FUNCTIONAL ANALYSIS OF TRANSGELIN IN BREAST CANCER

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IN

MOLECULAR BIOLOGY AND GENETICS

By

Nazlı Değer

June, 2018

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Abstract

FUNCTIONAL ANALYSIS OF TRANSGELIN IN BREAST CANCER

Nazlı Değer

M.S. in Molecular Biology and Genetics

Advisor: Işık Yuluğ

June, 2018

Transgelin (TAGLN) is an actin-binding protein. It is highly expressed in fibroblasts and smooth muscle cells. In smooth muscle cells, it takes part in processes including motility and differentiation and also it has a role in the formation of stress fibers. TAGLN gene has been found to be downregulated by promoter hypermethylation in breast and colon tissues and in these tissues, it acted as a tumor suppressor gene. However, in a study on nerve sheath tumors, TAGLN expression was found as upregulated via hypomethylation and in nerve sheath tumors, it acted as a proto-oncogene. To the best of our knowledge, the functional effect of TAGLN gene expression has not been studied in detail in breast carcinoma cell lines. The aim of this study was therefore to identify the functional role of TAGLN in breast cancer development. Hence, TAGLN gene expression was silenced or overexpressed and functional analysis was performed in selected breast cancer cell lines. Breast cancer cell lines were chosen according to their subtypes such as basal, HER2 positive or triple negative; their migratory properties; epithelial or mesenchymal characteristics and the expression level of TAGLN. Therefore, triple negative and mesenchymal MDA-MB-157 cells and MDA-MB-231 cells that express TAGLN at medium level were selected to silence TAGLN expression. The same cell lines and HER2 positive and epithelial MDA-MB-361 cells which express TAGLN at very low level were selected to overexpress TAGLN gene. Immunofluorescence and western blot analysis showed that in MDA-MB-157 and MDA-MB-231 cells mesenchymal marker Vimentin expression is correlated with TAGLN gene expression level. On the contrary, a reverse relationship exists in MDA-MB-361 cells where E-Cadherin expression increased and Vimentin expression decreased in TAGLN overexpressing MDA-MB-361 cells. TAGLN silencing in MDA-MB-157 cells increased the cell

spreading potential and viability capacity while TAGLN upregulated cells did not show any significant change. TAGLN silencing in MDA-MB-231 cells decreased the cell spreading potential and cell viability of the cells, TAGLN overexpression in MDA-MB-231 cells increased these properties of cells. MDA-MB-361 cells behaved differently with TAGLN overexpression; cells were able to form less colonies and cell viability decreased in TAGLN overexpressing cells. TAGLN gene silencing affected the cell cycles of MDA-MB-157 and MDA-MB-231 cells but TAGLN overexpression had no effect on the cell cycle.

In conclusion, TAGLN expression has an effect on Epithelial to Mesenchymal Transition (EMT) by altering the expression of established EMT markers E-Cadherin and Vimentin and its effect is based on the original morphology of the respective cell lines. In this study, the effect of TAGLN expression on cell proliferation was also studied and TAGLN seems to be acting as a tumor suppressor in MDA-MB-157 and MDA-MB-361 cells and as an oncogene in MDA-MB-231 cells. This might be due to the invasive character of MDA-MB-231 cells and the underlying mechanisms for this outcome should be investigated. Also, in vivo experiments can be performed to see whether changes in the expression of TAGLN gene has a role in tumor formation or metastasis capacity of cells.

