INVESTIGATING ONCOGENIC ROLE OF SEMA6D IN BREAST CANCER CELLS

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ABSTRACT

INVESTIGATING ONCOGENIC ROLE OF SEMA6D IN BREAST CANCER CELLS

Breast cancer, the most commonly diagnosed cancer type and the leading cause of cancer-associated deaths, is the major health issue among women worldwide. In many cancer types, the expression of the semaphorins and their receptors such as plexins and neuropilins are dysregulated.

SEMA6D is a member of class-6 family transmembrane semaphorin proteins and acts through Plexin-A1 receptor. It was previously shown that overexpression of SEMA6D in breast cancer cell line MCF-7 leads to a reduction in proliferation and an increase in migration. On the other hand, in the MDA-MB-231 breast cancer cell line, overexpression of SEMA6D had no significant effect on proliferation but enhanced migration.

In this study, we aimed to analyze the effects of SEMA6D overexpression in normal breast cell line MCF10A and investigate the invasive behavior and transformation capacity of SEMA6D overexpressing breast cancer cell lines. We demonstrated that overexpression of SEMA6D leads to elevated proliferation, viability and migration in MCF10A cells, whereas it did not trigger their anchorage-independent growth. On the other hand, MDA-MB-231 and MCF7 cells stably expressing SEMA6D showed reduced colony formation in the soft-agar assay. Furthermore, the invasiveness of MDA-MB-231 cells was elevated with SEMA6D overexpression, whereas SEMA6D overexpression did not stimulate the invasiveness of MCF-7 cells through matrigel microenvironment, whereas slightly trigger invasion through bone microenvironment.

In conclusion, SEMA6D overexpression has cell-specific effects on breast cancer. The exact role of SEMA6D in breast cancer development remains undefined and must be further investigated.

ÖZET

SEMA6D'NİN ONKOGENİK ROLÜNÜN MEME KANSERİ HÜCRELERİNDE İNCELENMESİ

Meme kanseri, kadınlarda en sık teşhis edilen kanser türü olmakla birlikte dünya çapında kansere bağlı ölümlerin ikinci önde gelen nedenidir. Bir çok insanda bulunan kanser çeşitlerinde semaphorinler ve receptörleri olan plexin ve neuropilinler anormal bir şekilde ekspres edilmektedir.

SEMA6D, grup 6 transmembran semaphorin proteinlerinin bir üyesidir ve sinyalini hedef yerlere reseptörü olan Plexin-A1 ile iletir. Önceki çalışmalarımızda, SEMA6D aşırı ekspresyonunun MCF-7 meme kanseri hücrelerinin proliferasyonunda azalmaya sebep olduğu ancak hücre göçünde artışa sebep olduğu gösterilmiştir. MDA-MB-231 meme kanseri hücresinde ise, SEMA6D fazla ekspresyonu proliferasyonu anlamlı olarak etkilemezken hücre göçünü arttırdığı gözlemlenmiştir.

Bu çalışmada, SEMA6D aşırı ekspresyonunun MCF10A normal meme epitel hücrelerindeki etkisinin araştırılması ve meme kanseri hücrelerindeki invazif davranışları ve transformasyon kapasitesi üzerine etkisinin incelenmesi hedeflenmiştir. MCF10A hücrelerinde SEMA6D fazla ekspresyonunun proliferasyonda ve hücre göçünde artışa sebep olduğu gösterilmiştir, ancak yumuşak agarda herhangi bir koloni oluşumu gözlenmemiştir. Meme kanseri hücreleri olan MDA-MB-231 ve MCF-7 da SEMA6D fazla-ifadesi yumuşak agarda koloni oluşumunu azaltmıştır. SEMA6D fazla-ifade edildiğinde MDA-MB-231 hücrelerinin invazif karakterini arttırmıştır ancak invazif olmayan MCF-7 hücre hattında matrigel mikro çevresine doğru invasyonu tetiklememiştir fakat kemik mikro ortamına doğru invazyon görülmüştür.

Sonuç olarak SEMA6D kanser ilerlemesinde hücre spesifik olarak önemli bir role sahiptir. SEMA6D' nin meme kanseri gelişimindeki net rolü halen belirsizliğini korumaktadır ve daha detaylı olarak incelenmelidir.

To my family…

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CHAPTER 1

INTRODUCTION

1.1. Breast Cancer

Breast cancer that is originated from tissues of breast, especially from milk ducts or lobules, leads to growth of the cells an uncontrolled manner. Female breast cancer is the leading type of cancer worldwide with regards to the number of newly diagnosed cases and responsible for 30 percent of whole new cancer diagnoses among women [1]. Breast cancer accounts for 11.6% of total cancer mortality and is the second leading cause of death after lung cancer among both sexes [2]. Metastasis of breast cancer, in which the cells spread to distant organs as being predominantly to the bone and other organs such as lung, regional lymph nodes, brain, and liver is responsible for the majority of breast cancer-related deaths and commonly stem from its incurability. Metastasized and recurrent breast cancer accounts for the majority of breast cancer-related deaths as the incidence of over 90%. However, the five-year survival rate is above 90% in early diagnosed cases and patients having localized tumor, which not spreading to distant regions [3]. Early detection of cancer enhances the chance of the curability and leads to high survival rates [4]. The rate of breast cancer mortality decreased between 2007 to 2016 as 1.8% each year thanks to improvements in breast cancer diagnosis and awareness and also effective treatment [5]. The treatment of breast cancer varies relying on types of breast cancer and how tumor advanced whether it has metastasized or not. Treatments applied to people having breast cancer include local treatments as surgery (lumpectomy and mastectomy as removal of the breast cancer or whole breast, respectively) or radiation and systemic treatments as hormone therapy, chemotherapy, immunotherapy, and targeted therapy [6]. There are also several other approaches that aid to elevate the therapeutic effect of drugs against cancer such as nanocarriers like liposomes, quantum dots, and nanoparticles. Thus, developments in

current therapeutics can enable long-lasting survival rates and enhanced life quality for patients suffering from breast cancer [7].

1.1.1. Molecular Categorization of Breast Cancer

Breast cancer comprises heterogeneity that contains several distinct entities as the difference in molecular, histological and pathological features. Classification of breast tumors provides insights for prognosis and determination of treatment strategy. Categorization of breast cancer varies depending on gene expression profile called intrinsic subtypes as its hormone receptor expression as estrogen and progesterone or expression status of HER2 (Human epidermal growth factor receptor 2, ERBB2) and origin of tumor cells as luminal or basal. There is also traditional classification based on histopathological properties of the tumor [8]. Molecular profiling subcategories of breast cancer are divided into five different classes as luminal A and B type, triple-negative (basal like, TNBC), normal-like and HER2 enriched. Additionally, breast tumors can develop from different parts of breast tissue as in ducts and lobules or tissues in between them [9]. Breast cancer can also be defined as in two broad classes such as being invasive and non-invasive. In invasive type also called infiltrating, cells of the breast can travel from its primary location through a distant part of the body through the bloodstream or via lymph nodes called metastasized [10]. Adenocarcinomas possess the majority of breast cancers that account for 95% of breast malignancies that originated from glandular tissues by virtue of breast duct and lobules consisting of many glandular tissues [11]. Based on World Health Organization (WHO) classification of breast carcinoma, it has categorized into 19 distinct main subtypes which majority of them comprised by invasive carcinoma no special type (NST) also called as invasive ductal carcinoma constitutes 70-75% of diagnosed cases and lobular carcinoma accounts for approximately 10-14% of the cases [12]. The other types of invasive carcinomas include mucinous, tubular, papillary, cribriform carcinoma of the breast, medullary carcinoma (MC) which holds morphological differences [13, 14]. In non-invasive breast cancer, cells do not spread from lobules or ducts. Ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (means in its precursor place) fall under the category of non-invasive

type breast cancer. It is defined as an atypical group of cells that evolved from milk ducts whereas it holds malignant transformation and can be a precursor for invasive breast cancer [11, 15].

Gene expression patterns facilitate distinct features of breast cancer and provide an estimation of efficient treatment strategy and beneficial for prognosis. Five intrinsic subtypes of breast tumors each possess distinct features. Luminal tumors are named as Luminal A and Luminal B, which express hormone receptors for both estrogen and progesterone and are negative or positive for human epidermal growth factor receptor 2 (HER2), respectively [16]. The expression of proliferative markers, such as Ki-67 is elevated in Luminal B breast cancer whereas it is low in Luminal A type which leads to worse prognosis in Luminal B type. The breast tumor hormone can be negative for both hormone receptors as estrogen-receptor (ER-), progesterone-receptor (PR-) and also the absence of human epidermal growth factor receptor 2 (HER2-) defined as triple-negative breast cancer (TNBC) also called as basal-like. It accounts for 15% of entire invasive breast cancer [17, 18]. The TNBC incidence advanced in patients having a mutation in *BRCA1* gene [19]. HER2 enriched breast cancer constitutes 15% of whole invasive breast cancers. The expression profile can be defined as HER2 positive and ER and PR receptors commonly negative. Tumors holding HER2-overexpression leads to poor prognosis and having a high rate of lymph node metastasis [20]. Normal-like breast cancers resembles to Luminal A type as being positive for estrogen-receptor (ER+), progesterone-receptor (PR+) and having low levels of the proliferative marker as Ki-67 whereas its prognosis still not better than Luminal A-type breast cancer prognosis [16].

1.2. Breast Cancer Metastasis

The majority portion of cancer-related mortality and morbidity arises not only because of the primary tumor but also heavily caused by metastasis. Even breast cancer starts as a local disease it demonstrates a high probability to disseminate through lymph nodes and distant organs [21]. Early detection of metastasis is crucial for the management of cancer and the selection of effective treatment strategies. In clinics, several detection methods are utilized for the diagnosis of metastatic lesions such as organ biopsies,

protein-based biomarkers, and histopathology tests, several imaging techniques as magnetic resonance imaging (MRI), computerized tomography (CT), positron emission tomography (PET) and blood tests [22].

Malignant transformation of cancer is defined as a multi-step and complex cascade and several distinct steps aid to the development of secondary tumors (Figure 1.1). The primary tumor undergoes sequentially distinct steps that initiate the metastasis process basically listed as local invasion, epithelial to mesenchymal transition, intravasation, and transportation through circulation, extravasation, ultimately the formation of pre-metastatic niche and generation of macro metastasis [23].

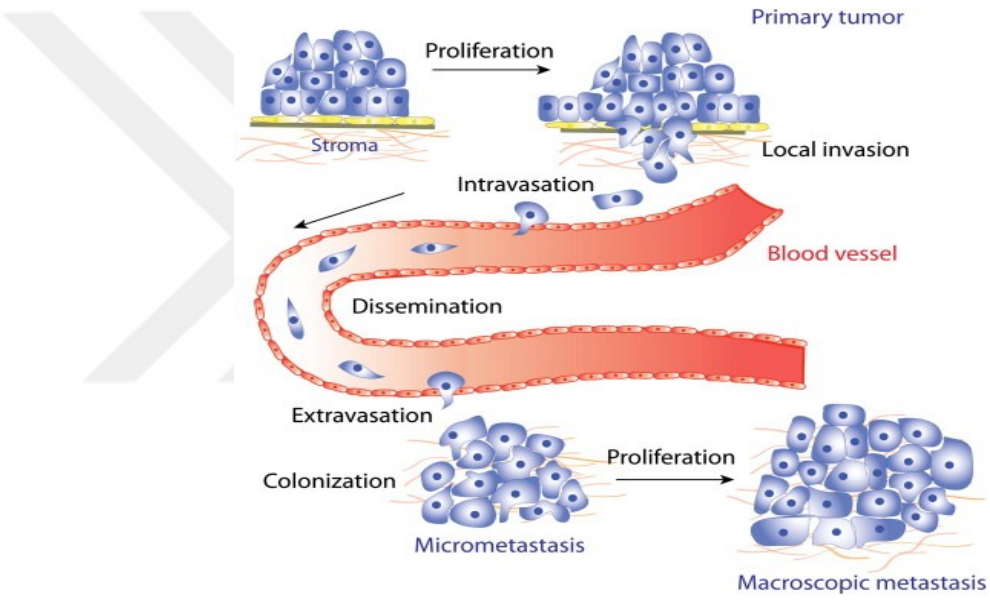

Figure 1. 1. General representation of multistep metastasis dissemination[24].

1.2.1. Tumor Cell Invasion and Epithelial-to-Mesenchymal Transition

The initial step for the dissemination of tumor cells is a local invasion that the cells invade through surrounding tissue as extracellular matrix (ECM) and stromal tissues. During malignant transformation, tumor cells utilize cellular plasticity that drives reprogramming of their morphological and phenotypical features including

altering mesenchymal and epithelial-like status and mesenchymal to the amoeboid transformation which remarkably sustains heterogenic feature of breast cancer [25, 26]. Invasive cancer cells begin to penetrate through the basement membrane due to altering cell to cell and cell to matrix adhesion molecules. The basement membrane is the specialized type of extracellular matrix (ECM) that possesses a crucial role in structural support to tissues and the organization of epithelial tissues. Additionally, the basement membrane takes part in signal relaying that induce switching the polarity of cells [27, 28]. Deprivation of cell to cell or cell to matrix adhesion molecules lead to altering tissue structure employed by mainly cadherin family proteins. The change of characteristics of the cells from epithelial to mesenchymal state is defined as epithelial-to-mesenchymal transition (EMT) by means of downregulation of epithelial transcription factors as E-cadherin and upregulation of mesenchymal transcriptional factors like N-cadherin (Figure 1.2) [29]. Although, epithelial to mesenchymal transition indicated as a direct switching between two states whereas malignant tumor cells acquire mesenchymal traits and also allowing the conservation of part of epithelial phenotype so the cells present in mixed phenotype reflecting mesenchymal and epithelial traits [30].

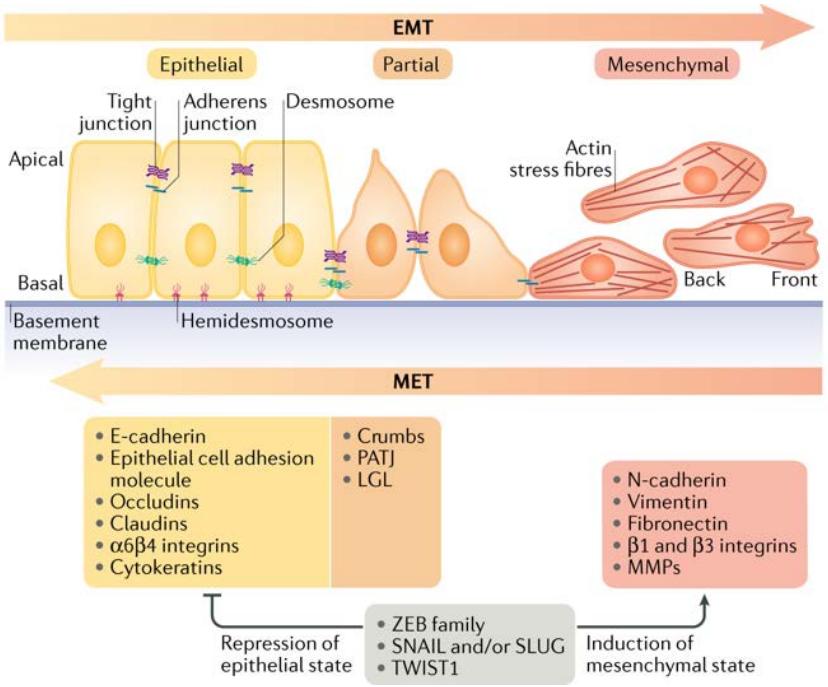

Figure 1. 2. Overview of epithelial-to-mesenchymal transition including EMT-Transciption Factors (TF) [31]

Several transcription factors a play role as EMT inducers, such as zinc-finger E-box binding homeobox as ZEB1/2, Snail family zinc finger TFs as Snail/Slug and Twist. EMT-transcription factors also regulate the activity of proteases (matrix degradation enzymes) as several matrix-metalloproteinases (MMP-1, MMP-2, MMP-7, MMP-9) which aids to disruption of apical-basal cell polarity and remodeling of the extracellular matrix. Eventually, tumor cells gain migratory capability and become motile can invade through the surrounding tissue [32, 33].

