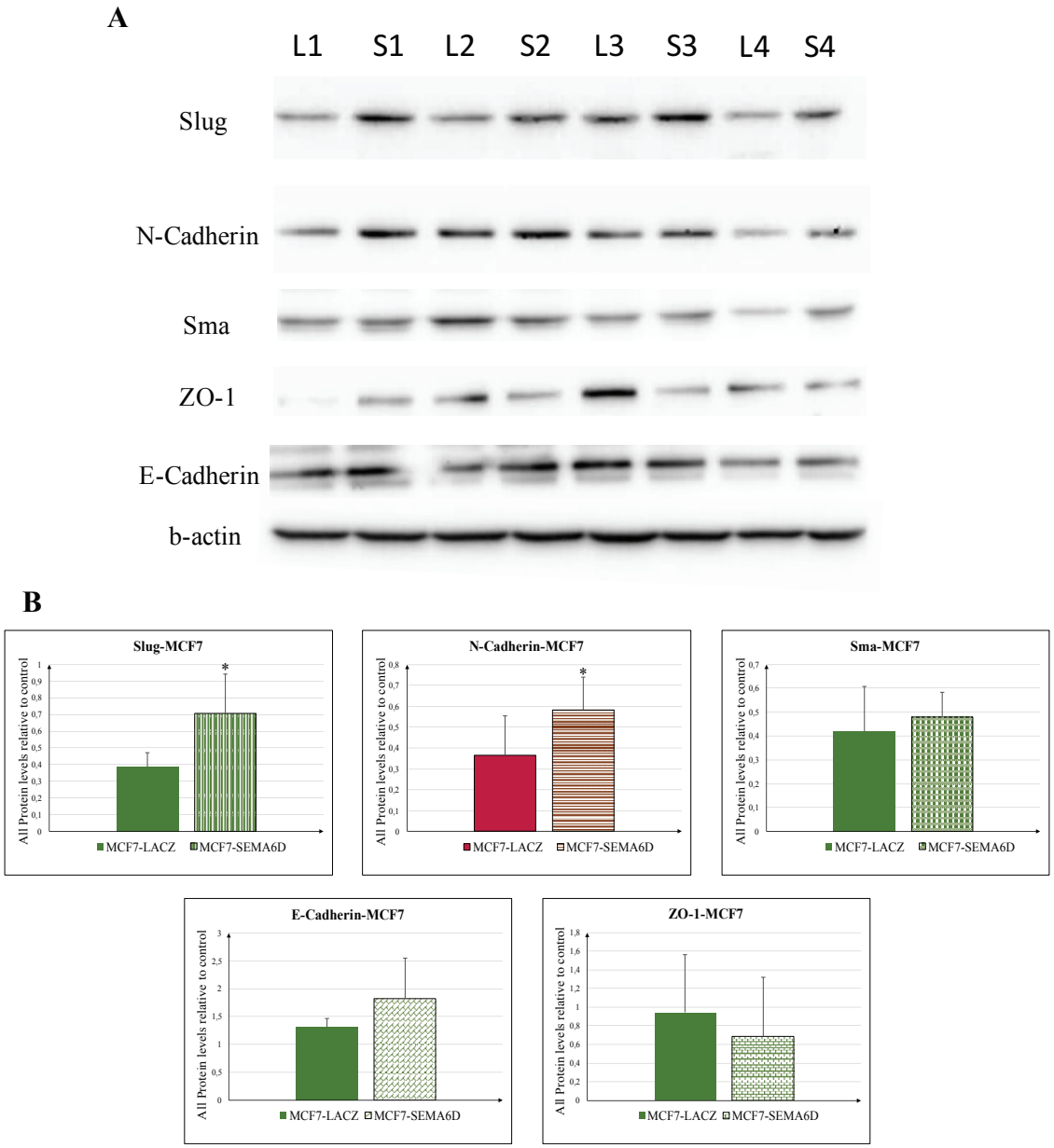

Figure 3.3.1.2 A) Western Blot images of MCF7 cells with four different stable clones B) Quantification of four stable clones: N-Cadherin, 140 kDa; Slug (Snai2), 30 kDa; Sma, 43 kDa; ZO-1 220kDa; E-cadherin, 135 kDa. Labels represent short description of LACZ as L and SEMA6D as S; Numerical letter after labels represents biological repeats of cell lines by infection; e.g. LACZ infection #1 as labelled L1, SEMA6D infection #3 as labelled S3. (* $p<0.05$)

According to qRT-PCR, MCF7 stable cell lines did not have statistically significant results in their epithelial markers; ZO-1 and E-cadherin, according to at least 3 independent experiments and 4 different biological repeats (Figure 3.3.1.2 A, B). However, mRNA expression levels of the most of mesenchymal markers, including

Snai1, Snai2, Vimentin and N-Cadherin significantly decreased (Figure 3.3.1.2 C-F). In contrast to to Western blot analysis, Snai1 and Snai2(Slug) had significant 20% and 40% decreases, respectively (Figure 3.3.1.2 C and D). Vimentin and N-Cadherin have significantly decreased by almost 20% of both mesenchymal markers (Figure 3.3.1.3 E and F). Other mesenchymal markers; Zeb1 and Twist do not have significant change in between control group and SEMA6D overexpressed cells (Figure 3.3.1.3 G and H).