Keywords: Breast cancer, Breast carcinoma cell line, TAGLN, Transgelin

Özet

MEME KANSERİNDE TRANSGELİN GENİNİN

FONKSİYONEL ANALİZİ

Nazlı Değer

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Işık Yuluğ

Haziran, 2018

Transgelin (TAGLN) aktin proteinine bağlanan bir proteindir. Fibroblastlarda ve düz kas hücrelerinde yüksek seviyede ifade edilir. Düz kas hücrelerinde, hareketlilik ve farklılaşmada görev alır ve stres liflerinin oluşumunda rol oynar. TAGLN geninin ifadesi, meme ve kolon kanseri dokularında promoter hipermetillenmesiyle düşürülmüştür ve TAGLN geni bu dokularda tümör baskılayıcı gen olarak davranmaktadır. Bununla birlikte, sinir kılıfı tümörleri üzerinde yapılan bir çalışmada, TAGLN geninin ifadesinin hipometilasyon ile artırıldığı ve bu dokularda, onkogen olarak davrandığı görülmüştür. Bildiğimiz kadarıyla, TAGLN geninin fonksiyonel etkisi daha önce meme karsinoma hücre hatlarında detaylı olarak çalışılmamıştır. Bu çalışmanın amacı, meme kanseri gelişiminde TAGLN geninin fonksiyonel rolünü ortaya çıkarmaktır. Bu sebeple, seçilen meme kanseri hücre hatlarında TAGLN geninin ifadesi azaltılmış ya da artırılmış ve sonrasında fonksiyonel analizler yapılmıştır. Meme kanseri hücre hatları seçilirken, hücre hatlarının alt tipleri, hücre göçü karakterleri, epitel ya da mezenkimal yapıda olma statüleri ve TAGLN geninin işlem yapılmamış hücrelerdeki ifade seviyeleri göz önüne alınmıştır. Bu sebeple, TAGLN genini orta seviyede ifade eden üçlü negatif mezenkimal MDA-MB-157 ve MDA-MB-231 hücreleri TAGLN geninin ifadesinin azaltılması için kullanılmıştır. Bu hücre hatları ve TAGLN genini az miktarda ifade eden HER2 pozitif ve epithel MDA-MB-361 hücreleri TAGLN geninin ifadesinin artırılması için kullanılmıştır. Immun floresan ve western blotlama analizleri MDA-MB-157 ve MDA-MB-231 hücrelerinde Vimentin ifadesinin TAGLN geninin ifadesiyle doğru orantılı olduğunu göstermiştir. Bu hücrelerden farklı olarak MDA-MB-361 hücrelerinde ters bir ilişki gözlenmiş ve

TAGLN geninin yüksek ifadesiyle E-Cadherin ifadesinin arttığı ve Vimentin ifadesinin azaldığı görülmüştür. TAGLN geninin sessizleştirilmesi MDA-MB-157 hücrelerinde hücrelerin yayılma potansiyelini ve yaşama yeteneğini artırırken, TAGLN geninin ifadesinin artırılmasının bir etkisi olmamıştır. TAGLN geninin sessizleştirilmesi MDA-MB-231 hücrelerinde hücrelerin yayılma potansiyelini ve yaşama yeteneğini azaltırken, TAGLN geninin ifadesinin artırılması hücrelerin bu özelliklerinde artışa sebep olmuştur. MDA-MB-361 hücreleri TAGLN geninin yüksek ifadesinde daha farklı davranarak daha az koloni kurmuşlar ve yaşama yeteneklerinde azalma göstermişlerdir. MDA-MB-157 ve MDA-MB-231 hücrelerinde, TAGLN geninin sessizleştirilmesi hücre döngüsünde değişikliğe sebep olurken TAGLN geninin yüksek ifade edilmesi bir değişikliğe sebep olmamıştır.

Sonuç olarak, TAGLN geni E-Cadherin ve Vimentin ifadelerini değiştirerek epitel mezenkimal değişim (EMD) üzerinde bir etkiye sahiptir ve bu etki ilgili hücre hatlarının orijinal morfolojisine bağlıdır. Bu çalışmada, TAGLN ifadesinin proliferasyon üzerinde etkisi de çalışılmıştır ve TAGLN geninin MDA-MB-157 ve MDA-MB-361 hücrelerinde tümör baskılayıcı gen olarak, MDA-MB-231 hücrelerinde ise onkogen olarak işlevi olduğu görülmüştür. Bu farklılık MDA-MB-231 hücrelerinin invasif karakterinden kaynaklanıyor olabilir. Bu sebeple altta yatan mekanizmalar incelenmelidir. Ayrıca, TAGLN geninin ifadesindeki değişimlerin hücrelerin tümör oluşturma ve metastaz kapasitelerinde bir değişikliğe neden olup olmadığını anlamak için hayvan deneyleri yapılabilir.

Anahtar kelimeler: Meme kanseri, meme karsinoma hücre hattı, TAGLN, Transgelin

To my mother and father…

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Abbreviations

ANLN	Annilin		NT	Non-tumorigenic
BC	Breast carcinoma		OD	Optical density
CDH1	E-Cadherin		OS	Overall survival
CDH2	N-Cadherin		PBS	Phosphate buffered saline
DBC	Ductal breast carcinoma		PR	Progesterone receptor
DCIS	Ductal carcinoma *in situ*		RT	Room temperature
ddH2O	Double distilled H2O		shRNA	Short hairpin RNA
DMSO	Dimethyl sulfoxide		siRNA	Small interfering RNA
EMT	Epithelial to mesenchymal transition		SMC	Smooth muscle cell
ER	Estrogen receptor		SNAI2	Snail family transcriptional repressor 2
FACS	Fluorescence assisted cell sorting		TAGLN	Transgelin
FBS	Fetal bovine serum		TBS	Tris buffered saline
FN	Fibronectin		TCA	Trichloroacetic acid
IDC	Invasive ductal carcinoma		TN	Triple negative
IF	Immunofluorescence		TSG	Tumor suppressor gene
ILC	Invasive lobular carcinoma		TSS	Transcription start site
KRT18	Keratin 18		um	Micrometer
LBC	Lobular breast carcinoma		UTR	Untranslated region
LCIS	Lobular carcinoma *in situ*		VIM	Vimentin
MET	Mesenchymal to epithelial transition		WB	Western blotting
MMP9	Matrix metallopeptidase 9		ZO1	Occludin

Chapter 1. Introduction

1.1. Cancer

Cancer is the one of leading causes of death worldwide. According to World Health Organization Statistics, in 2012, there were 14.1 million new cancer cases and 8.2 million cancer mortality worldwide. While cancer cases occurred throughout the world in people at different socioeconomic status, in 2012, 57% of new cancer cases occurred in less developed regions of the world and 65% of cancer mortality was observed in these regions (**1**).