1.2.2. Intravasation and Survival in Circulatory System

Local invasion of tumor cells is followed by intravasation into the circulatory system either entrance to the lymphatic or venous vessel which aids to the dissemination of breast carcinoma cells. Transendothelial migration of tumor cells is crucial for intravasation leading to the generation of distant metastases. EMT is one of the major factor that triggers intravasation of tumor cells and the formation of circulating tumor cells and enhance their survival in circulatory system [34, 35]. Intravasated tumor cells undergo several structural and molecular changes that facilitate escaping from the host's immune system, apoptosis and anoikis and resistance to hydrodynamic flow and cope with shear stress [36]. Circulating tumor cells can also be protected with non-tumor structures as platelets that cover the circulating tumor cells in order to preserve from shear stress [37].

1.2.3. Extravasation and Metastatic Colonization

Emigrating cancer cells gain the ability to cross blood vessel lumina by means of penetrating endothelial barrier that left from bloodstream called extravasation. After extravasation from the bloodstream, tumor cells proliferate at the secondary site. Metastatic colonization occurs when the tumor cells find a suitable microenvironment

which is supported by several signaling factors [38]. The seed and soil hypothesis proposed by Steven Paget that states a convenient microenvironment is required for tumor cells (seed) to pose secondary sites of a distant organ (soil). It is also assumed that malignant cells can able to modify their soil as host microenvironment to constitute pre-metastatic niche by means of secreting distinct molecules before tumor dissemination [39, 40].

Several chemokines can play role in the secretion of homing signal for tumor cells such as elevated CXCR4 receptor expression in breast cancer and target organs that have upregulated expression of CXCR4 ligand referred to be the prevalent region of metastases formation [23].

1.3. The Semaphorin Family Proteins

The semaphorin family proteins comprise both secreted and transmembrane associated proteins that their role implicated in key biological processes including migration of neuronal cell, axon guidance, tumor angiogenesis, regulation of immune cells and heart morphogenesis. There are 20 members of semaphorins that present in human and Drosophila possesses 5 of them [41]. The classification of semaphorins is based on their cysteine-rich sema domain which indicates each family. Semaphorin glycoproteins are divided into eight groups depending on species and their protein motifs; as class 1 and 2 in invertebrates, classes 3 to 7 in vertebrates and class 8 in viruses [42]. The Semaphorin family classes 1, 4, 5 and 6 comprise transmembrane semaphorins whereas 2, 3 and 5 defined as secreted proteins. Glycosylphosphotidylinisitol-linked (GPI) class of semaphorin is class 7 (Figure 1.3.a) [43].

Semaphorin signaling is relayed by means of its receptor as plexins and neuropilins that are transmembrane proteins containing 4 subclasses depicted as Class A, B, C, D. Secreted semaphorin protein is the member of class 3 family that cannot interact with plexins rather signal through neuropilin 1 or 2 receptors. Neuropilin family receptors are able to form a stable complex with plexin family proteins in order to mediate binding and signaling processes (Figure 1.3.b) [42, 44].

Each subgroup of semaphorin family proteins represents a particular structural feature whereas entire members share structural homology in their extracellular sema domain among all species [41]. The signal transduction of semaphorins through plexins achieved by intrinsic plexin-GAP activity and following with relaying signaling towards downstream elements like protein-kinases, cytoskeleton related molecules and small GTPases [45].

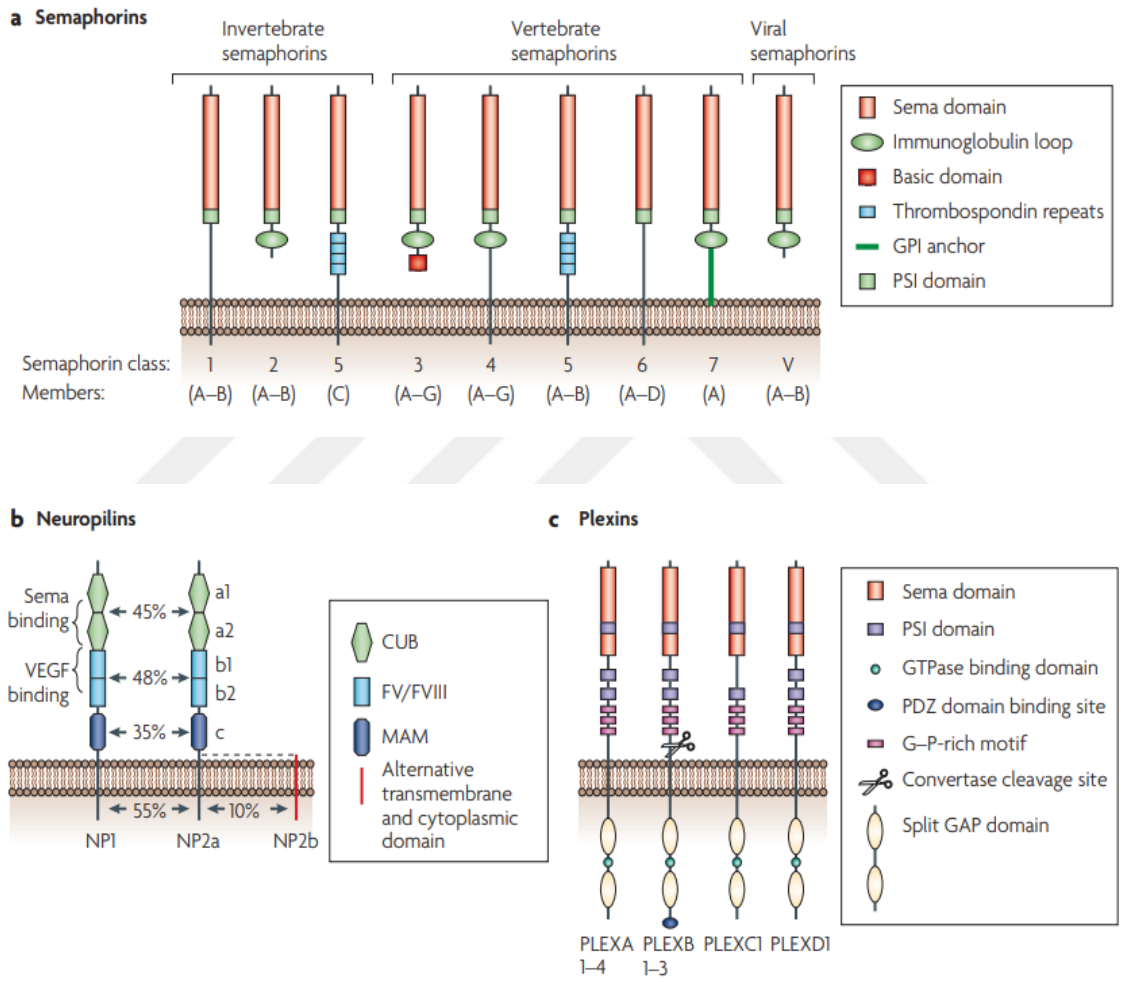

Figure 1. 3. (a) Schematic representation of Semaphorin family proteins and their receptors (b) Neuropilins and (c) Plexins [44].

1.4. The Semaphorins and Cancer

Accumulating evidences showed that, although semaphorins are commonly characterized in developmental processes, whereas they are also implicated in the modulation of tumor cell behavior in the concept of cancer progression, tumor angiogenesis, and cancer-associated inflammation [44, 46]. There are several studies that demonstrated the significance of semaphorins in either inhibition or enhancement of tumor progression, the survival of tumor cells and metastasis. A variety of tumors expresses semaphorins, the roles of which are diversified in a tissue-specific manner. SEMA3F and SEMA3B act as a tumor suppressor in small cell lung cancer even though their intrinsic role indicated in the building of neuronal network and development of nervous system [47]. Additionally, another study demonstrates that semaphorins might participate in preventing tumor development via altering the tumor microenvironment by the recruitment of macrophages [48]. Other than class 3 semaphorins, the members of class 4 and 5 semaphorins such as SEMA4D and SEMA5A were shown to enhance angiogenesis [49]. Additionally, gene silencing of SEMA4D by shRNA results in the attenuation of tumor growth and lowers its vascularization [50]. In prostate cancer, it was shown that SEMA3C regulates epithelial to mesenchymal transition (EMT) and promotes malignancy by elevated invasiveness and tumor cell dissemination [51]. Recent research related to SEMA6A also demonstrates that decreased levels of SEMA6A in lung cancer are correlated with enhanced probability of relapse in patients [52].

1.5. The Semaphorin 6D (SEMA6D)

Semaphorin 6D is a member of the class-6 semaphorin family which is single-pass membrane-tethered semaphorin relaying their signaling by means of their receptors Plexin A1. SEMA6D is fundamentally depicted in axon guidance factor in neurogenesis and also is a key regulator of heart development, vascular growth and immune cell regulation [53]. There are several types of researches that indicate the role of SEMA6D as expressing pro-angiogenic and pro-tumorigenic features [54].

During cardiac chamber formation which includes the myocardial organization, constitutively activated SEMA6D signaling promotes the migration of myocardial cells through trabeculae. Also, SEMA6D or Plexin-A1 knockdown results in abnormal trabeculation and reduction in the formation of ventricular compact layer [55].

The overexpression SEMA6D enhances the expansion of the ventricular chamber, on the contrary silencing of SEMA6D leads to the narrowing of the ventricular chamber during cardiac morphogenesis of chick embryo. Additionally, SEMA6D which demonstrates as site-specific function on cardiac explant promotes migration in outgrowing cells, whereas inhibits migration in cells from the ventricle. Also, it was shown that the site-specific activity of SEMA6D depending on the Plexin-A1 receptor structure which generates receptor complex with VEGF-R2 (vascular endothelial growth factor receptor type-2) present in the conotruncal segment or forms a receptor complex with Off-track in ventricle segment. The formed complexes aid to exerting function of SEMA6D in distinct regions [56].

In the aspect of role of SEMA6D in cancer, it has been shown that SEMA6D was found in elevated levels in gastric carcinoma that is associated with stimulation of tumor progression, angiogenesis and also metastasis. Additionally, the elevated angiogenesis has been indicated in gastric cancer, which was associated with enhanced SEMA6D expression [53, 57].

SEMA6D expression was correlated with enhanced patient survival in invasive breast cancer based on genomic expression analysis of public data sets of breast cancer-derived from The Cancer Genome Atlas. The analysis showed that higher SEMA6D expression in patients with triple-negative breast cancer (TNBC) might be associated with better survival rates [58].

According to *Sleeping Beauty (SB)* transposon-based forward genetic screen in mice, common insertion site (CIS) analysis from primary tumors and metastatic nodules was shown that new osteosarcoma-associated genes. SEMA6D and SEMA4D have been represented and validated as an oncogene in human osteosarcoma [59].

In malignant pleural mesothelioma (MPM), SEMA6D and its receptor Plexin-A1, are found to be highly expressed and stimulate pro-survival capability which enhances anchorage-independent growth. It was also shown that Plexin-A1 and VEGF-R2 (vascular endothelial growth factor receptor type-2) constitute a complex which then SEMA6D signaling enhances the tyrosine phosphorylation of vascular endothelial growth factor receptor type-2 through Plexin-A1 dependent way. It was also

demonstrated that VEGF-R2 can elevate tumor cell survival by nuclear factor-kappaB (NF-kappaB) activation [60].

In recent research, it was shown that SEMA6D is a novel signaling regulator that manages cardiomyocyte proliferation during fetal-to-neonatal transition process [61].

These researches elicit an unknown biological function of semaphorins in cancer development and progression through several undescribed pathways. There were limited researches about semaphorins in cancer and the function of SEMA6D in cancer especially in breast cancer still controversial. Studies indicating the role of semaphorins in breast cancer have predominantly focused on class 3 secreted Semaphorins and SEMA4D [58]. There was no elaborative research apart from bioinformatics analysis that expanding the function of SEMA6D in breast cancer.

CHAPTER 2

AIM OF THE PROJECT

The aim of the project was to investigate the oncogenic role of SEMA6D in immortalized non-tumorigenic breast cell line MCF10A, and breast cancer cell lines MDA-MB-231 and MCF-7.

We had previously shown that SEMA6D overexpression is associated with enhanced migration in MCF-7 and MDA-MB-231 cells, whereas overexpression of SEMA6D leads to a decreased proliferation in MCF-7 cells but has no significant effect on proliferation of MDA-MB-231 cells.

Semaphorins and their receptors are expressed in several types of tumor cells, yet their signal transduction mechanism and biological function in tumor progression are not fully elucidated and mostly remain unknown. Specifically in breast cancer, SEMA6D research is limited to a silico correlation study that showed a relation between SEMA6D expression and patient survival. Therefore, in this study, it was aimed to understand the emerging role of SEMA6D in breast cancer in the context of migration, proliferation, transformation, and invasion. Understanding the distinct role of SEMA6D in cancer progression may aid to unravel distinct molecular mechanisms in breast cancer which might lead to elicit new therapeutic approaches.

CHAPTER 3

MATERIALS & METHODS

3.1. Cell Line and Cell Culture

In this study, 6 cell lines were used which are MCF10A (American Type Culture Collection, ATCC) human breast epithelial non-tumorigenic cell line, MDA-MB-231 (ATCC) "basal" type triple-negative human breast cell line (TNBC), MCF-7 (ATCC) "luminal" type human breast adenocarcinoma, NIH3T3 embryonic mouse fibroblast cell line, HEK293T human embryonic kidney cell line, and hFOB human embryonic osteoblast cell line.

MCF10A cells were mainted in high glucose DMEM-F12 (Gibco, catalog no. 31330-038) supplemented with 5% Horse Serum (Gibco, catalog no. 16050-122), 1% Penicillin/Streptomycin (Thermo-Fisher Scientific, catalog no. 15140-122), 20ng/mg EGF (Sigma, catalog no. E9644-.5MG), 0.5µg/mg Hydrocortisone (Sigma, catalog no. H0888-1G), 100 ng/ml Choleratoxin (Sigma, catalog no. C8052-1MG), 10µg/mg Insulin (Sigma, catalog no. I1882-100MG).