Overall, mRNA levels of mesenchymal markers Snai2, N-Cadherin and Vimentin were significantly decreased in qRT-PCR in opposite to protein level of these markers.

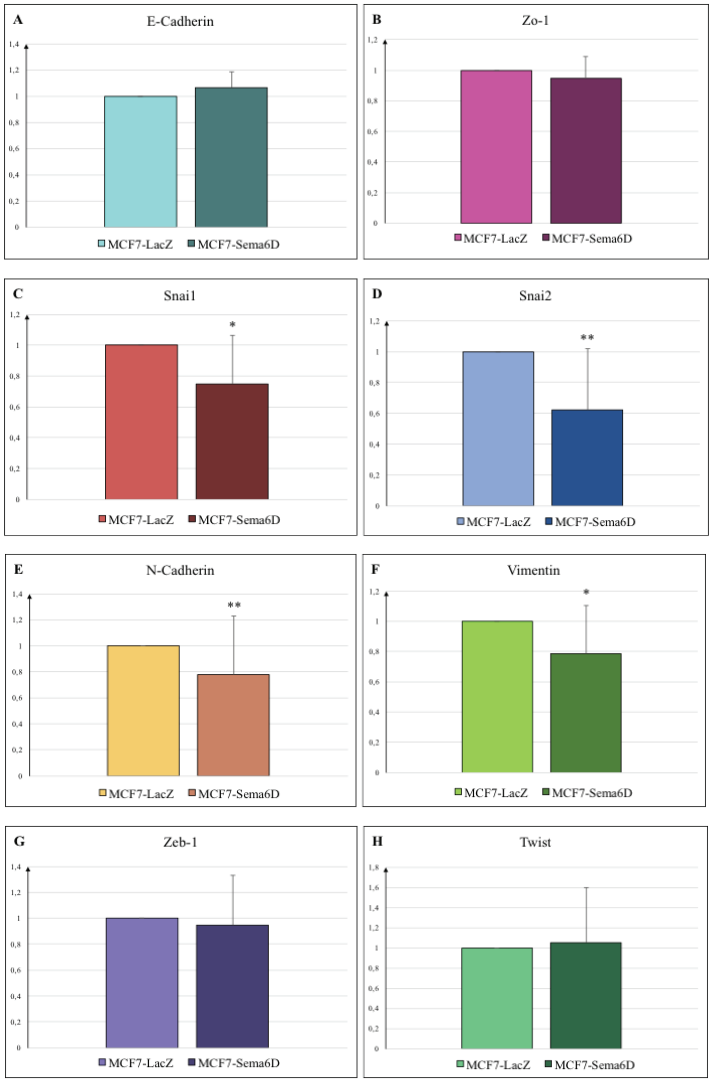

Figure 3.3.1.3 qRT-PCR quantifications of MCF7 cell lines. MCF7-LacZ is control group and MCF7-SEMA6D is SEMA6D overexpressed MCF7 cells. A) E-Cadherin, B) ZO-1, C) Snai1, D) Snai2 (Slug), E) N-Cadherin, F) Vimentin, G) Zeb-1, H) Twist. At least six independent experiments and four biological repeats were performed. (* $p<0.05$, ** $p<0.005$, *** $p< 0.0005$)

3.3.2 Effects of SEMA6D Overexpression on EMT of MDA-MB-231 Cells

EMT markers were also tested for MDA-MB-231 cells by Western Blotting and qRT-PCR. ZO-1 and E-cadherin were used as epithelial markers, whereas Sma, Twist, Vimentin, Zeb1, Zeb2, Snai1, Snai2(Slug), N-Cadherin were used as mesenchymal markers.