1.2. Breast Cancer

Breast cancer is the most common cancer observed among women followed by lung and bronchus cancer. In 2018, 266,120 new cases were estimated to occur in U.S. For cancer mortality, breast cancer stands at the second position behind lung and bronchus cancer. In U.S., in 2018, there were an estimated 40,920 breast cancer related deaths. Breast cancer is common in middle-aged and older women while it can rarely develop in men. Survival is more likely among younger women when compared to women at an advanced age (**2** and **56**).

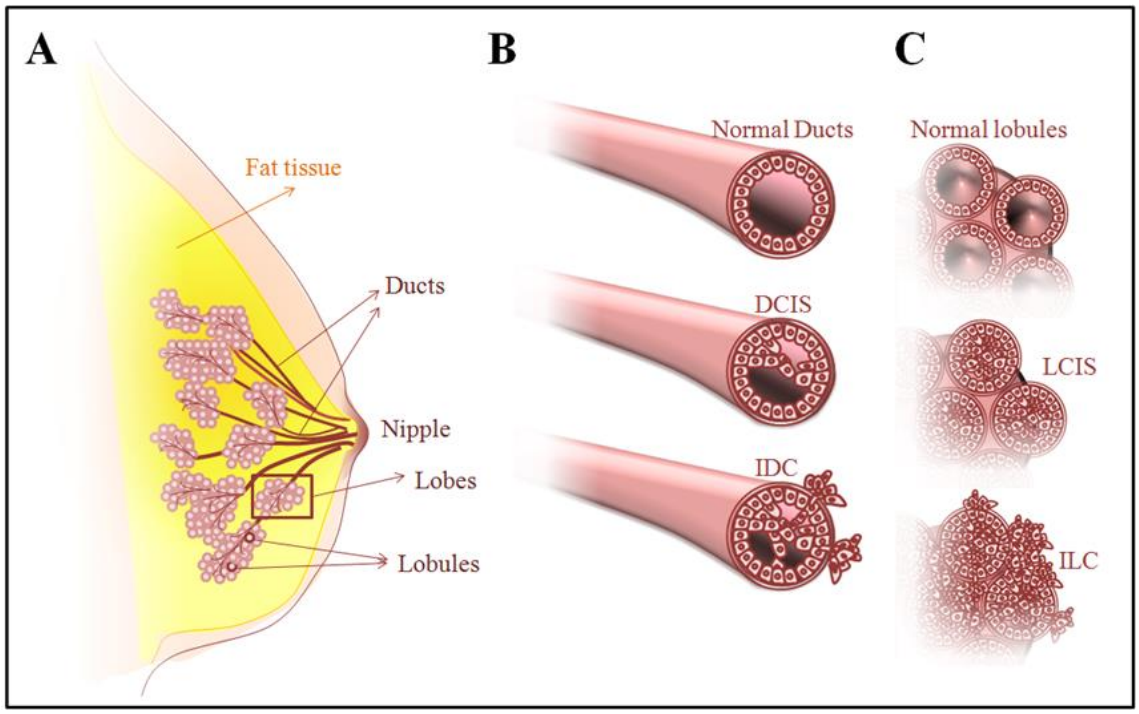


Figure 1.1. Structure of normal breast tissue and development of breast cancer. *(A) shows the structure of the normal breast tissue. (B) shows the development and progression of ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). (C) shows the development of progression of lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC) (Figure taken from (***3***)).*

1.3. Human Mammary Gland

Human mammary glands have a unique and specific function to synthesize, secrete and deliver milk to a new-born baby. During pregnancy and lactation, the mammary glands reach their mature development states under the influence of hormones through which the mammary glands are remodeled into milk-secreting organs. During reproductive life, human mammary glands have the capacity to cycle from resting state to lactation and with the cessation of lactation to the resting state. Tight hormonal regulation is critical for the normal functioning of the glands (**4**).

1.4. Anatomy of Human Mammary Gland

The human breast is composed of secretory (glandular), fatty (adipose) tissue and a supporting connective tissue (Cooper's ligaments) consisting of blood and lymph vessels. Secretory tissue is composed of a branching ductal-lobular system in which

milk glands (lobules) and milk ducts exist. Milk ducts are responsible for carrying milk from milk glands to the nipple during lactation. Milk ducts branch into smaller units called ductules and they channel into the lobules of a lobe (**4**).