MDA-MB-231, MCF-7, and HEK293T cell lines were cultured in high glucose DMEM (Gibco, catalog no. 41966-029) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, catalog no. 10270106) and 1% Penicillin/Streptomycin (Thermo-Fisher Scientific, catalog no. 15140-122).

NIH3T3 cells were grown in high glucose DMEM (Gibco, catalog no. 41966-029) supplemented with 10% New Born Calf Serum (NBCS) (BI, catalog no. 04-102-1A) and 1% Penicillin/Streptomycin (Thermo-Fisher Scientific, catalog no. 15140-122).

hFOB cells were cultured in DMEM-F12 nutrient (Ham) included 10% FBS and 1% Penicillin/Streptomycin.

Each cell line was maintained in 5% CO_2 at 37 ˚C.

NIH3T3 and HEK293T cell lines were provided by Prof.Dr. Cathrin Brisken Laboratory (EPFL, ISREC).

3.2. Lenti-Virus Production

Lentivirus based system is commonly used to transduce cells for gene expression analysis researches. For overexpression of SEMA6D, lentiviruses including SEMA6D cDNA were generated in a host as HEK293T cell line. pCMV-dr8.74 as packaging and pMD2-VSVG as envelope plasmid vectors were used in order to produce lentivirus. $3x10^6$ HEK293T cells were plated to 10 cm plate 24 hours before transfection. pLX304-SEMA6D plasmid vector and pLX304-LacZ plasmid vector as control were used. Following the optimized ratio (1:3), 1.3 µg pCMV-dr8.74 and 0,7 µg of pMD2-VSVG and 2 µg desired lentiviral plasmid vector and 12 µl FuGene HD (Promega, catalog no. E2311) were mixed in 500 µl serum-free medium and allowed for incubation for 30 minutes at room temperature. After incubation, the mixture was added to HEK293T cells. After 24 hours of transfection, the medium was changed. After 48 hours and 72 hours of transfection, virus containing medium was collected and stored at 80 ˚C.

3.3. Virus Titration

Virus titration is performed to figure out virus efficiency before infection. $15x10^4$ NIH3T3 cells were plated into each well of the 6-well plate prior to infection. The next day, cells were infected with serially diluted viruses as 10^{-3}, 10^{-4}, 10^{-5} supplemented with 8µg/ml polybrene (Sigma Aldrich, catalog no. 107689). The plate was centrifuged at 2500 rpm 32 ˚C for 2h and then the medium was changed. After 48h of infection, NIH/3T3 cells were transferred to 10 cm plates and selection done by 4 µg/ml Blasticidin. The selection was continued until non-infected cells died. After the selection was completed, cells were washed with 1X PBS and 0.5% Crystal Violet dye was added and incubated 20 minutes on the shaker at room temperature. Then, the excess dye was aspirated and washed with 1X PBS. The colonies of cells that were

counted indicate the efficiency of viruses and the amount of virus for infection was determined.

3.4. Infection of MCF10A Cells by Viruses

Lentiviruses are commonly used to generate stable expression cell lines. $25x10^4$ MCF10A cells were seeded into a 6-well plate prior to infection. After 24h, cells were infected with pLX304-LacZ used as control and pLX304-SEMA6D lentiviruses supplemented with 8µg/ml polybrene (Sigma Aldrich, catalog no. 107689) to enhance the efficiency of infection and plate was centrifuged at 2500 rpm, 32 ˚C for 2h. Then, the virus-containing medium was changed with the culture medium. After 48h of infection, stable cell lines were obtained by 2 µg/ml blasticidin (Santa Cruz, catalog no. sc-204655A) selection until non-infected cells completely died.

3.5. MTT Assay

MTT assay is utilized to determine the influence of alteration on cell proliferation. MTT assay is commonly utilized for a rough indicator of cell proliferation. Yellow tetrazolium (MTT) is converted to formazan as the purple insoluble product in metabolically active (viable) cells by the medium of mitochondrial dehydrogenases [62]. Stable MCF10A cells as expressing SEMA6D and its control were plated at $6x10^3$ cells per well in 24-well plate. At days 2, 4, 6 and 8 MTT measurement was taken. Stable MCF10A were incubated 4h with 10% tetrazolium dye (MTT) prepared in complete medium at 5% CO_2 atmosphere at 37 ˚C. After 4h incubation, DMSO was added in order to dissolve formazan product and transferred to flat bottom 96- well plate. Spectrophotometric measurement was performed at 570 and 650 nm by Thermo Multiskan Spectrum. For statistical analysis, two-tailed student t-test was applied.

3.6. Wound Healing

$75x10^4$ stable MCF10A cells were seeded into each well of the 12-well plate. Following day, cells were treated with 10 µg/ml Mitomycin C (Santa Cruz, catalog no. CAS 50-07-7) prepared in complete medium and were incubated 2 hours at the incubator. Mitomycin-C which causes DNA-crosslinking ultimately leading to inhibition of DNA synthesis also used as a chemotherapeutic agent in several cancer types. To avoid the toxicity of Mitomycin-C, cells treated with a low-dose and in a short time scale as 2hours only and to further minimize the proliferation of cells, starvation medium containing low serum concentrations was used throughout experiment [63]. Then, the scratch was introduced in the middle of the plate and 1% Donor Horse Serum (Gibco, catalog no. 16050-122) and 1% Penicillin/Streptomycin containing medium was added to cells. The scratched gap was monitored under Leica DMI8 confocal microscope supplemented with the incubation chamber through 72 hours at 37 ˚C with 5% CO_2. At least three positions for each well were imaged and open regions were quantified with ImageJ. Percent open areas for each condition and position were quantified in three different biological replicates. Statistical analysis was conducted by applying the two-tailed Student's t-test.

3.7. BrdU Assay

BrdU (Bromodeoxyuridine/5-bromo-2'-deoxyuridine) is a thymidine analog which is incorporated into actively replicated cell's DNA and enables to indication of proliferating cells. $15x10^4$ cells were seeded for each condition as a positive control, negative control and starvation condition. 20 µM BrdU (Abcam, catalog no. ab221240) was added to the culture medium and incubated 4h at 37 ˚C with 5% CO_2. Then, cells fixed by 4% PFA (Paraformaldehyde in 1X PBS) for 1 hour. After washing with 1X PBS 3 times, fixed cells were treated with 1.5M HCI for 30 min, and followed by blocking non-specific epitopes with 5% Donor Horse Serum in 1X PBS with 0.2% Triton X-100 for an hour. Then, cells were treated with anti-BrdU antibody prepared in

1X PBS with 2% horse serum and 1% Triton X-100 and incubated overnight at room temperature. The next day, after washing three times, the nucleus of cells was stained with DAPI and mounted on a microscope slide. Fluorescent images were captured using an Olympus U-LH100HG fluorescent microscope (Tokyo, Japan).

For each trial, negative controls were conducted to indicate the absence of non-specific binding of primary antibody. At least four different regions were analyzed in each sample. Statistical significance was calculated by using a two-tailed Student's test.

3.8. Soft Agar Colony Formation Assay

Colony formation assay allows to the investigation of anchorage-independent growth of transformed cells in a solid surface which is associated with tumorigenic potential. $3x10^4$ cells were mixed with 0.35% noble agar (BD Difco Noble Agar, catalog no. 12185-010) and seeded to on top of solidified 0.5% noble agar prepared in complete medium in each well of 6-well plates. After solidification of the top layer, the growth medium was added to each well and changed with a fresh growth medium every week for up to 6 weeks. Then, 0.05% crystal violet was used to stain colonies and the images were taken from each well with 3 different Z layers under a Leica DMI8 confocal microscope. The sizes of colonies that are higher than 30 µM in diameter were counted with ImageJ. Statistical analysis was conducted by applying the two-tailed Student's t-test.

3.9. Invasion Assay

Lab-on-a-chip system (Initio Biomedical) was utilized to investigate the invasion capabilities of cells. Growth factor reduced matrigel (Corning, catalog no. 356230) was mixed with the serum-free medium as 1:1 ratio and loaded into mid-channel and allowed for polymerization for 30 min at 37 ˚C with 5% CO_2. After that,

20% serum-containing medium was loaded to the below channel and $1x10^6$ MDA-MB-231 and MCF-7 cell/ml in serum-free medium was loaded to upper channel. The chips were incubated vertically in Hellendahl jar at 37 ˚C with 5% CO_2 in a humidified incubator for three days. Beginning from day 0, each day chips were imaged under Leica DMI8 confocal microscope and each position was visualized throughout the matrigel for 500 µM (z-stack images were produced). Images were quantified based on the distance of each bright pixel to the starting line segment that constituted the border of growth-factor reduced matrigel channel, which was calculated by a Python program. For each position, data is normalized to d0 and distribution of distances was visualized by box-plot on R-Studio version 1.1.442. For statistical analysis, two-tailed student t-test was applied.

3.10. mRNA Isolation and Semi-Quantitative Real Time RT-PCR (qRT-PCR)

Total mRNA was isolated by using Pure-link RNA mini kit (Invitrogen Thermo Scientific, catalog no. 12183018A) and treated with PureLinkTM DNase (Invitrogen, catalog no. 12185-010) to eliminate DNA contamination. The concentration of RNAs was determined by NanoDrop (Thermo Scientific). Complementary DNA (cDNA) was synthesized by RevertAid first-strand cDNA synthesis kit (Thermo Scientific, catalog no K1622) from 1 µg total RNA by using random hexamer primers. mRNA expression levels of desired genes were identified by Roche-Light Cycler 96 Real-Time PCR Detection system by using FastStart Essential DNA Green Master (Roche, catalog no. 06402712001). FastStart Essential DNA Green Master comprise of FastStart Taq DNA Polymerase and double-stranded DNA specific SYBR Green I dye. TATA-box binding protein (TBP) was chosen for normalization as being housekeeping gene and the delta-delta Ct method was performed to calculate relative mRNA expressions. Non-template controls were used in each trial. For statistical analysis, two-tailed student t-test was applied.

Following primer pairs were used:

TBP-Forward Primer 5'-TAGAAGGCCTTGTGCTCACC-3'

TBP-Reverse Primer 5'-TCTGCTCTGACTTTAGCACCTG-3'
SEMA6D-Forward Primer 5'-TTTCCCAGTTGAGGGCAGTC-3'
SEMA6D-Reverse Primer 5'-AGGGCGTCCTCTAAAAACCG-3'

3.11. Stem Cell Markers-Flow Cytometry

$50x10^4$ cells were resuspended in 1X PBS and incubated with CD44 antibody (Thermo Scientific, catalog no. MA1-10229, FITC conjugated, 2µg/ml) and CD24 antibody (Thermo Scientific, catalog no. MA1-10154, PE conjugate, 20µl/10^6 cells) at room temperature in dark for 30 minutes. After that, cells were washed with 1X PBS and spun down at 1400 rpm for 2 minutes. The supernatants were eliminated and 1X PBS was added to cells. Cells were sorted by BD FACS Canto flow cytometry and analyzed by BD original software.

3.12. Propidium Iodide Stain

For cell cycle analysis, propidium iodide stain was used. $20x10^4$ MCF10A SEMA6D and MCF10A LACZ infected cells were seeded in a 6-well plate. After 72 hours of seeding, cells were trypsinized and collected in falcon tubes. After that, cells were centrifuged for 10 minutes at 1200 rpm. The pellet was resuspended in cold 1X PBS very gently and falcons were placed on ice. Then, 4ml cold absolute ethanol was added and was mixed gently by pipetting. The falcons were placed at -20 ˚C at least overnight for up to 1 month. After overnight incubation, the falcons were centrifuged at 1500 rpm for 10 minutes and the following centrifugation at 2000 rpm for 1 minute at 4 ˚C. The supernatants were aspirated and the pellet was resuspended in cold 1ml PBS. Then, solutions transferred to eppendorf tubes and tubes were centrifugated at 1500 rpm for 10 minutes at 4 ˚C. The supernatant was removed and the pellet was mixed with 200 µl 0.1% Triton-X-100/PBS and 20 µl Rnase A (200 µg/ml) and incubated at 37 ˚C for

30 minutes. After incubation, 20 µl PI (1mg/ml) was added and incubated for 15 minutes in dark. The cell cycle was analyzed with BD FACS Canto flow cytometry.

3.13. Protein Isolation and Western Blotting

After stable cell lines generated, the protein expression level of the interested gene as SEMA6D were analyzed by western blotting method.

3.13.1. Protein Isolation

Total protein from flash-freezed cells was isolated by RIPA lysis buffer supplemented with 1mM DTT, 1X protease inhibitor, phosphatase inhibitors, 50 mM NaF and 1mM sodium orthovanadate (Na_3VO_4). Then, cells were scraped with the presence of RIPA lysis buffer and the lysate was collected in eppendorf tubes. After that, lysed cells were homogenized with 26G syringe and lysates were incubated on ice for 20 minutes. Then, the tubes were centrifuged at 14000 rpm at 4 $^{\circ}$C for 20 minutes. After centrifugation, supernatants were transferred to clean eppendorfs and kept at 80 $^{\circ}$C.

3.13.2. Protein Quantification

Total protein content was determined with Bradford assay. For protein standard, 20 mg/ml BSA was diluted to different concentrations as 0.5, 1, 2, 4, 8 mg/ml into water. 10 µl from diluted standards and protein samples were dissolved in 800 µl water in 1 cm cuvette. Then, 200 µl 5X Bradford dye solution was added to each sample and mixed by pipetting well. The absorbance of each sample was determined at 595 nm by a

spectrophotometer. The relative protein concentration of isolated proteins was calculated according to the standard curve equation.

3.13.3. Western-Blot Procedure

60 µg of total protein, 6 µl 5X loading dye and water up to 30 µl were mixed in eppendorf and boiled at 95 ˚C for 5 minutes. Protein samples and 5 µl protein marker (NEB, catalog no. P7719S) were run on 5% stacking and 10% resolving SDS-polyacrylamide gel. After that, the proteins were transferred to PVDF membrane (MilliporeSigma Immobilon-P-transfer membrane, pore size 0.45 µM, catalog no. IPVH00010). Blocking of membranes was performed with 5% milk powder in TBS-Tween-20 (TBS-T) for either 2 hours at room temperature or overnight at 4 ˚C. Then, after blocking, membranes were incubated with primary antibody diluted in 5% milk powder in TBS-T for 2 hours at room temperature. After that, membranes were washed with 1X TBS-T for 10 minutes 3 times and membranes were incubated with secondary antibody for 2 hours at room temperature. After secondary antibody incubation, membranes were washed for 10 minutes, 3 times and the interested proteins were detected with Clarity Western ECL Substrate (Bio-Rad, catalog no. 170-5061) by using Vilber Fusion SL Imaging System. Images were quantified by ImageJ gel analysis tool and Beta-actin was used to housekeeping protein for normalization.

The following primary antibodies were used against targeted protein: rabbit polyclonal anti-β-actin (Abcam, catalog no. ab75186, 1:2000 diluted), rabbit polyclonal anti-SEMA6D (Abcam, catalog no. ab191169, 1:250 diluted). For statistical calculations, a two-tailed student t-test was used.