According to Western Blot results, MDA-MB-231 cell lines do not have significant chance in their protein level of SEMA6D overexpression. E-Cadherin decrease 40% in SEMA6D overexpressed MDA-MB-231 cells but this data was not statistically significant (Figure 3.3.2.1 B). As it is in MCF7 Western Blot results, Snai2(Slug) increased by 1.8-fold in SEMA6D overexpressed cells, but this data does not reflect a significance in MDA-MB-231 cells (Figure 3.3.2.1 C). Other markers; N-Cadherin, Vimentin, Sma and ZO-1 do not demonstrate significant results according to unpaired t-test results (Figure 3.3.2.1 A, B, D, E).

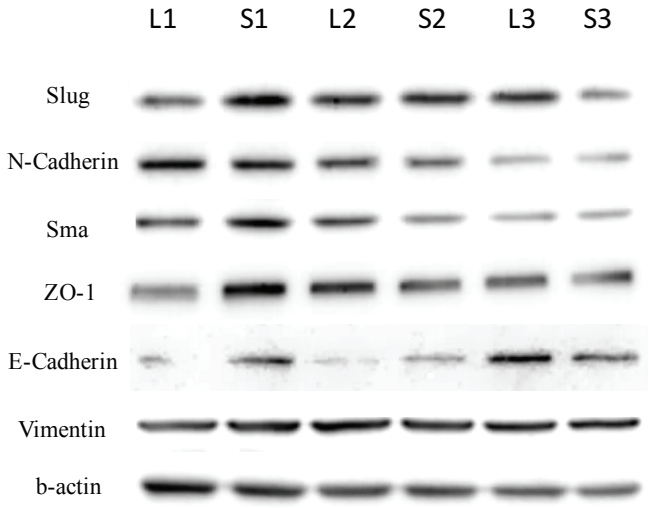

Figure 3.3.2.1 Western Blot images of MDAMB231 cells A) Vimentin antibody, 57 kDa B) E-Cadherin, 135 kDa, C) Snai2(Slug), 30 kDa D) ZO-1, 220 kDa E) Sma, 43 kDa Labels represent short description of LACZ as L and SEMA6D as S; Numerical letter after labels represents biological repeats of cell lines by infection; e.g. LACZ infection #1 as labelled L1, SEMA6D infection #3 as labelled S3. Graph near the image represent quantification of image. Graph below the image represents average of all proteins of control and SEMA6D overexpressed MDAmb231 cells. Red arrows indicate where protein bands are. (* $p<0.05$)

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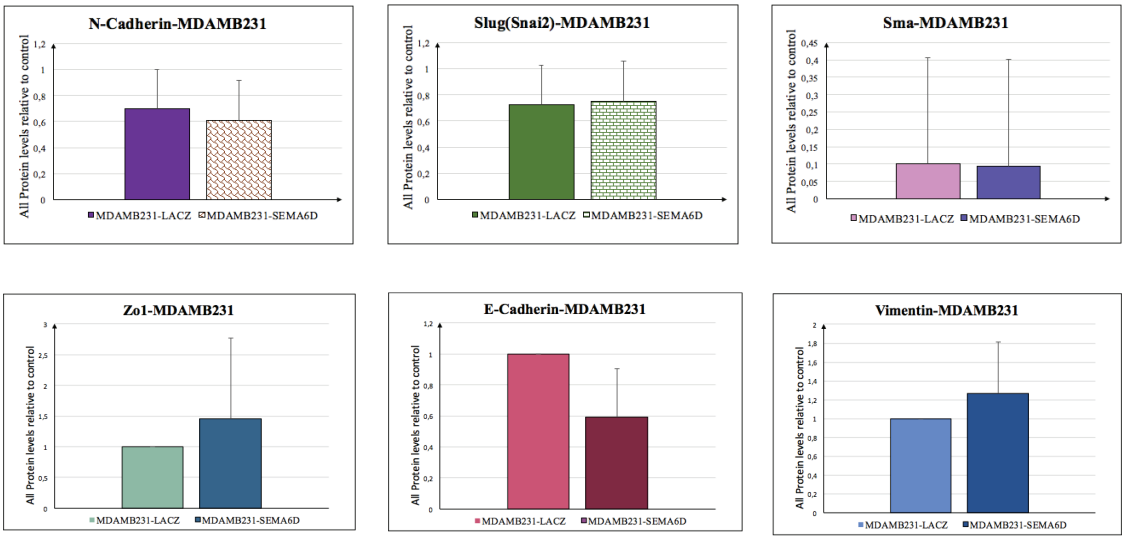

Figure 3.3.2.1 (Cont.)

According to qRT-PCR results, no statistically significant difference was observed in mRNA expression levels of epithelial markers ZO-1 and E-Cadherin in MDA-MB-231 cells (Figure 3.3.2.2 A and B). Also, Snai2, N-Cadherin, Vimentin, Zeb1 and Twist do not have significant change in mRNA levels of MDA-MB-231 cells (Figure 3.3.2.2 D-H). Only mesenchymal markers Snai1 and Zeb2 had significantly increased by 1.1-fold and 2.5-fold, respectively (Figure 3.3.2.2 I).