1.5. Histological Classification of Breast Cancer Subtypes

To date, several pathological subtypes of breast cancer have been defined. Among them, some are widely occurring while others are classified as rare. Breast cancer is a heterogeneous disease which is also the reason for high numbers of breast cancer related mortality. This heterogeneity means that the breast tumor can be composed of more than one type at the pathological level (**5**). Breast cancer is mainly classified as in situ carcinoma and invasive carcinoma. Breast carcinoma in situ is classified as ductal or lobular carcinoma. Ductal carcinoma in situ (DCIS) is observed more commonly than lobular carcinoma in situ (LCIS). DCIS includes tumors from a heterogeneous origin. DCIS can be further classified as Comedo, Cribiform, Micropapillary, Papillary and Solid (6).

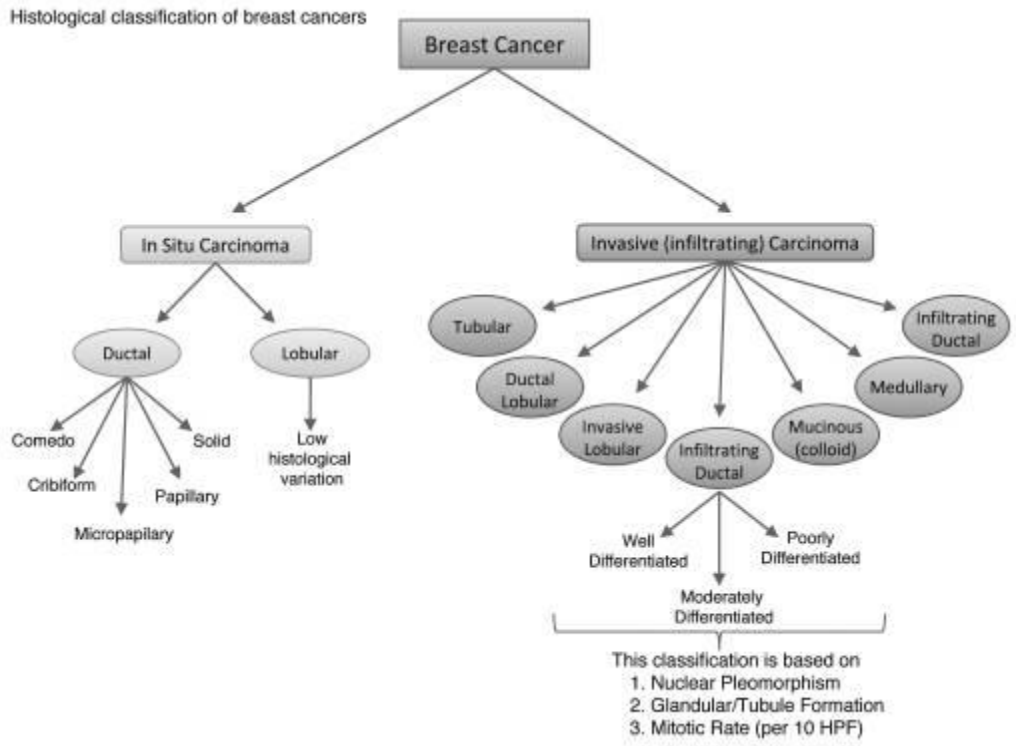


Figure 1.2. Histological classification of breast cancer subtypes.

*Breast cancer is mainly classified as in situ carcinoma or invasive (infiltrating) carcinoma which are further classified into groups. Classification is done according to growth patterns and architectural features (Figure taken from (**6**)).*

1.6. Molecular Classification of Breast Cancer

Breast tumors are classified into separate groups based on their global expression profiles. They are generally grouped into subtypes based on the prognosis of patients, aggressiveness of the disease and treatment selection.

Table 1.1. Molecular classification of breast cancer.

*Breast cancer tumors can be categorized based on their expression profiles. Several subtypes shows different occurrences and disease progression (Table adapted from (**7**)).*

Subtype	**Alias**	**Biomarker Status**	**Grade**	**Outcome**	**Prevalence**	
Luminal	Luminal A	[ER+|PR+] HER2-KI67-	1|2	Good	23.7%	
	Luminal B	[ER+|PR+] HER2-KI67+	2|3	Intermediate	38.8%	
		[ER+|PR+] HER2+KI67+		Poor	14%	
HER2 positive	HER2 overexpression	ER-PR-HER2+	2|3	Poor	11.2%	
Triple negative	Basal	ER-PR-HER2-, basal marker+	3	Poor	10-25%	12.3%
	Claudin-low	ER-PR-HER2-, EMT marker+, Stem-cell marker+, claudin-	3	Poor	7-14%	
	Metaplastic breast cancer (MBC)	ER-PR-HER2-, EMT marker+, Stem-cell marker+	3	Poor	1%	
	Interferon-rich	ER-PR-HER2-, interferon regulated genes+	3	Intermediate	~10%	
Molecular apocrine cancer (MAC)	Molecular apocrine cancer (MAC)	ER-PR-AR+	2|3	Poor	13.2%	