CHAPTER 4

RESULTS

4.1. Overexpression of SEMA6D Gene in MDA-MB-231, MCF-7 and MCF10A Cells by Lenti-virus Based System

To understand the effect of SEMA6D in breast cancer cell lines as MDAMB231 and MCF-7 and immortalized non-tumorigenic breast cell line as MCF10A, ectopic expression of SEMA6D was introduced by lentivirus based system. After stable cell lines were generated by the antibiotic selection, the amount of SEMA6D expression in mRNA and protein level was quantified via RT-PCR and Western blot, respectively. SEMA6D overexpression in MDAMB231 and MCF-7 cell lines were performed in our lab previously. LacZ was used as a control. Overexpression of SEMA6D leads to elevated expression levels such as 1400 times in MCF-7 cells, 429 times in MDA-MB-231 cells and 742 times in MCF10A cells (Figure 4.1, Figure 4.2). The protein level was 2.8 times increased with SEMA6D overexpression (Figure 4.3).

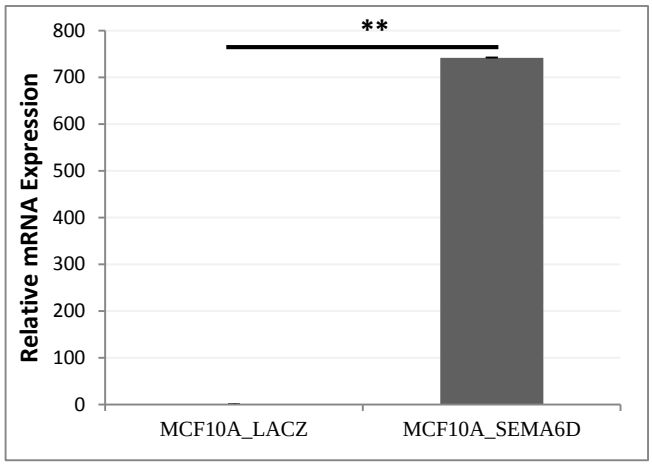

Figure 4. 1. Semi-Quantitative RT-PCR results of overexpression after stable MCF10A cell line was selected with 4 µg/ml blasticidin. TATA-binding protein (TBP) was used as internal reference gene for normalization. (n=3, **p<0.01)

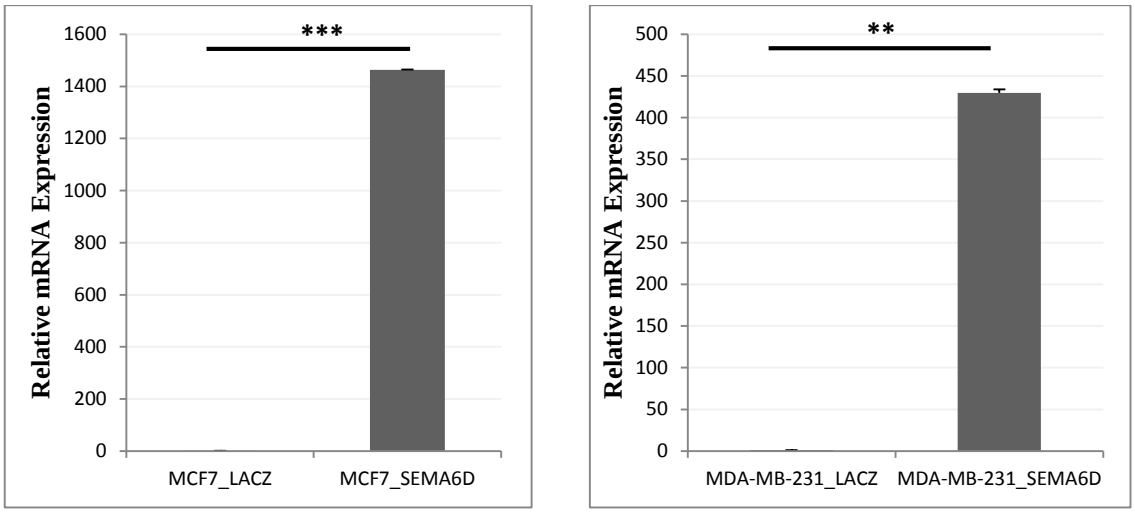

Figure 4. 2. Semi-Quantitative Real time PCR results of SEMA6D overexpression after stable MCF-7 and MDA-MB-231 cell lines were selected with 4 µg/ml blasticidin. TATA-binding protein (TBP) was used as housekeeping gene. (n=3, **p<0.01, ***p<0.001)

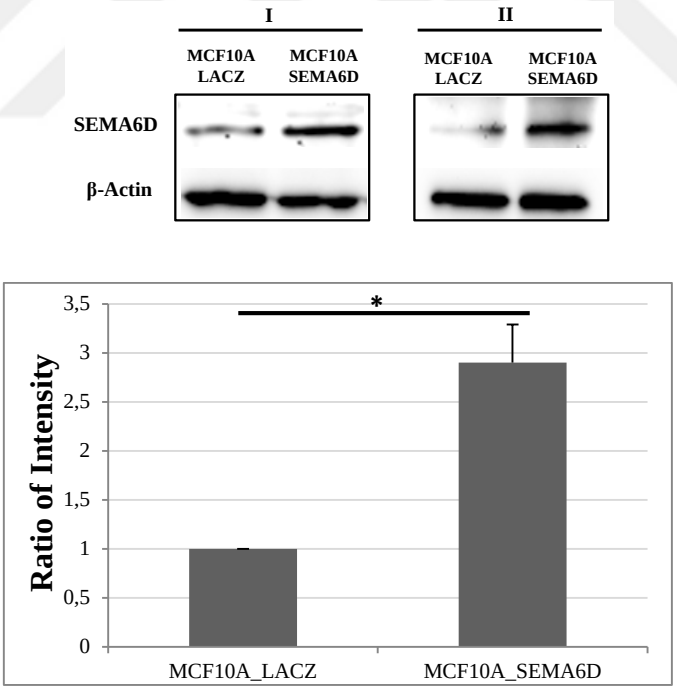

Figure 4. 3. Western Blot results of SEMA6D overexpression in MCF10A cells. Relative expression levels of SEMA6D were normalized to its control. (n=2, *p<0.05)

4.2. Effect of SEMA6D Gene on Proliferation in MCF10A Cells

To investigate the impact of SEMA6D overexpression on the proliferation of non-tumorigenic breast epithelial cell line MCF10A, MTT assay was performed. SEMA6D regulates cardiomyocyte proliferation during fetal-to-neonatal transition process [61]. So, SEMA6D may also promote proliferation in the non-tumorigenic breast cell line. According to Figure 4.4, cell proliferation was enhanced significantly with regards to SEMA6D overexpression in MCF10A cells on day 4, day 6 and day 8. During normalization of overall data, each day normalized to day 2 for two cells separately. Then, MCF10A SEMA6D overexpressing cells were normalized to its control MCF10A LACZ.

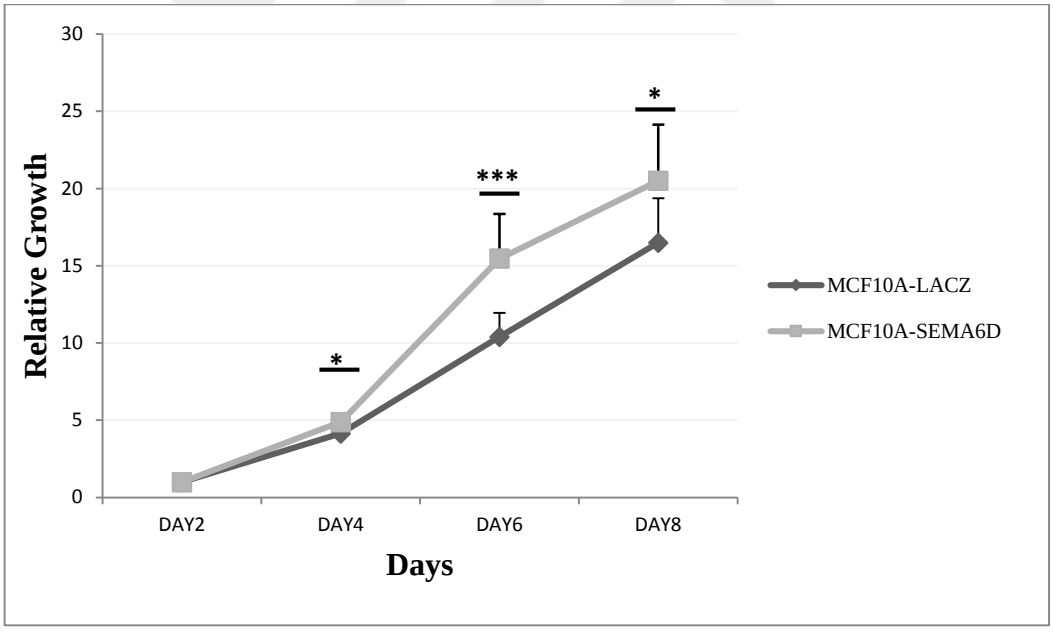

Figure 4. 4. Results of MTT assay representing proliferation of MCF10A cells overexpressing SEMA6D and its control LACZ. All days normalized with respect to day 2 in itself. (n=3, *p<0.05, **p<0.01, ***p<0.001)

BrdU assay was performed to understand the effect of SEMA6D expression on proliferation in MCF10A cells. BrdU (5-Bromo-2'-Deoxyuridine) functions as a

thymidine analog that is incorporated into DNA during replication and consequently can be used to assess the proliferation rate of cells. There was a significant difference in BrdU incorporation among SEMA6D overexpressing MCF10A and its control (Figure 4.5). Negative control was conducted as only primary antibody treatment no BrdU treatment.

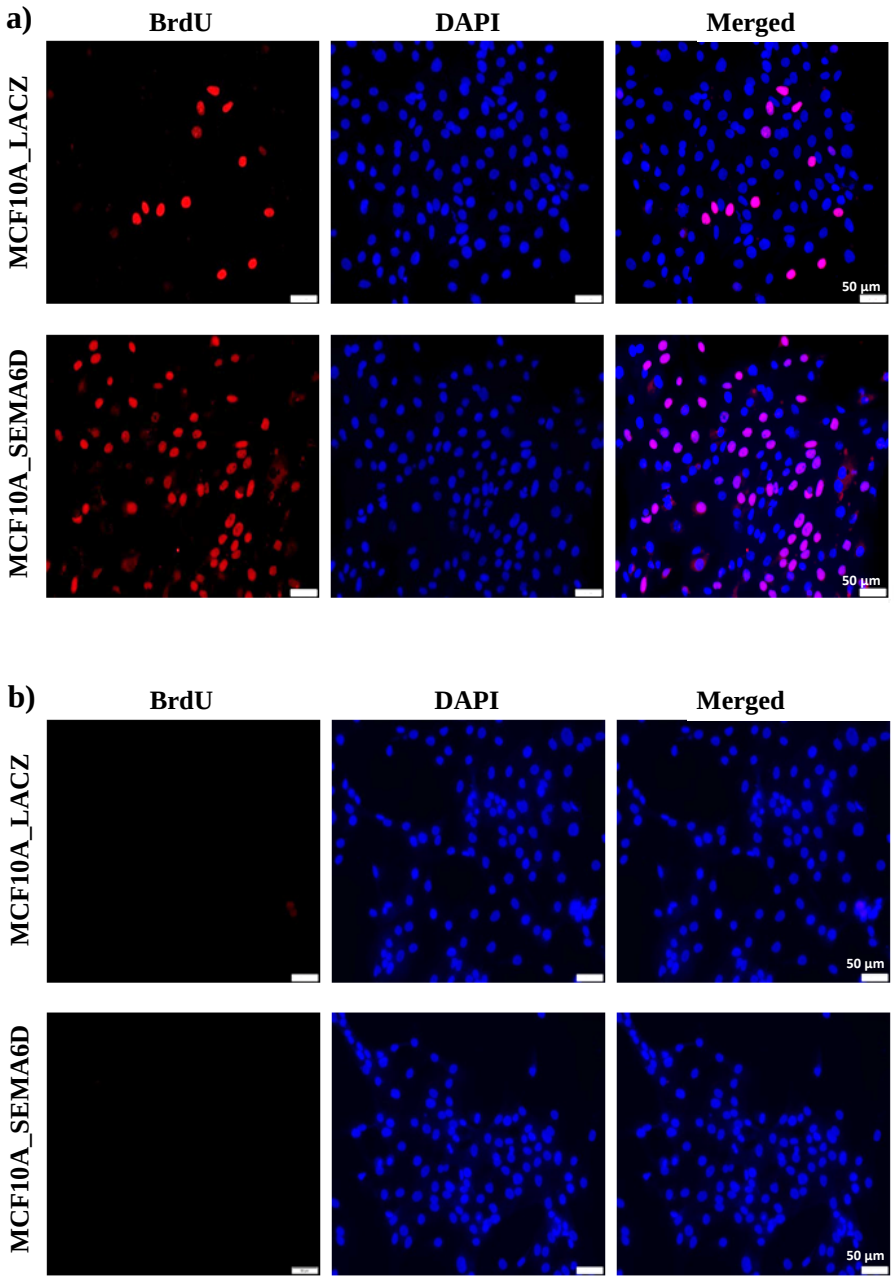

Figure 4. 5. (a) Photomicrographs of BrdU positive cells in MCF10A_LACZ (Control) and MCF10A_SEMA6D cells and (b) their negative controls to demonstrate antibody specificity against BrdU. (scale bar:50 µm)

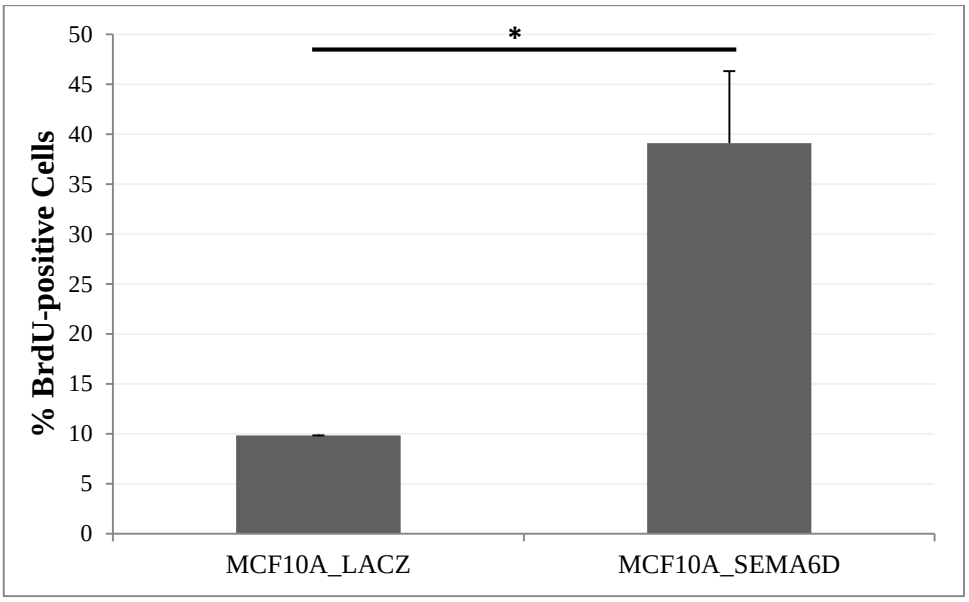

Figure 4. 6. Percentage of BrdU positive cells in MCF10A_LACZ (Control) and MCF10A_SEMA6D cells 48h after culturing (n=3, *p<0.05)

4.3. Cell Cycle Assessments in MCF10A Cells

To further investigate the effect of SEMA6D overexpression in the proliferation of MCF10A cells propidium iodide (PI) staining was performed and the percentage of cells present in cell cycle phases as G1, S, G2 was calculated. The cells were harvested plating after 72h and were stained with propidium iodide following day.