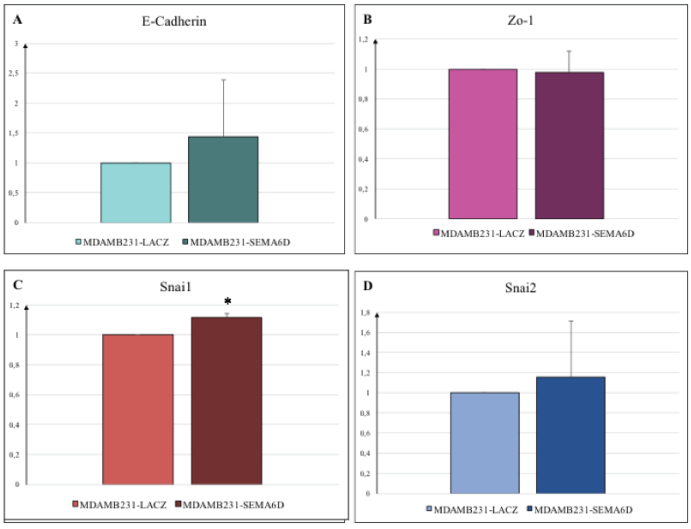

Figure 3.3.2.2 qRT-PCR quantifications of MDA-MB-231cell lines. MDA-MB-231-LacZ is control group and MDA-MB-231-SEMA6D is SEMA6D overexpressed MDA-MB-231cells. A) E-Cadherin, B) ZO-1, C)Snai1, D)Snai2 (Slug), E)N-Cadherin, F)Vimentin, G) ZEB-1, H) Twist and I) ZEB-2. One independent experiment and three biological repeats were performed. (*$p<0.05$, ** $p< 0.005$)

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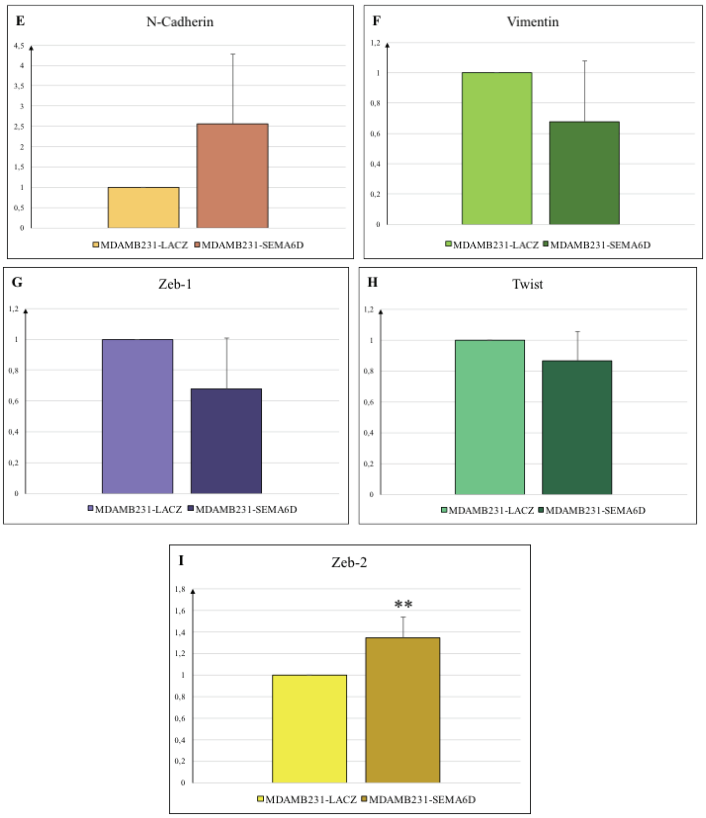

Figure 3.3.2.2 (Cont.)

All in all, SEMA6D overexpression on MDA-MB-231 cells does not reflect much significant data compared to MCF7 cells.

3.4 Effect of SEMA6D Overexpression on Migration of Breast Cancer Cells

Migration ability of MCF7 and MDA-MB-231 cells were analyzed by Wound-Healing assay and quantified by ImageJ program (Figure 3.4.2 A-E). Mitomycin C (MMC) was used for inhibiting cell proliferation. In order to confirm that MMC inhibits proliferation at indicated conditions, BrdU proliferation assay was performed in 6^{th}, 24^{th} and 48^{th} time periods separately.