1.7. Epithelial to Mesenchymal Transition

Epithelial-mesenchymal transition (EMT) is a biological process through where epithelial cells undergo multiple biochemical changes and then transform into a mesenchymal cell phenotype. During this process, several transcription factors are activated, specific cell-surface proteins are expressed, cytoskeletal proteins are expressed and reorganized, ECM-degrading enzymes are produced and changes in the expression of specific microRNAs occur. These changes can be used as tools in other words as biomarkers to distinguish the phenotype of cells and any change (**8**).

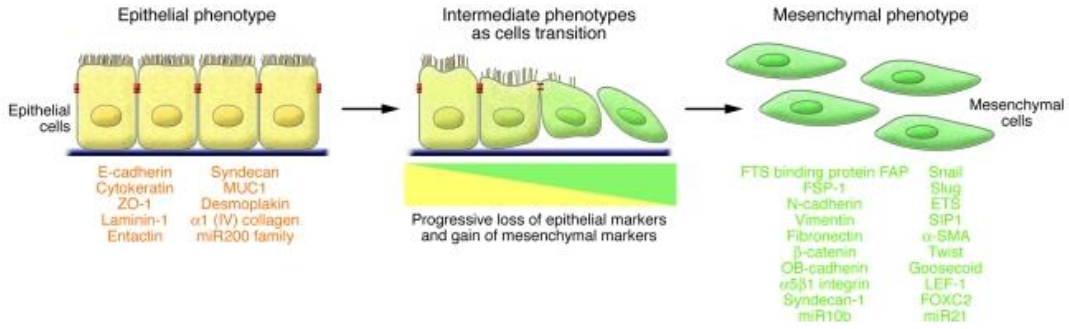


Figure 1.3. Epithelial to mesenchymal transition.

*During EMT, changes in cell morphology and in the expression of established biomarkers occur (Figure taken from (***8***)).*

During EMT, while epithelial markers decrease in cells, the expression of mesenchymal markers increases, supporting cells to take on the mesenchymal phenotype.

The reverse of EMT called Mesenchymal to Epithelial Transition (MET) is also possible and has been observed in fibroblasts during the generation of induced pluripotent stem cells (**9**). During the process of MET, cells lose mesenchymal markers and the expression of epithelial markers increase.

EMT in cells can occur in three distinct biological settings. Each setting has different outcomes and they require different signals to emerge. These different EMT processes are called Type 1 EMT, Type 2 EMT and Type 3 EMT. Type 1 EMT occurs during

implantation, embryogenesis and organ development. Structures formed by EMT can be further induced to differentiate into other types of epithelial tissues via MET and also with subsequent EMT, cells of connective tissue can be generated. Type 2 EMT is associated with wound healing, tissue generation and organ fibrosis. Type 2 EMT starts as a repair event to reconstruct tissues after a trauma or inflammatory injury. During this event, new fibroblasts are generated. With the case of wound healing and tissue generation, inflammation occurs and then ceases. However, with organ fibrosis, Type 2 EMT continues with ongoing inflammation which at the last causes organ destruction. Type 3 EMT is associated with cancer prognosis and metastasis (**8**). Excessive proliferation of epithelial cells and angionesis are characteristics of primary epithelial cancers (**10**). Newly gained abilities such as invasiveness and increased proliferation rate through EMT might cause threatening outcomes. Several studies show the carcinoma cells to gain a mesenchymal phenotype and express mesenchymal markers (**8**).