The percentage of cells in the G1 phase reduced for SEMA6D overexpressed MCF10A cells when compared to its control. Also, there was an elevated percent of cells in the S phase significantly in SEMA6D overexpressing cells as 23% than control as 18%. Additionally, in the G2 phase, the percentage of cells slightly decreased in MCF10A LACZ control cells (Figure 4.7).

So, based on propidium iodide staining results, SEMA6D overexpression caused an enhanced percentage of cells in the S phase which can be associated with upregulated proliferation due to SEMA6D overexpression in MCF10A cells.

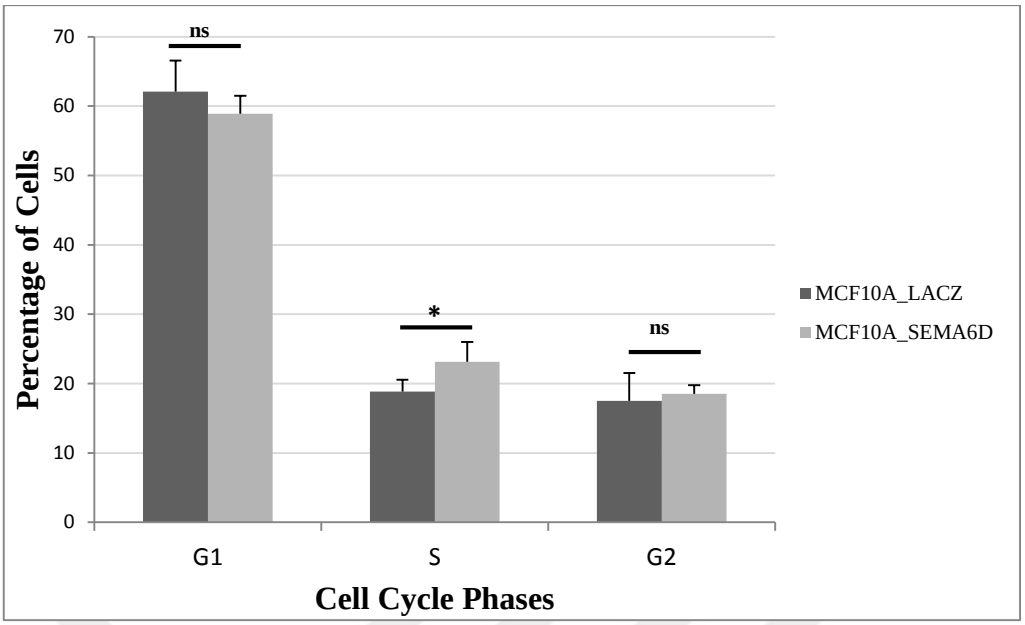

Figure 4. 7. Percentage of population of stable MCF10A cells at G1, S, G2 phases were demonstrated. (n=4, * $p<0.01$; ns, not significant)

4.4. Overexpression of SEMA6D Gene Enhance Migration in MCF10A Cells

MCF10A cells which stably express the SEMA6D gene was seeded for wound healing experiment also called scratch assay. Coordinated movement of cells and cell to cell interaction can be distinguished with wound healing assay. In order to prevent any effect of proliferation on wound closure, cells were treated with low-dose of the anti-proliferative drug as Mitomycin-C.

Overexpression of SEMA6D in MCF10A cells induce migration considerably compared to its control LACZ. At 12h, 14% of the gap was closed by the control group while 33% of the gap was closed by SEMA6D overexpressing MCF10A cells. At 24h, the gap closed by MCF10A cells stably overexpressing SEMA6D was 46% whereas in the control group 23% of the gap closed (Figure 4.8 b). Consequently, the percent of open area decreased much rapidly in MCF10A cells overexpressing SEMA6D with respect to its control (Figure 4.8 a).

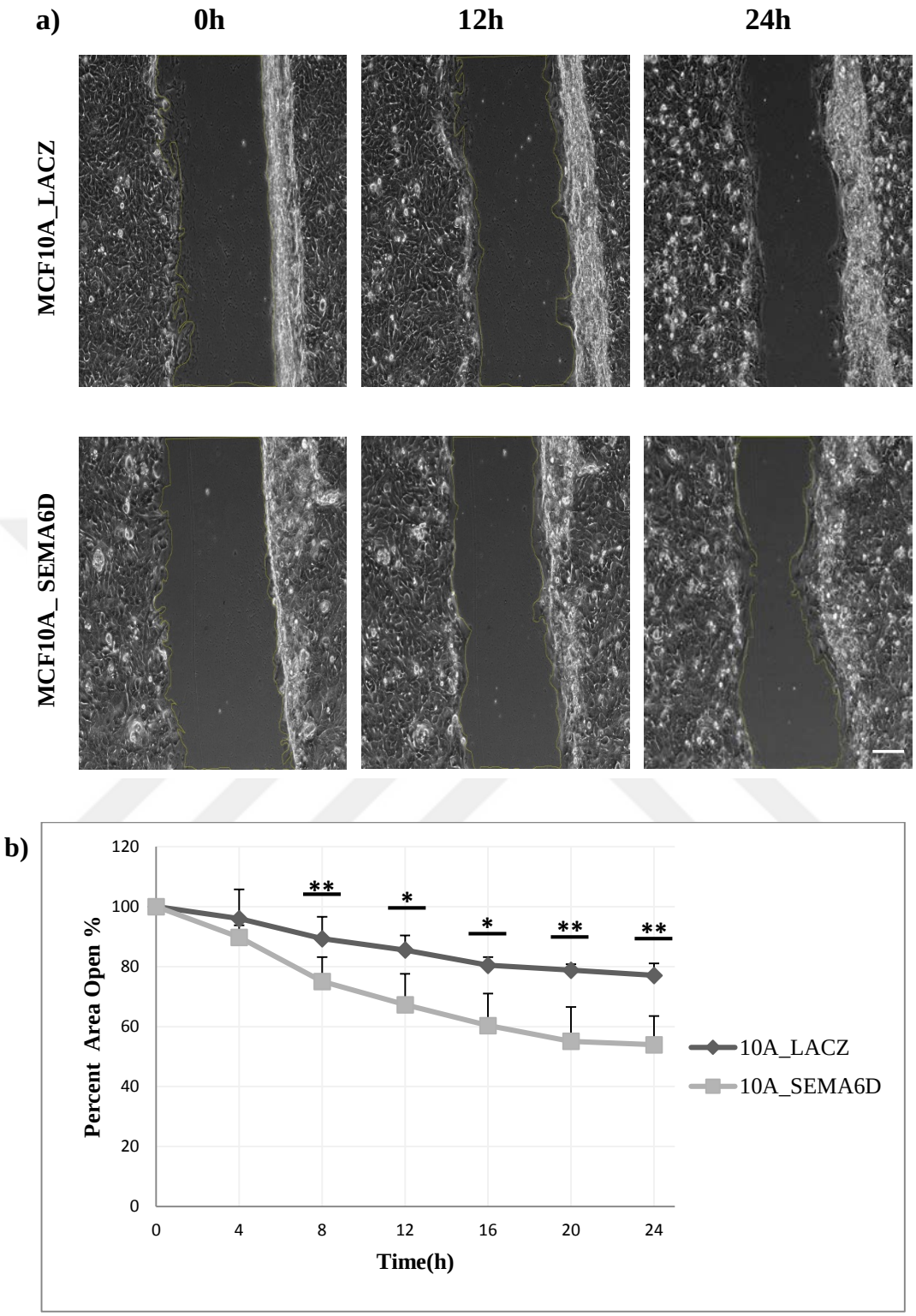

Figure 4. 8. (a) Representative results of wound healing assay and (b) Quantification of percent open area in SEMA6D overexpressing MCF10A cells and its control MCF10A LacZ. (n=3, *p<0.05, **p<0.01, scale bar:100 µm)

4.5. Effect of SEMA6D Overexpression on Invasion of MDA-MB-231 and MCF-7 Cells

Local invasion of tumor cells which occur breaching through the basement membrane and extracellular matrix trigger initiation of metastasis. To mimic the invasion process, the lab-on-a-chip system was utilized. There were three channels in the lab-on-a-chip system. Growth factor reduced Matrigel was loaded to the middle channel and after polymerization cells were loaded to the upper channel and also below channel was used as chemoattractant serum rich medium reservoir. After each day the distance of each pixel to the starting point as borderline of matrigel was measured by Python program and box-plot representation indicates the distribution of distances plotted with R Studio. SEMA6D overexpressing MDA-MB-231 cells demonstrate enhanced invasion through the matrigel than its control (Figure 4.9).

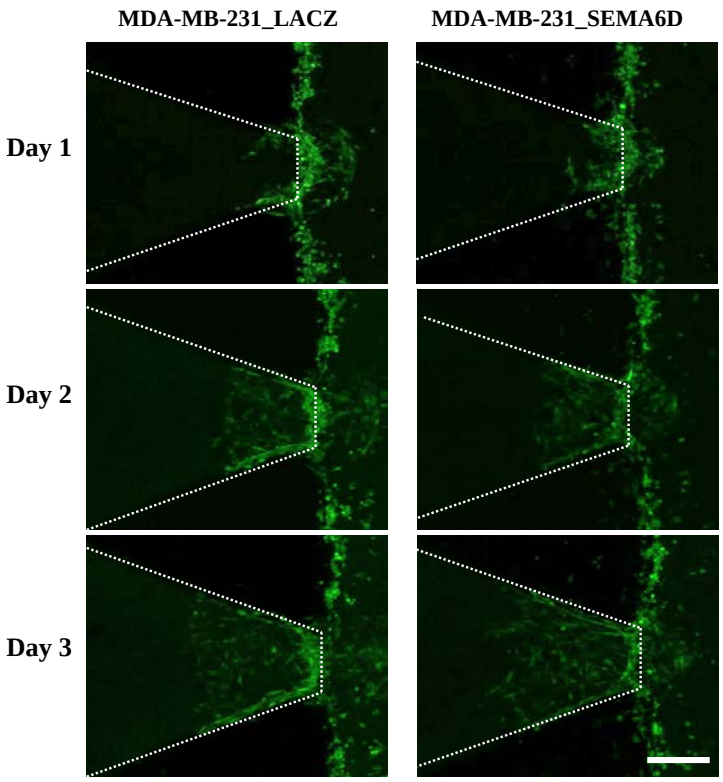

Figure 4. 9. Photomicrographs demonstrates that effect of SEMA6D on invasion on MDA-MB-231 at day 1, 2 and 3. (Scale bar: 300 µm)

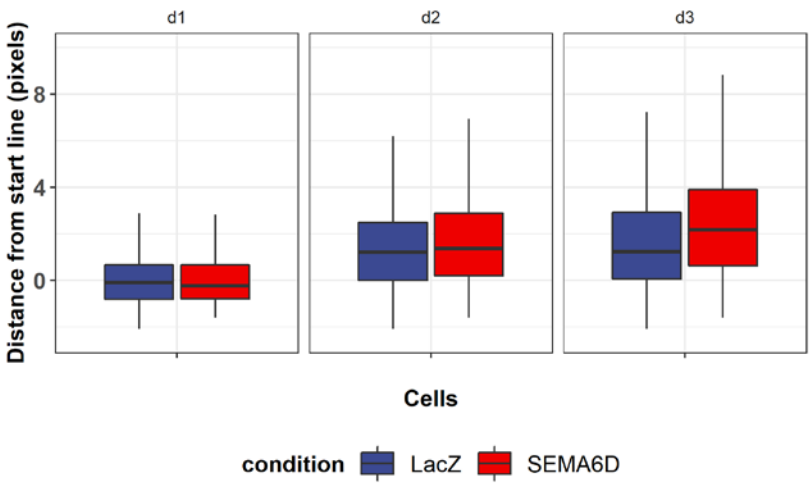

Figure 4. 10. Box-plot representation of distance of each pixel of MDA-MB-231 SEMA6D and its control MDA-MB-231 LACZ in lab-on-a-chip system with respect to starting point in day 1, day 2, day 3. All days normalized to day 1.

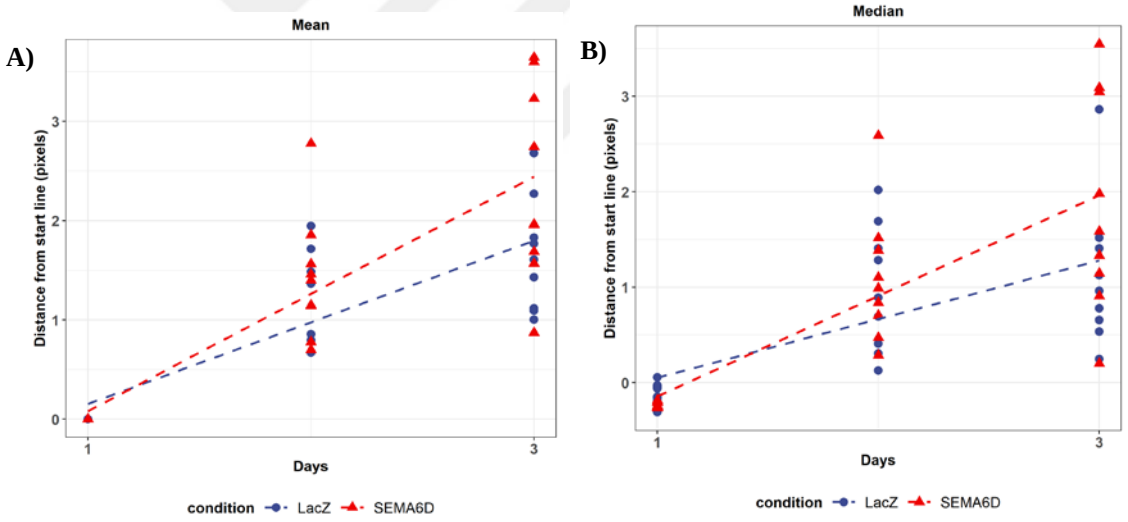

Figure 4. 11. (A) Mean and (B) Median of distance from starting line of each pixel in day 1, day 2, day 3. Each pixel obtained from 3 different positions (n=3) belong to MDA-MB-231 SEMA6D and its control MDA-MB-231 LACZ.