In Figure 3.4.1, except positive (+) control, all anti- BrdU images did not have BrdU signal in all time periods. We showed that MMC completely inhibits cell proliferation in 6^{th}, 24^{th} and even 48^{th} hours. (+) control group is performed from 24 hours' period (Figure 3.4.1).

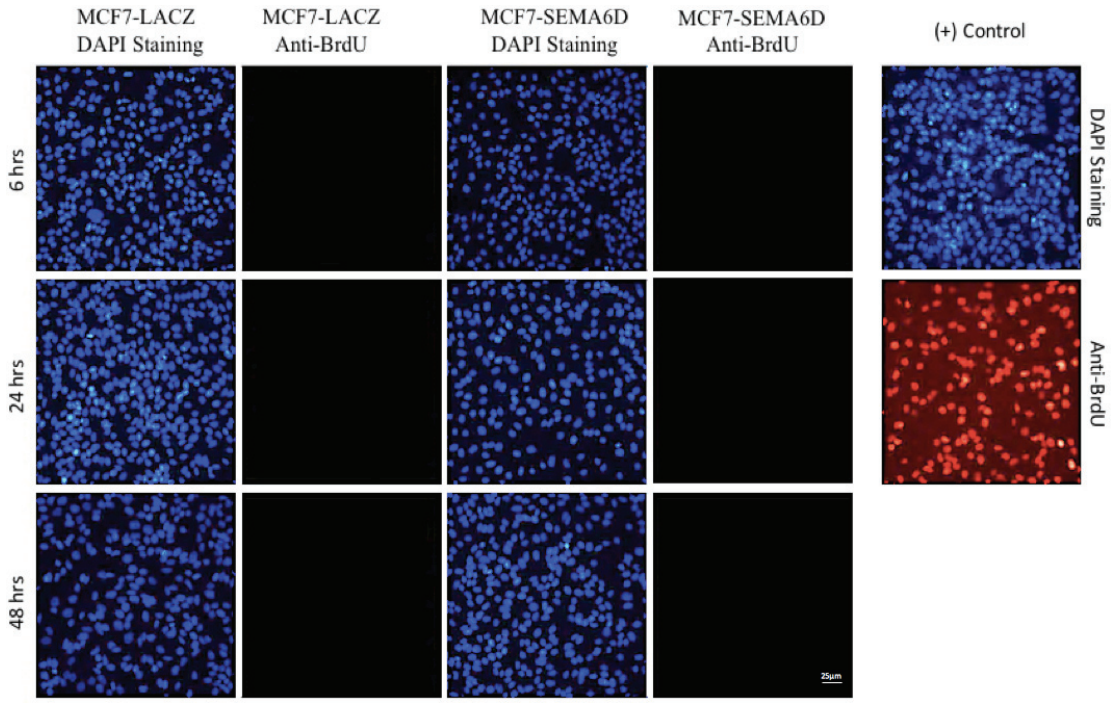

Figure 3.4.1 BrdU proliferation assay in time periods (6h, 24h, 48h) after 2 hours Mitomycin C (MMC) treatment of MCF7 cells. Nuclei counter staining, DAPI(blue); APC anti-BrdU antibody (red). All images were taken in 20X magnification under Fluorescence Microscope with 25µm scale bar. Negative Control was performed without BrdU treatment (not shown).

Figure 3.4.2 A and B represent both MCF7 and MDA-MB231 cell images of migration assay during time-lapse microscopy. Quantification of images is shown in Figure 3.4.2 C and D. Based on Wound-Healing experiments, migration ability of SEMA6D overexpressing MCF7 cells was significantly increased in all time periods (6^{th}, 12^{th}, 24^{th}, 36^{th} and 48^{th} hours) compared to control group, LacZ (Figure 3.4.2 C).

In opposite to MCF7 cell lines, migration ratio was reduced in SEMA6D overexpressing MDA-MB-231 cells in 12^{th}, 18^{th} and 24^{th} hours compared to control group (Figure 3.4.2 D). 6^{th} and 36^{th} hours did not have significant results but they tend to decrease as other time periods of SEMA6D overexpressing MDA-MB-231 cells (Figure 3.4.2 D). Since MDA-MB-232 cell lines are migrating faster than MCF7 cells, we could not measure 48^{th} time period for MDA-MB-231 cells.