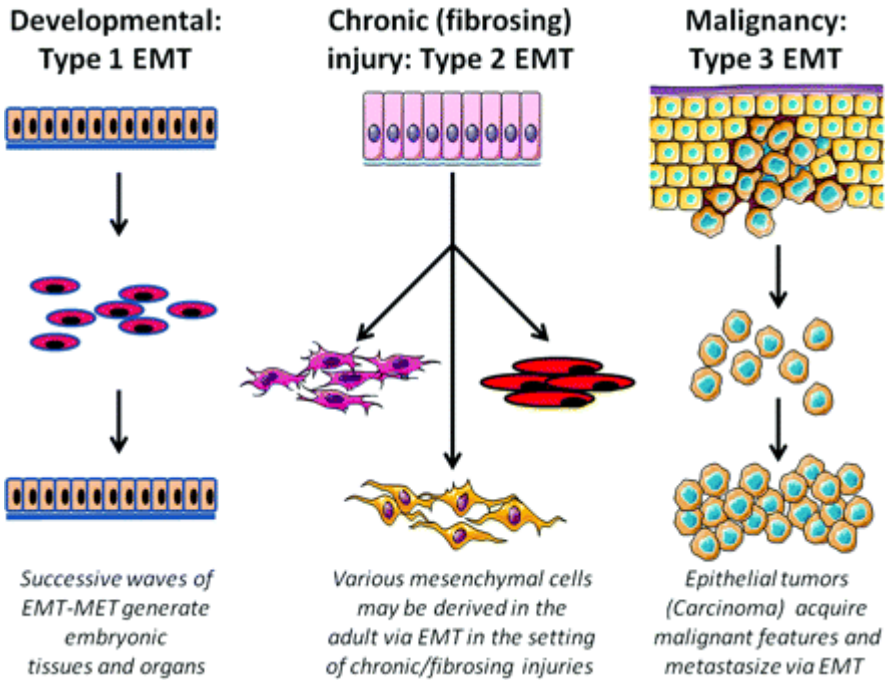


Figure 1.4. Types of EMT.

*Different types of EMT are associated with different outcomes in cells. While Type 1 EMT is mainly for developmental purposes, Type 2 EMT is associated with tissue regeneration and organ fibrosis whereas Type 3 is linked to cancerous outcomes (Figure taken from (**11**)).*

1.8. Transgelin (*TAGLN*) Gene

TAGLN (Transgelin) is a protein coding gene expressed in humans. It is also known by the name of SM22, SMCC, TAGLN1, WS3-10 and SM22-alpha. *TAGLN* gene has homologs in the chicken, chimpanzee, cow, dog, frog, mouse, rat and zebrafish. It is located on chromosome 11q23.3. It has 5 exons and 2 mRNA variants. Variant 1 is longer than Variant 2 which differs in its 5' Untranslated Region (UTR). These 2 variants encode for the same protein. Conserved domain of *TAGLN* includes Calponin homology domain which is an actin binding domain and therefore can facilitate actin binding. This Calponin domain can be as a single copy or in tandem repeats by which binding affinity is increased. This domain is found in cytoskeletal and signal transduction proteins, proposing some roles for TAGLN protein (**12**).

TAGLN is an actin binding protein though its previously stated Calponin domain. Therefore, it can interact closely with the actin cytoskeleton and it is highly expressed in smooth muscle cells (SMC) (**13** and **14**). In SMCs, TAGLN has roles in processes including motility and differentiation. Also, it takes part in the formation of stress fibers. *TAGLN* gene expression is differentially regulated in different diseases according to the different researches. While it is downregulated in breast, colon and prostate cancers (**15** and **16**), it is upregulated in pancreatic and gastric cancers and nerve sheath tumors (**17**, **18** and **19**). This behavior of *TAGLN* gene makes it undefinable as tumor suppressor or oncogene. A previous study in breast cancer has revealed that *TAGLN* gene is downregulated in 19 out of 21 tumors if compared to paired normal tissues due to DNA hypermethylation in tumors (**20**). In a study, TAGLN was shown to have a role in migration such that the reduced expression in REF52 fibroblasts with the disruption of actin organization results in the increase in the capacity of cells to invade. The same study also showed that re-expressing TAGLN in PC3 prostate epithelial cancer cells with undetectable TAGLN expression had reduced ability to migrate (**21**). TAGLN is also reported as a negative regulator of MMP9 which is a strong mediator of metastasis in HT1080 cells, connective tissue cells (**22**). MMP9 is found as a driver of malignant progression and metastasis in breast cancer (**23**) where TAGLN gene is known to be downregulated (**20**), proposing a role for TAGLN as a negative inducer of metastasis through the mechanism of inhibiting MMP9 expression.

Some research propose TAGLN protein to be a mesenchymal protein. Also, TGF- β has been identified as an inducer of *TAGLN* gene and also an inducer of EMT in cancer cells (**25** and **26**). This result brings the question of *TAGLN* expression being concurrent with EMT process.

1.9. Aim of the Study

The aim of this study was to identify the functional role of *TAGLN* gene in breast cancer. Different breast carcinoma cell lines were used in order to understand the functional role of TAGLN. Hence, *TAGLN* gene was upregulated in MDA-MB-157, MBA-MB-231 and MDA-MB-361 using an overexpression vector pCMV6 TAGLN and its empty vector as a control. Also, *TAGLN* gene was downregulated in MDA-MB-157 and MBA-MB-231 cell lines using TAGLN siRNA and Non-targeting siRNA as a control. Selected TAGLN overexpressing cell clones were used to analyze the role of TAGLN in cell cycle, cell growth, and EMT. The same analysis were also performed in *TAGLN* siRNA downregulated breast carcinoma cells.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. General Laboratory Chemicals and Reagents

General laboratory chemicals and reagents are routinely used substances which are used in common techniques such as PCR, western blotting, agarose gel electrophoresis, bacterial growth etc. **Table 2.1** lists these materials, their catalog numbers and the companies providing them.