In MCF-7 cells, overexpression of SEMA6D did not contribute to the invasiveness of the cells (Figure 4.12). Indeed, the MCF-7 cell line is defined as noninvasive and has less metastatic capability; accordingly, we tried to find out the effect of elevated levels of SEMA6D on invasion [64].

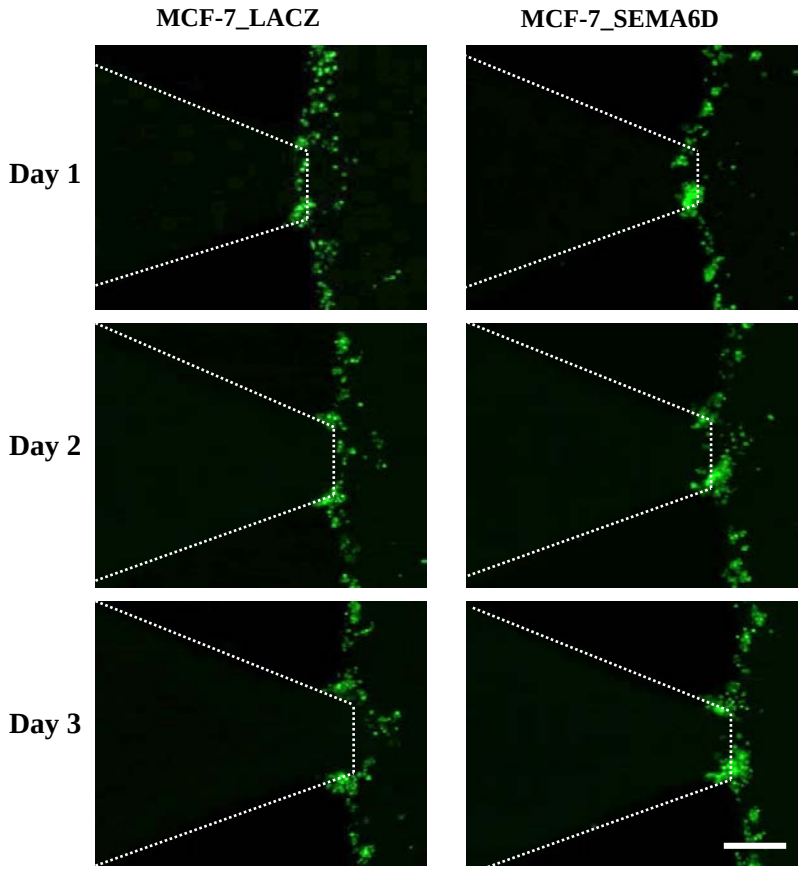

Figure 4. 12. Photomicrographs demonstrate that effect of SEMA6D on invasion on MCF-7 cells and its control LACZ at day1, 2 and 3. (Scale bar: 300 µm)

4.6. Microenvironment Dependent as Bone-Specific Invasion of SEMA6D Overexpressing MDA-MB-231 and MCF-7 Cells

SEMA6D is one of the driving factors of osteosarcoma and validated as an oncogene in the development of human osteosarcoma [59]. During the invasion modeling in the lab-on-a-chip system, in order to mimic the basement membrane the only matrigel was used. So, we hypothesized that secreted factors from the bone microenvironment might enhance the invasion of MDA-MB-231 SEMA6D overexpressing cells. Also, only matrigel microenvironment might not be enough for induction of invasion in MCF-7 cells stably expressing SEMA6D so further modification of matrigel microenvironment may be required to trigger invasiveness in

MCF-7 SEMA6D overexpressing cells. Moreover, SEMA6D signaling occurs both by means of locally via cell to cell interaction and ectodomain secretion [61]. So, secreted factors from bone-microenvironment or cell to cell interaction might contribute to invasion capability. Bone-microenvironment was generated by hFOB cells and mixed with matrigel before loading to lab-on-a-chip system and chips incubated 24h at incubator 5% CO_2 at 37 ˚C. The cells were loaded upper channel after 24h of incubation.

According to Figure 4.13, the bone-microenvironment enhances the migration of MDA-MB-231 SEMA6D overexpressing cells in comparison with its control and only matrigel microenvironment. Additionally, the bone-microenvironment is slightly inducing the MCF-7 cell invasiveness (Figure 4.18).

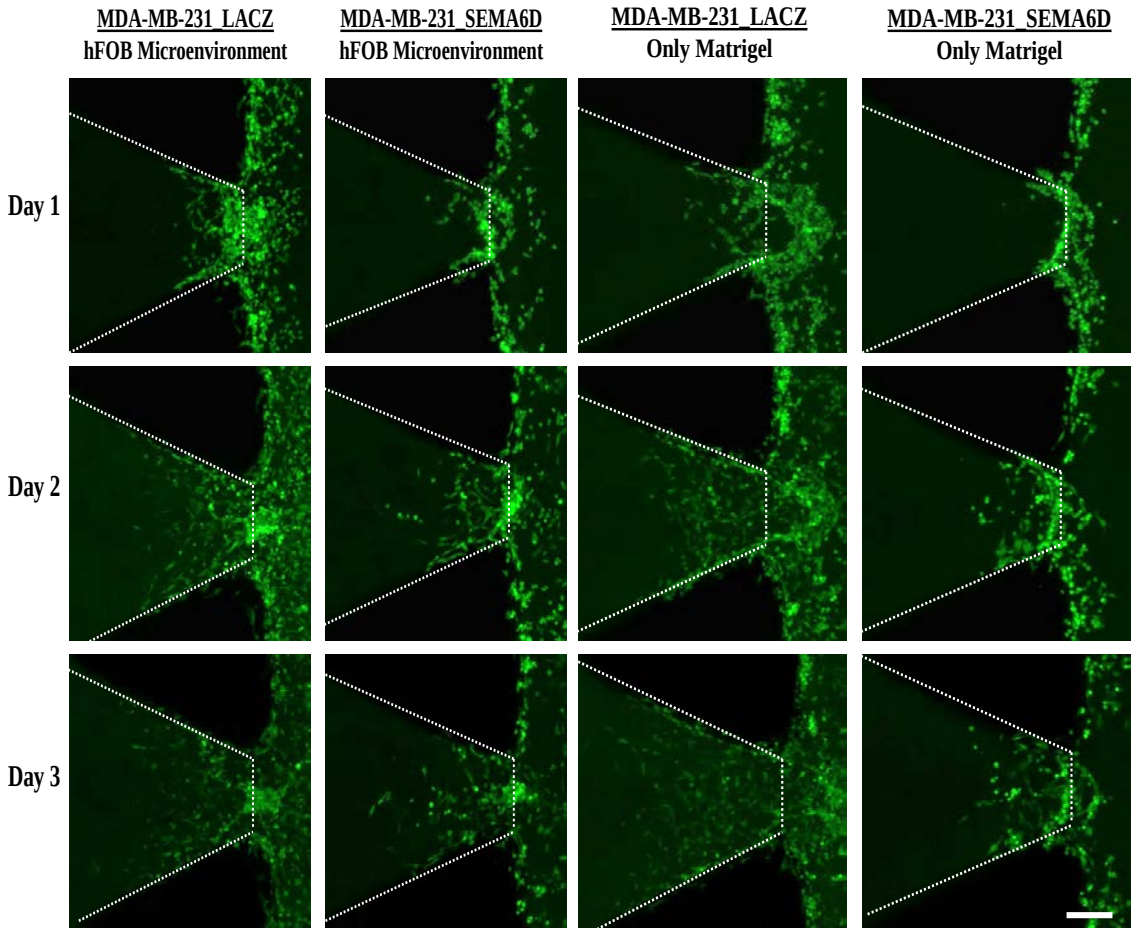

Figure 4. 13. Photomicrographs demonstrates that effect of SEMA6D on invasion of MDA-MB-231 cells through bone-microenvironment.(Scale bar: 300µm)

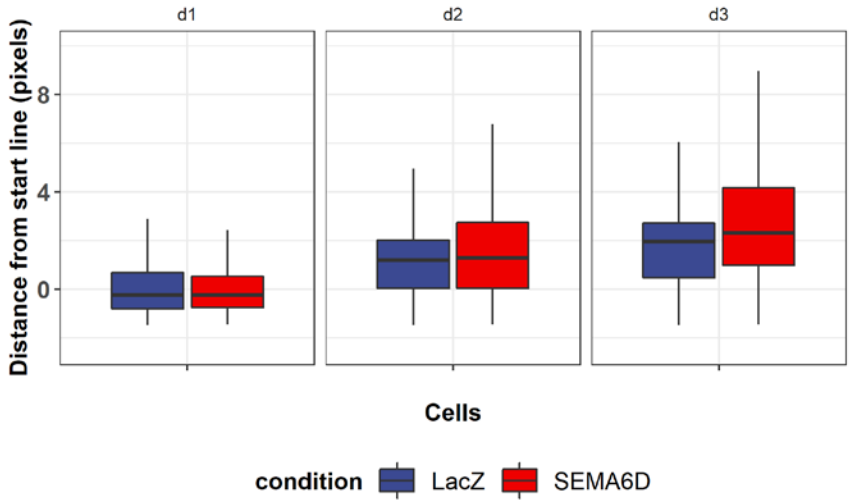

Figure 4. 14. Effect of SEMA6D overexpression on invasion of MDA-MB-231 cells demonstrated as box plot representation as distance of each bright pixel according to starting line through bone-microenvironment.

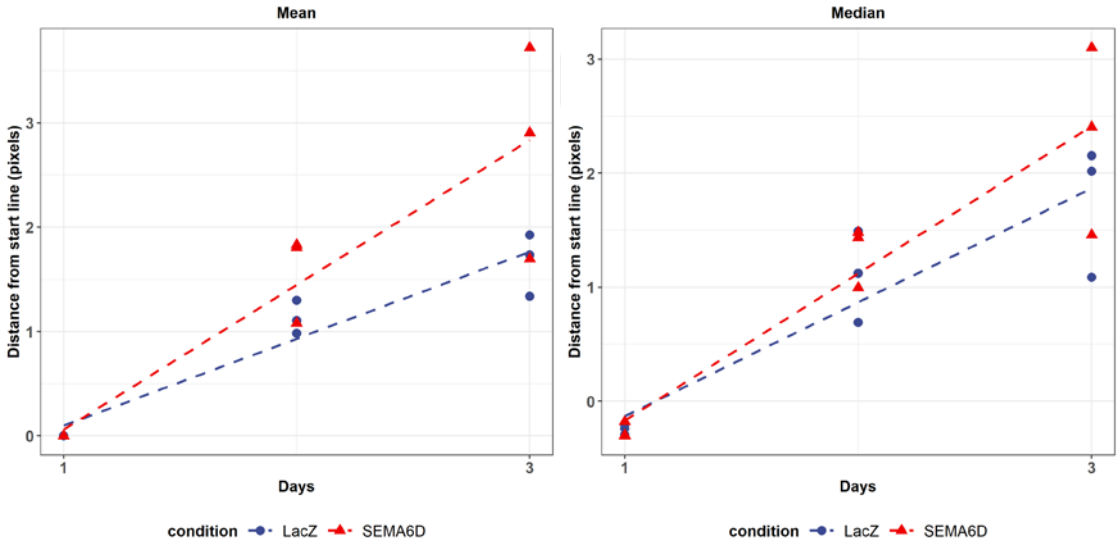

Figure 4. 15. A) Mean B) Median of all distance of each pixel of MDA-MB-231 SEMA6D and its control MDA-MB-231 LACZ from starting line in day 1, day 2, day 3 through bone-microenvironment.

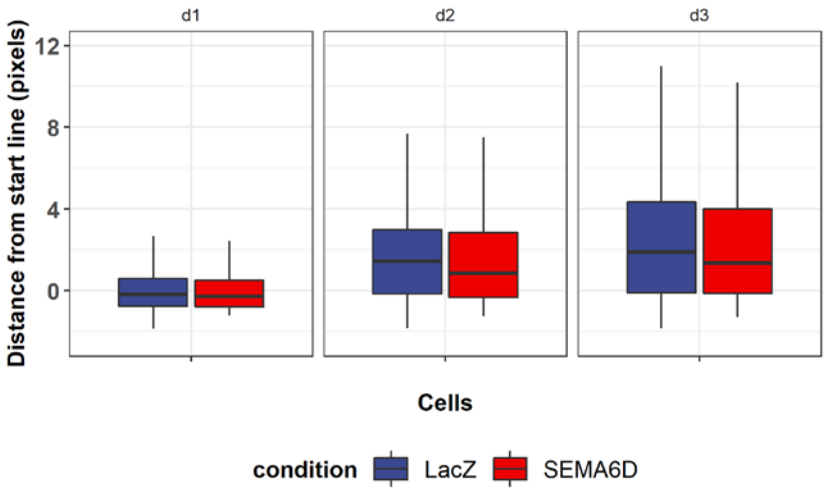

Figure 4. 16. Effect of SEMA6D overexpression on invasion of MDA-MB-231 cells demonstrated as box plot representation as distance of each bright pixel according to starting line through only matrigel containing microenvironment.

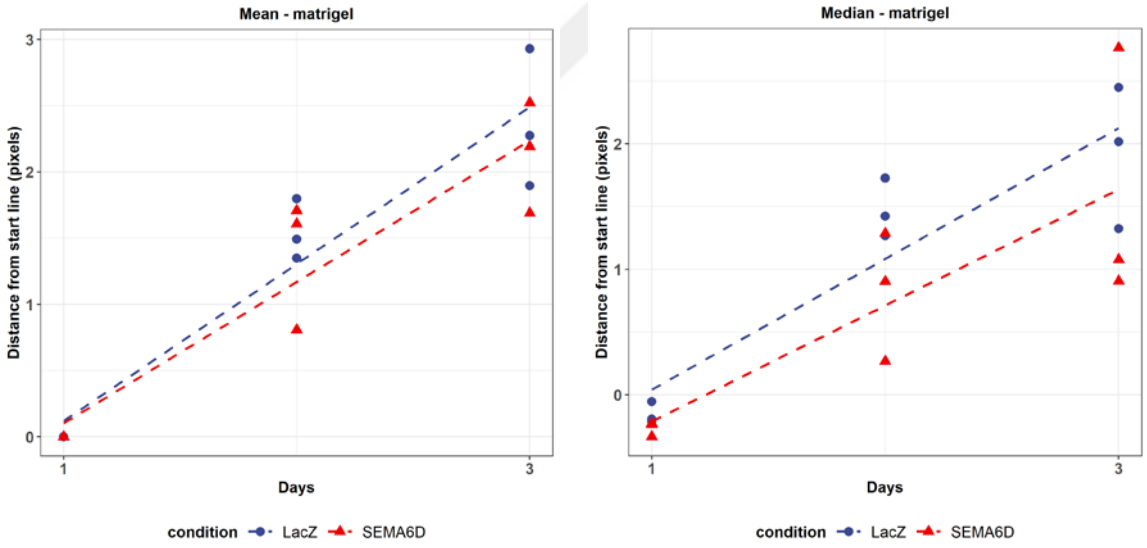

Figure 4. 17. Mean and Median of all distance of each pixel of MDA-MB-231 SEMA6D and its control MDA-MB-231 LACZ from starting line through matrigel microenvironment in day 1, day 2, day 3.