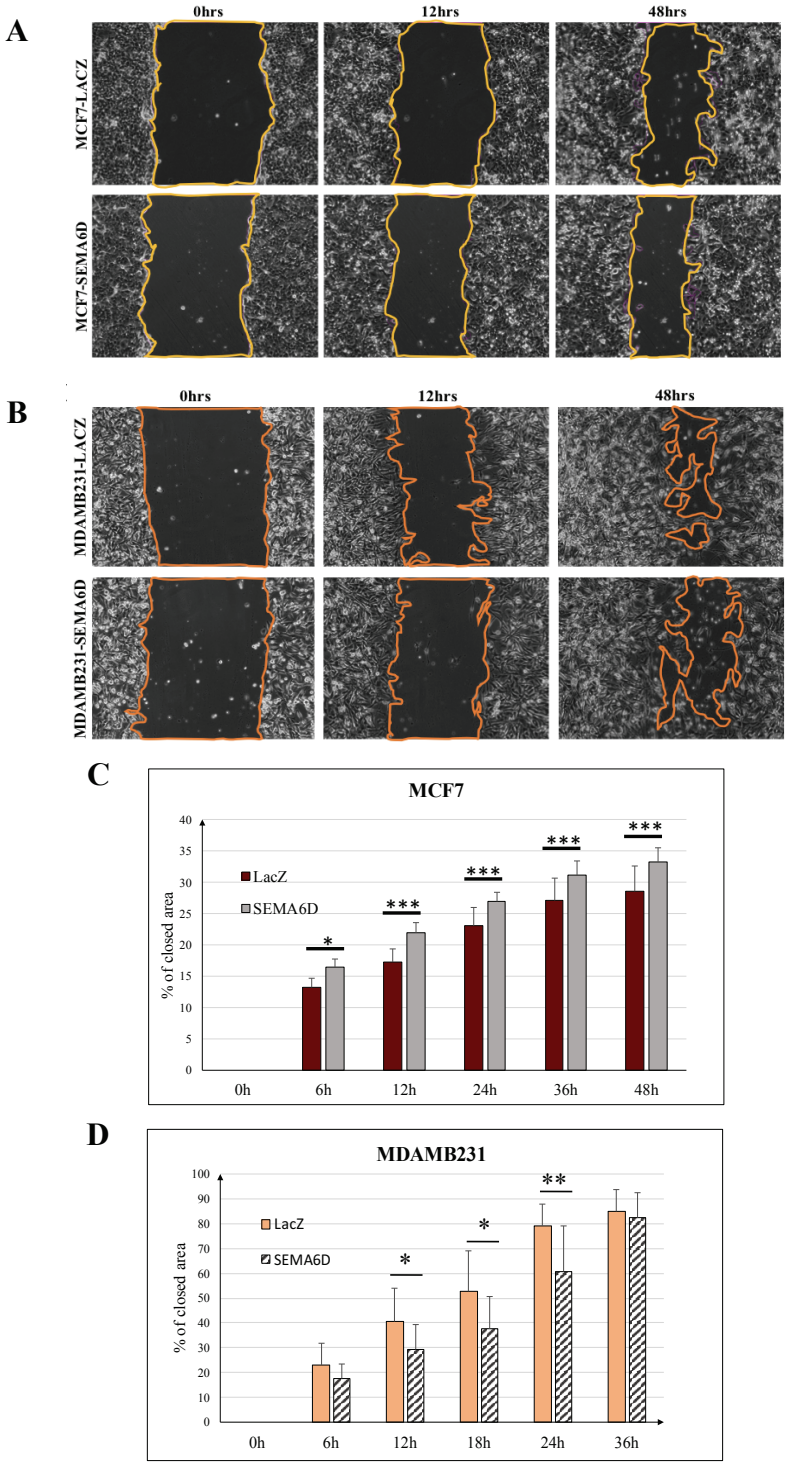

Figure 3.4.2 Migration Assay images at the indicated time points (0hrs, 6hrs, 12hrs, 24hrs, 36hrs and 48 hrs), A) MCF7 stable cell line with four biological repeats and eight independent experiments B) MDA-MB-231 stable cell line with three biological repeats and two independent experiments. All images were taken in 10X magnification and at least three different positions were taken from same cell line in one experiment. Statistical analysis and quantification of closed area for all wound-healing experiments of C) MCF7 cells, D) MDA-MB-231 cells. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$)

It was observed that some MCF7 cells tend to dissociate from the border and migrate as individual cell, therefore in addition to calculation of closing area, individually migrating cells are also counted at the same time points (Figure 3.4.3). Similar to rate of closing area, individually migrating cells are found to be significantly higher in number in SEMA6D-overexpressing MCF7 cells compared to control group, particularly in 12th, 24th, 36th and 48th hours. (Figure 3.4.3).