Table 2.1: Chemicals, reagents, enzymes and kits used for general laboratory purposes.

Name	**Catalog #**	**Company (Country)**
Molecular Biology Grade Water	SH30538	Thermo Scientific (USA)
Nucleospin RNA extraction kit	740955	Macherey Nagel (Germany)
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Scientific (USA)
DyNAmo HS SYBR Green qPCR Kit	F-410L	Thermo Scientific (USA)
Taq DNA Polymerase and buffers	EP0402	Thermo Scientific (USA)
Agarose	BHE500	Prona (Spain)
Ethidium Bromide	17898	Thermo Scientific (USA)
Gene Ruler 1 kb DNA Ladder	SM0311	Thermo Scientific (USA)
Gene Ruler 50 bp DNA Ladder	SM373	Thermo Scientific (USA)
Yeast extract	1702	Conda (Spain)
Agar (microbiology grade)	05039	Sigma Aldrich (USA)
Bacto-tryptone	1612	Conda (Spain)
Ampicillin	A0839	Applichem (Germany)
Kanamycin	60615	Sigma Aldrich (USA)

Table 2.1: Chemicals, reagents, enzymes and kits used for general laboratory purposes.		
Name	**Catalog #**	**Company (Country)**
PureLink Quick Plasmid Miniprep Kit	K210011	Thermo Scientific (USA)
Proteinase inhibitor cocktail	P8340	Sigma Aldrich (USA)
Triton X-100	T8787	Sigma Aldrich (USA)
2-mercaptoethanol	M3148	Sigma Aldrich (USA)
EDTA	A3562	Applichem (Germany)
SDS	71725	Sigma Aldrich (USA)
Ammonium per-sulfate	A3678	Sigma Aldrich (USA)
TEMED	1610801	Biorad (USA)
PageRuler Prestained Protein Ladder, 10 to 170kDa	26616	Thermo Scientific (USA)
Glacial acetic acid	27225	Sigma Aldrich (USA)
Tris (Trizma Base)	T1503	Sigma Aldrich (USA)
Glycine	G8898	Sigma Aldrich (USA)
Roche PVDF Membranes 0.2uM	3010040001	Roche (USA)
Bovine Serum Albumin Fraction V (BSA)	10735078001	Roche (USA)
Tween-20	822184	Merck /Germany)
ECL Prime System	RPN2232	Life Sciences (USA)
Mounting medium for IHC	CS70330	Dako (Denmark)
Sulforhodamine B (SRB)	230162	Sigma Aldrich (USA)
Trichloroacetic acid (TCA)	33731	Sigma Aldrich (USA)

2.1.2. Cell Culture Chemicals and Reagents

Chemicals, reagents and media which are used in the cell culture experiments are listed in **Table 2.2**.

Table 2.2: Chemicals, reagents, kits and media used in cell culture.		
Name	**Catalog #**	**Company (Country)**
DMEM, High Glucose	SH30243	GE Healthcare (UK)
DMEM, Low Glucose	SH30021	GE Healthcare (UK)
DMEM:HAM's F-12 1:1	SH30023	GE Healthcare (UK)
OptiMEM I	11058	Thermo-Fischer Scientific (USA)
Fetal Bovine Serum	CH30160	GE Healthcare (UK)
L-glutamine	SH30034	GE Healthcare (UK)
Non-Essential Amino Acids	SH30238	GE Healthcare (UK)
Penicillin/Streptomycin	SV30010	GE Healthcare (UK)
Sodium Pyruvate (100mM)	11360	Thermo-Fischer Scientific (USA)
Hydrocortisone	H4001	Sigma Aldrich (USA)
Epidermal Growth Factor	E9644	Sigma Aldrich (USA)
Insulin	I9278	Sigma Aldrich (USA)
PBS	SH30256	GE Healthcare (UK)
Trypsin/EDTA (0.25%)	SV30031	GE Healthcare (UK)
Dimethyl sulfoxide (DMSO)	A1584	Applichem (Germany)
UltraCruz™ Mounting Medium for IF	SC24941	Santa Cruz Biotechnology (USA)
Lipofectamine 2000 transfection reagent	11668	Thermo-Fischer Scientific (USA)

2.1.3. Nucleic Acids

Nucleic acids such as siRNAs, shRNA and overexpression vectors which are used in the experiments are listed in **Table 2.3**. Description of vectors and sequences of siRNAs are also provided.