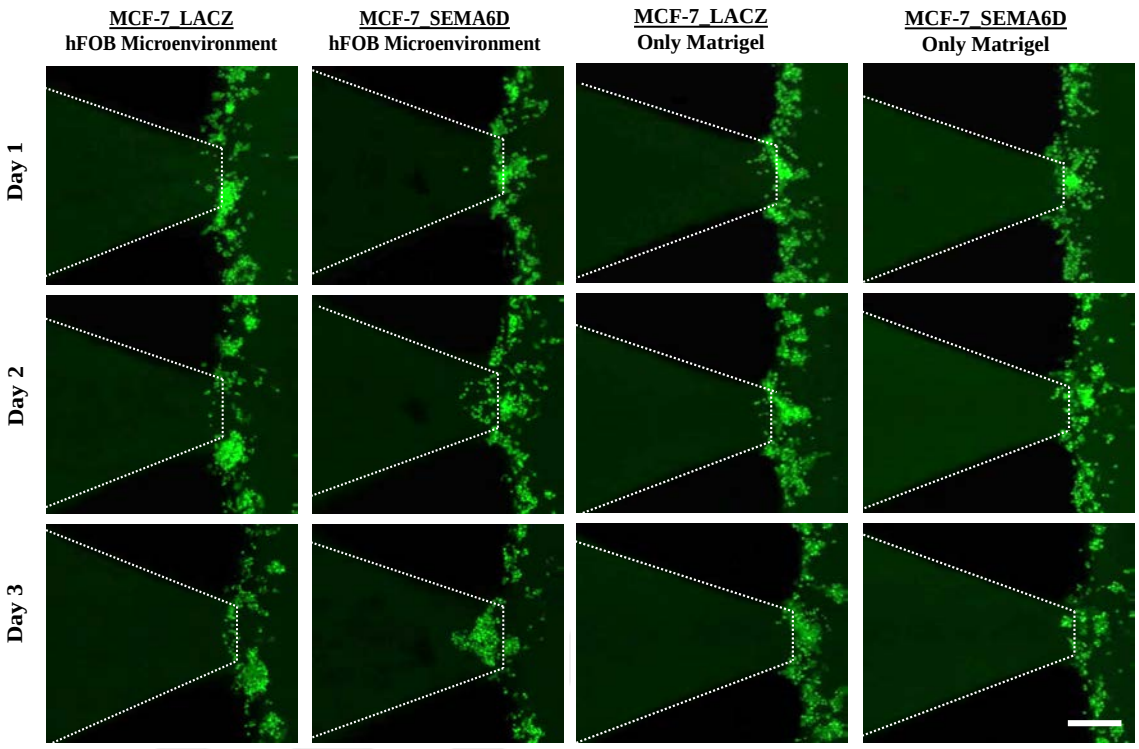

Figure 4. 18. Photomicrographs demonstrates that effect of SEMA6D on invasion of MCF-7 cells through bone-microenvironment (Scale bar: 250 µm)

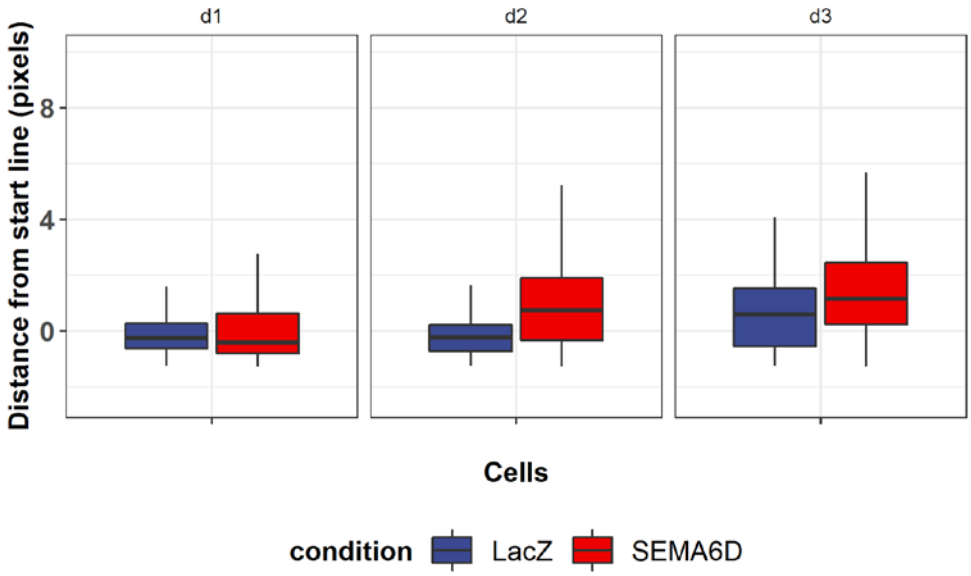

Figure 4. 19. Effect of SEMA6D overexpression on invasion of MCF-7 cells demonstrated as box plot representation as distance of each bright pixel according to starting line through bone microenvironment.

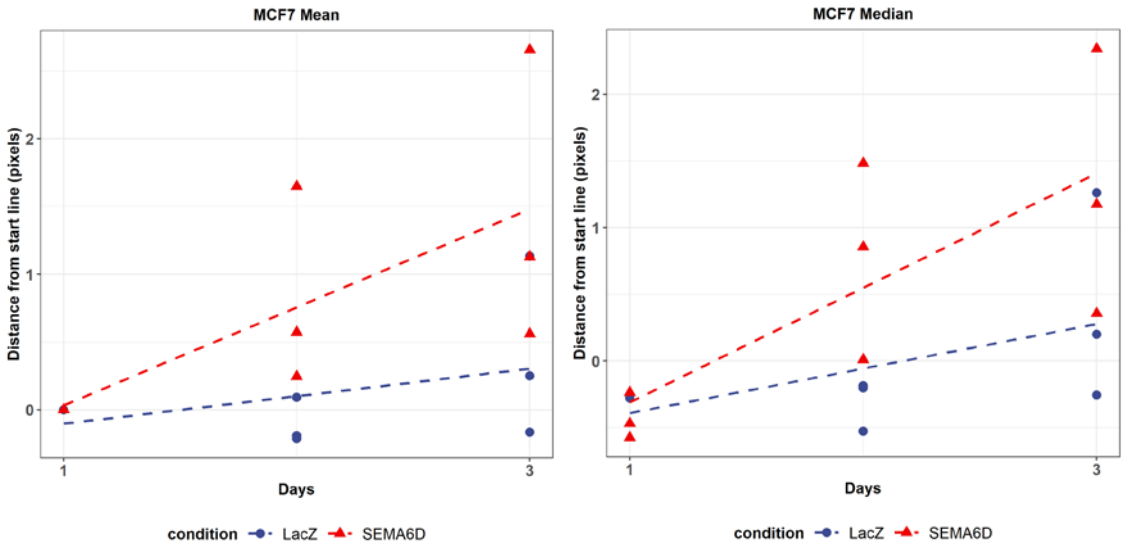

Figure 4. 20. Mean and Median of all distance of each pixel of MCF-7 SEMA6D and its control MCF-7 LACZ from starting line through bone microenvironment in day 1, day 2, day 3.

4.7. Detection of Transformation of Breast Cancer Cells Depends on SEMA6D Overexpression in MDA-MB-231 and MCF-7 Cells by Soft-Agar Assay

The soft-agar assay is a widely used in vitro detection method for carcinogenesis in virtue of anchorage-independent growth of cells. Cells that gain the ability to proliferate in the solid surface indicate the malignant transformation of cells [65]. Analysis of soft agar assay was performed by counting colonies bigger than 30 µm. For each cell and condition, colonies from 25 images with 3 different z layers per well were quantified using ImageJ software. To understand how SEMA6D overexpression affects tumorigenicity and cellular transformation of breast cancer cells, three independent experiments were established and quantified for MDA-MB-231 while for MCF-7 colony number from two independent experiments were calculated. Colony formation in SEMA6D overexpressed MDA-MB-231 cells was roughly two-fold decreased with respect to the control group (Figure 4.22). Furthermore, in MCF7 cells overexpression of SEMA6D led to decreased colony formation (Figure 4.24).

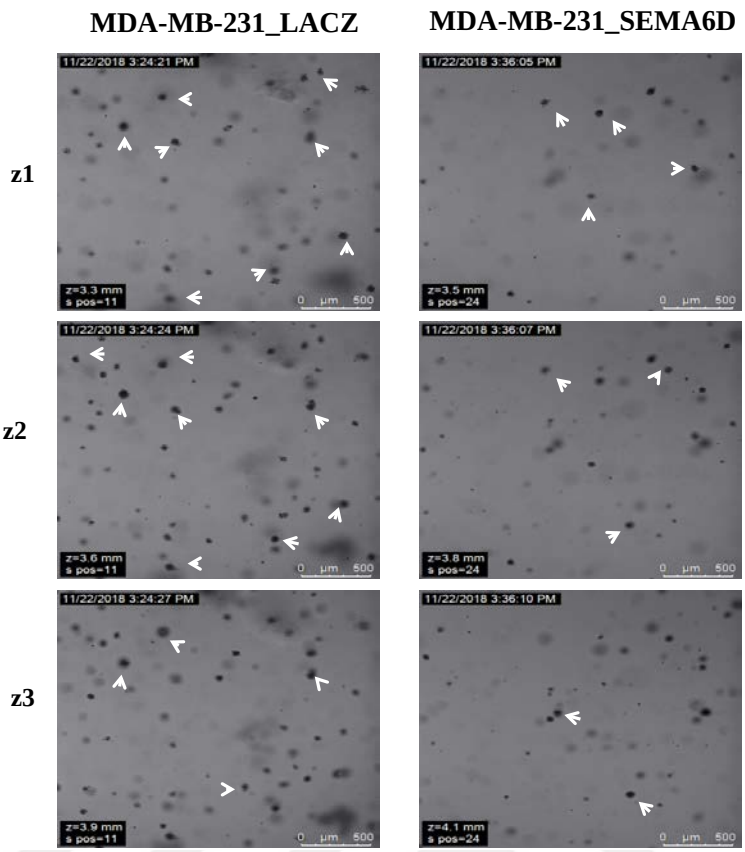

Figure 4. 21. Photomicrographs of stable MDA-MB-231 cells demonstrates colonies grown in solid agar for 5 weeks and representative counted colonies is pointed out by arrows in three different focal places of same area. (Scale bar: 500 µm)

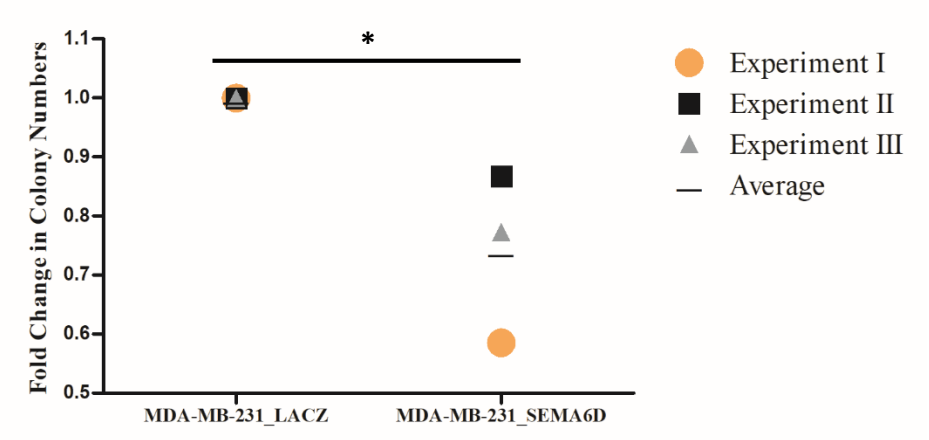

Figure 4. 22. Quantification of number of colonies in soft agar images of infected MDA-MB-231 bigger than 30 µm. (n=3, *p<0.05)

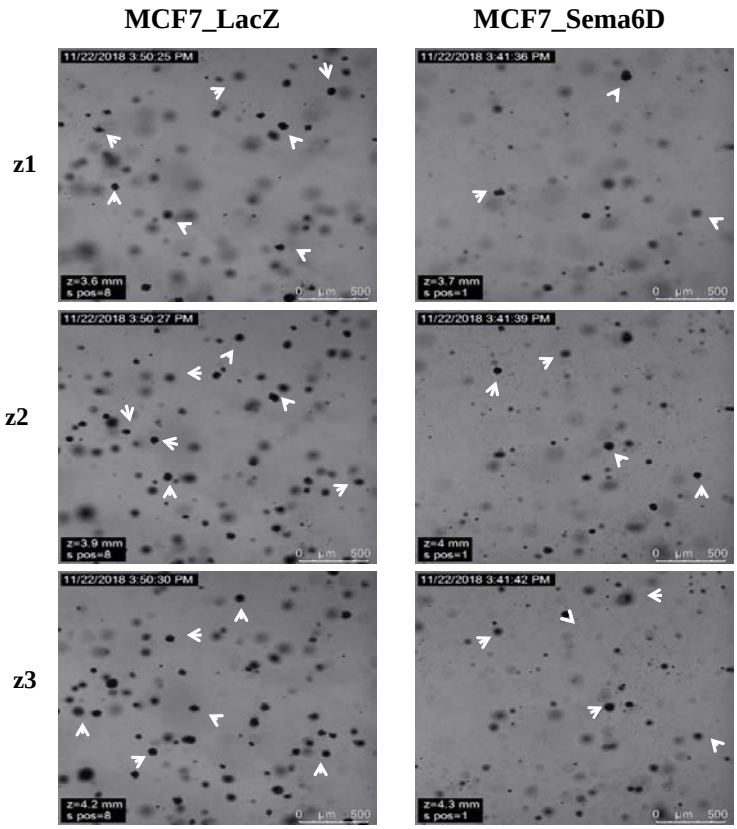

Figure 4. 23. Photomicrographs of SEMA6D overexpressed MCF-7 cells and its control reveals colonies grown in solid agar for 6 weeks and representative counted colonies is pointed out by arrows in four different focal places of same area. (Scale bar: 500 µm)

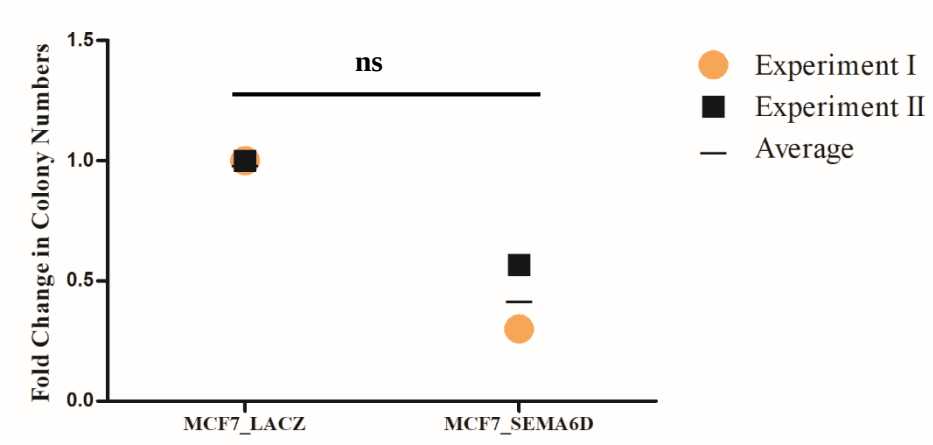

Figure 4. 24. Quantification of number of colonies of MCF-7 SEMA6D and its control MCF-7 LACZ, colonies bigger than 30 µm were counted (n=2, p=0,05171)

4.8. Detection of Stem Cell Population in MDA-MB-231 and MCF-7 Cells by Flow Cytometry

Cancer stem cell population is the sub-population of cancer cells that possess heterogeneity and has the capability to regulate tumor growth, invasion, metastasis, and drug resistance. They can undergo self-renewal and differentiation. CD44/CD24 has mostly used cancer stem cell markers in breast cancer and defined on the basis of $CD44^{+}CD24^{-/low}$ expression [66]. Due to decreased colony formation with overexpression of SEMA6D in MDA-MB-231 and MCF-7 cell lines, we hypothesized that there might be a change in their stem cell population. MDA-MB-231 cell line expresses highly $CD44^{+}CD24^{-}$ that associated with enhanced invasive features. According to Figure 4.25, SEMA6D overexpression in MDA-MB-231 cells did not significantly change $CD44^{+}CD24^{-}$ population.