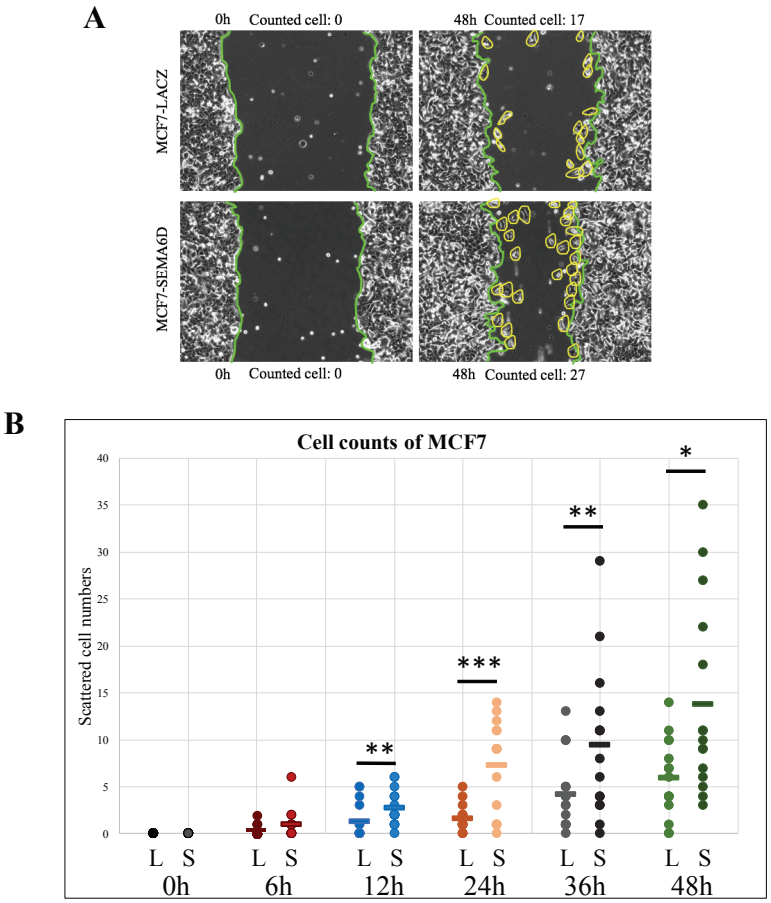

Figure 3.4.3 A) Example images of individually migrating cells in 0th and 48th hours of both control (LACZ) and SEMA6D overexpressed MCF7 cells. Images were taken in 10X magnification of time-lapse microscope. Yellow circles indicate migrating cells. Green line indicates area border. B) Individually migrating cell graph of MCF7 cells at the indicated time points. (0hrs, 6hrs, 12hrs, 24hrs, 36hrs and 48hrs). L represents a control group LacZ and S represents SEMA6D overexpressed MCF7 cell lines. Four independent experiments were performed and each cell line has three different positions in same image. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Overall, SEMA6D overexpression significantly increases migration in MCF7 cells whereas it significantly reduces in MDA-MB-231 cells. However, statistical significance of MCF7 cells is higher than MDA-MB-231 cells.

CHAPTER 4

DISCUSSION & CONCLUSION

Previous studies demonstrated that semaphorin family has important roles in development of organs and has roles in neural pathway [30] as well as semaphorins have roles in many cancer types [21]. Mostly, SEMA4D protein and its functions in immune system has been known [31]. SEMA6D functions and roles had not been known well but recently, importance of SEMA6D protein has been revealed in different types of cancer. SEMA6D is validated as an oncogene in human osteocarcinoma and its oncogenic roles were investigated in mesothelioma and gastric cancer [24–26]. There is only one study about SEMA6D gene in breast cancer and it was based on bioinformatic analysis from The Cancer Genome Atlas (TCGA) patient data [27]. Rather than only bioinformatics, focusing on in vitro or in vivo experiments of SEMA6D may help to figure out its functions and mechanisms better and investigating its roles in breast cancer may enlighten better solutions for patients who desperately waiting to be healed. For this reason, in this study, we aimed to investigate role of SEMA6D in proliferation, epithelial-mesenchymal transition and migration of breast cancer cell lines.

In order to make sure that experiments do not have coincident results, four different MCF7 stable clones were generated from 4 different viral infections and three different MDA-MB-231 stable clones were generated from three different infections. MCF7 cell line is neither normal breast cell line nor aggressive breast cancer cell line. It is luminal A type and it could give the best approach to understand whether SEMA6D increase or reduce metastatic ability of cells. Therefore, MCF7 cell line was chosen for further experiments in the first place. Then, MDA-MB-231 triple negative cell line was performed further experiments to compare with MCF7 cells and to understand SEMA6D in different breast cancer cell line.

According to proliferation and migration results, proliferation capacity of SEMA6D overexpressing MCF7 (MCF7-SEMA6D) cells was significantly decreased by MTT Assay whereas migration ability was significantly increased. This could demonstrate logical explanation for a signal of triggering metastasis, since migration ability should be increased in metastasis[8]. To make confirmation of decrease in proliferation, BrdU assay was also performed on MCF7 cells. According to both

proliferation assays; MTT and BrdU Assays, cell proliferation was significantly reduced in MCF7-SEMA6D cell lines. However, it should not be forgotten that decrease in proliferation may not mean a decrease in metastasis, oppositely it could be sign of initial steps of metastasis [32].

For metastasis, cell may gain epithelial to mesenchymal transition (EMT), so EMT markers were also performed in mRNA and protein level of MCF7 and MDA-MB-231 cells. According to Western Blot results Snai2 (Slug) and N-Cadherin, which were used as mesenchymal markers, were significantly increased in protein levels of SEMA6D overexpressing MCF7 cells and slight increase in MDAMB231 cells. In prostate cancer, it has been known that Snai2 reduce proliferation of cancer cells [32]. Thereby, decrease in proliferation might be caused by increase in Snai2, which is also a sign of epithelial to mesenchymal transition.

Surprisingly, Snai2 was significantly decreased in mRNA level of SEMA6D overexpressed MCF7 cells. This opposite situation can be caused by quick degradation of mRNAs compared to proteins. Overall, according to these results, migration ability of MCF7 may be related with epithelial-mesenchymal transition (EMT) and thereby SEMA6D can trigger metastatic ability in both cell lines, yet further experiments could be required.

Proliferation and migration capacities were also performed for MDA-MB-231 cells. Proliferation by both MTT assay and BrdU Assay did not have significant change whereas migration ability of SEMA6D overexpressing MDA-MB-231 cells (MDAMB231-SEMA6D) had significant decrease according to two independent and three biological repeats, especially 12^{th}, 18^{th} and 24^{th} hours. This decrease may be logical and consistent with TCGA bioinformatic analysis, because MDA-MB-231 cells are triple negative type and according to study of Chen et al., breast cancer patients, who had triple negative breast cancer type, had better survival rate in the presence of SEMA6D [27].

On the other hand, mesenchymal markers; ZEB2 and Snai1 were increased in mRNA level of SEMA6D overexpressing MDA-MB-231 cells. Due to having inconsistent results between Western Blotting, qRT-PCR and Wound-Healing assay, it is not possible to have sharp decision about SEMA6D reduce or enhance migration or proliferation in MDAMB231 cell line.

For metastasis, cell should also gain invasive ability, therefore; to understand role of SEMA6D on metastasis, invasion assay should be performed. Boyden Chamber Assay was performed before but there was no substantial data to evaluate because cells were

tiny and they could not be distinguished from membrane pores even if they have nuclei dye, DAPI. For this reason, before performing migration assay with Boyden Chamber Assay, cells might be dyed by fluorescence dye and migration period can be optimized to distinguish pores and cells from each other or other experimental setups, such as lab-on-a-chip (LOC) experiments would be performed with matrigel.

In this study, overexpression of SEMA6D had been performed by 2-dimensional (2D) cell cultures but 3D cell culture could give better results about proliferation and tumor progression since cancer cells in patients have formed 3D structure and metastasis occurs through blood vessels then migrated and invasive cells form 3D structure in the place of secondary tumor growth [33]. Moreover, experiments with primary cell culture, which are taken from breast cancer patients, may give us a substantial knowledge about SEMA6D role in patient's cells, which reflect tumor growth in real life and thereby, it can open new windows of diagnosis of breast cancer.

In a conclusion, MCF7 cell line guides us better to understand role of SEMA6D in breast cancer via its significant and consistent results. Besides, at the beginning of this project, role of SEMA6D were not known whether it triggers or reduces effects of metastasis. Therefore, choosing MCF7 cell line was logical since it is neither metastatic nor normal breast cancer cell. According to experiments performed with MDA-MB-231 cell line, overexpression of SEMA6D on MDA-MB-231 cells do not seem to have similar results with MCF7 cells and there were not significant changes in most of results. It should be considered that their metastatic capacity is already high compared to MCF7 cells. For this reason, instead of SEMA6D overexpression on highly metastatic breast cancer cell line, silencing SEMA6D might give more clear results to understand role of SEMA6D. For further experiments, different cell lines and also normal breast cells, such as MCF10A cell line, can be used to understand role of SEMA6D in breast cancer better. Besides, we only performed overexpression of SEMA6D, but silencing of SEMA6D may also guide us to understand roles of SEMA6D in breast cancer cell lines. In the future, understanding role of SEMA6D would provide us with better prognosis and diagnostic solutions for breast cancer patients.

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