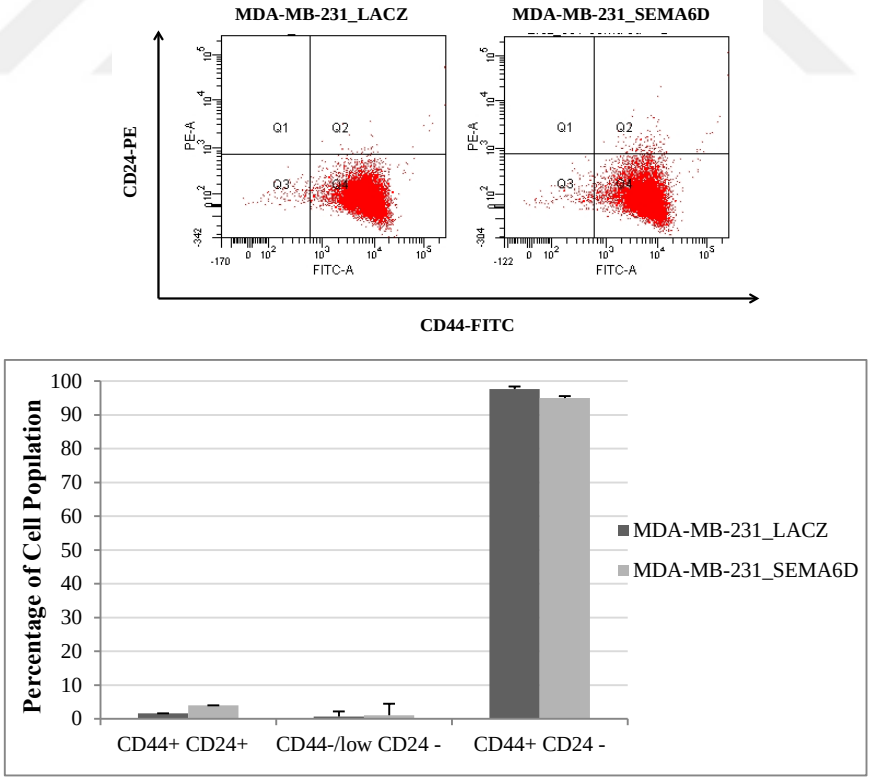

Figure 4. 25. Cancer stem cell subpopulations identified by CD24 and CD44 expression in MDA-MB-231 overexpressing SEMA6D and MDA-MB-231 LACZ control cells. (n=4)

In MDA-MB-231 SEMA6D overexpressing cells the change in $CD44^{+}CD24^{-/low}$ population was not significant, so it cannot be correlated with the effect of transformation of cells.

CD24 has also been found to take a role in a variety of malignancies such as breast cancer. It was shown that CD24 expression in MCF-7 was related to enhanced growth, invasion, and ability to adhesion [67]. Additionally, in MCF-7 cells overexpression of SEMA6D leads to significantly increased CD44+ CD24+ population whereas significantly decreased CD44- CD24+ population. The $CD44^{+}CD24^{-/low}$ population was slightly increased in SEMA6D overexpressing MCF-7 cells (Figure 4.26).

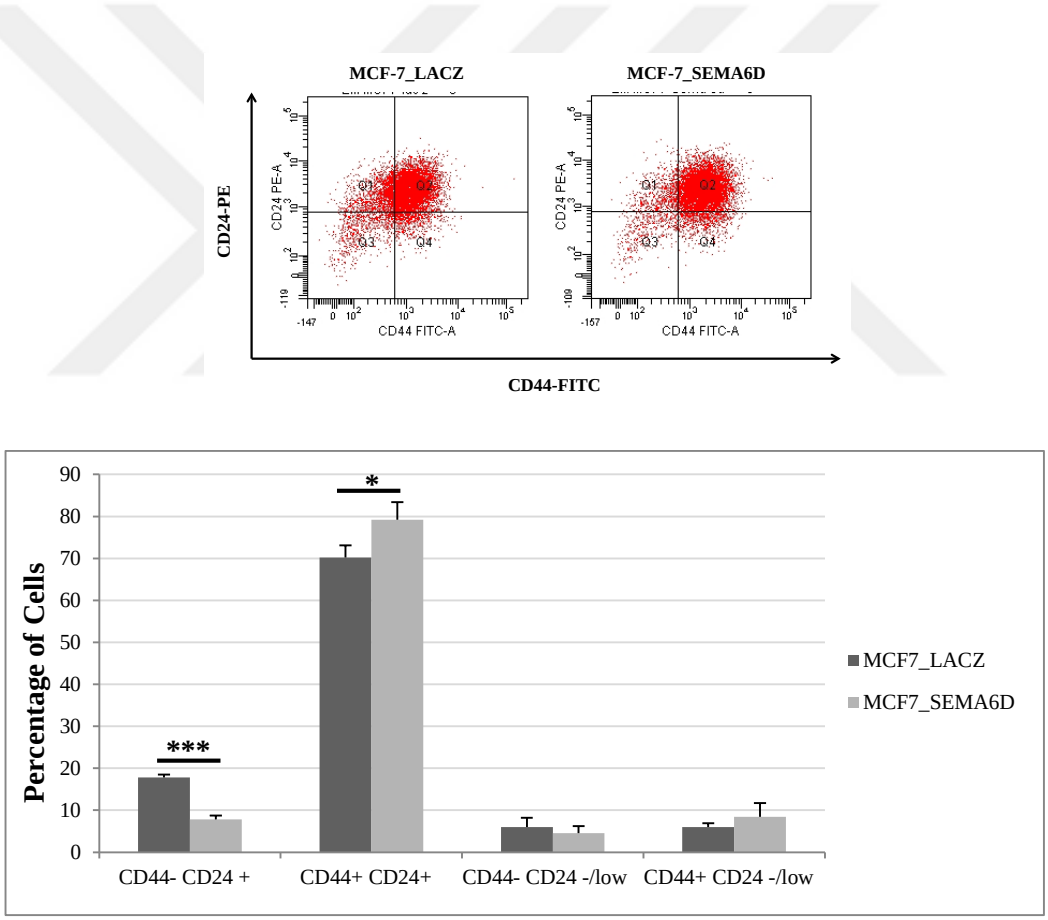

Figure 4. 26. Cancer stem cell subpopulations identified by CD24 and CD44 expression in MCF-7 overexpressing SEMA6D and MCF-7 LACZ control cells. (n=3, *p<0.05, ***p<0.001)

CHAPTER 5

DISCUSSION & CONCLUSION

Semaphorins have been found to regulate several developmental processes such as guidance of migrating cells during morphogenesis, axon guidance, and immune cell responses. Their regulatory roles in various human tumors have strikingly emerged in recent years [54]. SEMA6D has also been implicated in diverse processes such as neuronal guidance, cardiac development, tumor angiogenesis, regulation of immune cells and cancer progressions. Several studies showed that deregulated semaphorins were significant drivers of tumor progression [58]. It has been revealed that SEMA6D highly expressed in gastric carcinoma which induce tumor angiogenesis [53]. Additionally, according to genomic expression analysis of public data sets of breast cancer derived from The Cancer Genome Atlas (TCGA), high level of SEMA6D was found to be correlated with better survival in patients with triple-negative breast cancer (TNBC) in breast cancer [58]. However, there is no other data apart from bioinformatics analysis indicating role of SEMA6D in breast cancer. In this study, the pro-tumorigenic role of SEMA6D was demonstrated in normal breast epithelial cells (MCF10A) in context of proliferation, migration and transformation. Moreover in breast cancer cell lines MDA-MB-231 and MCF-7, invasion, stem cell markers and anchorage-independent growth were investigated.

We previously showed that overexpression of SEMA6D in MDA-MB-231 and MCF-7 cell lines is correlated with enhanced migration. In cancer progression, alteration of cell migration is predominantly correlated with ability of cancer cells, which gain ability to move away from primary tumor and toward neighboring tissues. To further investigate the role of SEMA6D in both breast cancer cell lines, their capability to invade through basement membrane and transformation capabilities in order to escape from anoikis to promote anchorage-independent growth were demonstrated. The invasion experiments were conducted with the lab-on-a-chip system to mimic 3D environment in vitro. SEMA6D overexpression was correlated with enhanced invasiveness in MDA-MB-231 cells whereas it did not trigger the

invasiveness of MCF-7 cells. Normally, MCF-7 cells have low metastatic capability and poorly invasive. The invasion experiments repeated with generation of bone microenviroment rather than usage of only matrigel in middle channel of lab-on-a-chip system. The reason for selecting bone microenvironment is that SEMA6D validated as oncogene in osteosarcoma and also breast cancer is frequently metastasize to bone[59, 68]. So, we hypothesized that secreted factors or cell-to-cell interaction might induce invasion in MCF-7 cells. It was observed that bone microenvironment promotes the invasion in MCF-7 overexpressing SEMA6D cells.

The chemokine receptor 4 (CXCR4) has been found to highly expressed in breast cancer tissue, whereas its ligand, chemokine ligand 12 (CXCL12) is mainly secreted in lung, bones, and lymph nodes which the breast tumors predominantly metastasize. Higher expression of CXCL12 in bone tissue that is correlated with being common sites for metastasis, also functions as a chemoattractant, lead to driving of CXCR4 positive primary tumor cells through secondary site resulting in metastatic lesions. Thus, the CXCR4-CXCL12 interaction promotes the migration of breast cancer cells toward common metastatic sites of breast cancer [23, 69]. Eventually, there might be distinct interaction with tissues which express SEMA6D receptor (Plexin-A1) in context of invasion and the role of SEMA6D in breast-to-bone metastasis should be further investigated.

The soft agar assay reflects that SEMA6D overexpressed MCF-7 cells generate reduced colony number with respect to its control. Anchorage-independent growth is one of the hallmark of carcinogenesis and transformation of cells [3]. Thus, the overexpression of SEMA6D in MCF-7 breast cancer cells might not be enough to transform their behavior of cells into being metastatic and aggressive.

In terms of non-tumorigenic breast cell line MCF10A, the role SEMA6D on migration, proliferation and anchorage-independent growth were investigated. The overexpression SEMA6D in MCF10A leads to enhanced migration, proliferation and viability. Additionally, it was shown that SEMA6D overexpression in MCF10A cells increased the ratio of BrdU-positive cells, which is associated with elevated proliferation. In addition, cell cycle analysis also supports the enhancement of proliferation. SEMA6D overexpression in MCF10A cells lead to increased S phase significantly. However, SEMA6D overexpression did not stimulate the anchorage independent growth so it might not enough to induce complete transformation of

MCF10A cells. The effects of SEMA6D on other processes involved in tumorigenesis, such as EMT, invasion and cancer stem cell population should be further investigated.

Alteration in cell migration is associated with ability of tumor cells that move away from primary tumor through neighbor tissues during cancer progression [70]. Enhanced level of SEMA6D provide an advantage in the sense of tumor progression to MDA-MB-231, MCF-7 and MCF10A due to increased migration in all three cell lines and significant increase in proliferation of MCF10A cells. Activation of SEMA6D chicken embryo promotes migration of myocardial cells during cardiac chamber formation[55]. So, SEMA6D demonstrates its activation as boosting the migration in breast cancer and normal breast cell lines during tumor progression and its function can be attributed to its role in development. However, SEMA6D alone was not enough to promote tumorigenesis because it did not stimulate the anchorage independent growth. Thus, overexpression of SEMA6D acts through context dependent way so it might need multiple hits. Additionally, overexpression of SEMA6D led to decrease in proliferation of MCF-7 cells and no significant effect on MDA-MB-231 cells, which can be correlated with reduction in colony formation in soft-agar assay.

It was shown that the SEMA6D gene expression was correlated with enhanced survival particularly in triple-negative breast cancer patients according to analysis of public datasets of breast invasive carcinoma obtained from The Cancer Genome Atlas (TCGA) [58]. According to soft agar and proliferation results, there was less colony formation in SEMA6D overexpressing MDA-MB-231, MCF-7 and no colony formation in MCF10A cell lines. Thus, SEMA6D impair transformation capacity of cells, which can be correlated with better survival in vivo.

In addition, flow cytometry analysis was performed for detection cancer stem cell population in MDA-MB-231 and MCF-7 in order to elucidate whether reduced colony formation in soft agar assay can be linked to reduced cancer stem cell population. It has been demonstrated that breast cancer stem cells have a $CD44^{+}CD24^{-}$ phenotype [71]. There was a slight decrease in $CD44^{+}CD24^{-}$ in MDA-MB-231 cells overexpressing SEMA6D, whereas there was a slight increase in $CD44^{+}CD24^{-}$ in MCF-7 SEMA6D activated cells but might not enough to boost the transformation capacity of MCF-7 cells. It was reported that luminal cell lines possess enriched $CD44^{-/low}$ $CD24^{+}$ cell population and shows properties of more differentiated luminal epithelial cells, yet basal type breast cancer cells comprise $CD44^{+}$ $CD24^{-/low}$ [72, 73]. Since, MCF-7 is a luminal A type, hormone-dependent (ER^{+}, PR^{+}, $HER2^{-}$) cell line and MDA-MB-231

cells basal type and triple negative cell line, the difference in their behavior in context of cancer stem cell population could be explained by their origin.

Consequently, SEMA6D has distinct effects on different cell lines and the emerging role of SEMA6D in cancer needed to be further elucidated focusing on different subtypes of breast cancer in order to understand the progression of tumor and development of efficient therapeutics. Our preliminary data suggests that SEMA6D could be involved in bone specific metastasis which should further be investigated in context of prognostic markers. Also, effect of SEMA6D overexpression might be correlated with patient data and association with distinct phenotypes should be further elucidated.

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