Table 2.3: List of nucleic acids used for gene editing.			
Name	**Catalog #**	**Company**	**Description/Target sequence**
pCMV6-Entry	PS100001	OriGene (USA)	Mammalian vector with C-terminal Myc- DDK Tag
pCMV6-TAGLN	RC215789	OriGene (USA)	TAGLN (Myc-DDK-tagged)-Human transgelin (TAGLN), transcript variant 2 (NM_003186)
siGENOME Non-Targeting siRNA #2	D-001210-02-20	Thermo Scientific (USA)	UAAGGCUAUGAAGAGAUAC
siGENOME Human TAGLN siRNA, SMARTpool	M-003714-02-0020	Thermo Scientific (USA)	siRNA1-AGAAAGCGCAGGAGCAUAA siRNA2-CCAGACUGUUGACCUCUUU siRNA3-CCAAAAUCGAGAAGAAGUA siRNA4-UGUCCUCCUUGGCGGCAAA
GIPZ Non-silencing Lentiviral shRNA Control	RHS4346	Dharmacon (USA)	Non-silencing shRNA
GIPZ Lentiviral Empty Vector shRNA Control	RHS4349	Dharmacon (USA)	Empty Lentiviral shRNA
GIPZ TAGLN shRNA	RHS5086-EG6876	Dharmacon (USA)	V2LHS_153751-ACATGTCAGTCTTGATGAC V3LHS_395056-TCAAAGAGGTCAACAGTCT V3LHS_395059-TGAAGACCATGGAGGGTGG

2.1.4. Antibodies

Antibodies which are used in the western blot and immunofluorescence experiment are listed in **Table 2.4**.

Table 2.4: Antibodies used in the study.		
Name of the antibody	**Catalog #**	**Company**
Rabbit anti-SM22 alpha (TAGLN)	ab14106	Abcam (UK)
Mouse anti-E Cadherin [HECD-1]	ab1416	Abcam (UK)
Mouse anti-Vimentin [V9]	ab8069	Abcam (UK)
Rabbit anti-Vimentin (D21H3) XP	5741	Cell Signaling Technology (USA)
Rabbit anti-Calnexin	C4731	Sigma Aldrich (USA)
Mouse anti-N-cadherin Antibody (3B9)	33-3900	Thermo Fisher (USA)
Mouse anti-Occludin Antibody (OC-3F10)	33-1500	Thermo Fisher (USA)
Anti-rabbit IgG-HRP	A6154	Sigma-Aldrich (USA)
Anti-mouse IgG-HRP	A0168	Sigma-Aldrich (USA)
Donkey polyclonal Secondary Antibody to Mouse IgG - H&L (Alexa Fluor® 488), pre-adsorbed	ab150109	Abcam (UK)
Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 568 conjugate	A10042	Life Sciences (USA)

Concentrations and secondary antibodies of primary antibodies are listed in **Table 2.5**.

Table 2.5. Concentrations and secondary antibodies of primary antibodies.			
Name of Primary Antibody	**Type of Secondary Antibody**	**Concentration for Western Blotting**	**Concentration for Immunofluorescence**
Anti-TAGLN	Rabbit	0.2 µg/mL	1 µg/mL
Anti-E-Cadherin	Mouse	1/2000	1/2000
Anti-Vimentin [V9]	Mouse	N/A	1 µg/mL
Anti-Vimentin [D21H3]	Rabbit	1 µg/mL	N/A
Anti-Calnexin	Rabbit	1/10000	N/A
Anti-N-Cadherin	Mouse	0.5 µg/mL	2 µg/mL
Anti-Occludin	Mouse	0.5 µg/mL	3 µg/mL

Concentrations and usage areas of secondary antibodies are listed in **Table 2.6**.

Table 2.6. Concentrations and usage areas of secondary antibodies.			
Name of Secondary Antibody	**Usage Area of Secondary Antibody**	**Concentration for Western Blotting**	**Concentration for Immunofluorescence**
Anti-Rabbit IgG-HRP	Western Blotting	1/5000	N/A
Anti-Mouse IgG-HRP	Western Blotting	1/5000	N/A
Anti-Mouse IgG H&L (Alexa Fluor® 488)	Immunofluorescence	N/A	4 µg/mL
Anti-Rabbit IgG H&L (Alexa Fluor® 568)	Immunofluorescence	N/A	4 µg/mL

2.1.5. PCR Primers

Primers which are used for qRT-PCR are listed in **Table 2.7**. Also, associated amplicon sizes, Tm values for the annealing step of PCR and qRT-PCR efficiency percentages are given. All primers were purchased from and synthesized by Iontek (Istanbul) or Sentromer (Istanbul). All primers were solved in nuclease-free water upon arrival to be 100 µM and later they were kept at -20°C for storage.

Table 2.7: Primers used in the study.

Primer Name	PCR[a]	Primer Sequence (5' to 3')	Tm[b] (°C)	Size[c] (bp)	Eff.[d] %
GAPDH F	RT	GGCTGAGAACGGGAAGCTTGTCAT	60	140	99
GAPDH R		CAGCCTTCTCCATGGTGGTGAAGA			
TAGLN F	RT	TGCGAGCCCTGAGGAAGCCT	60	220	104
TAGLN R		TCAGAATCACGCCATTCTTCAGCCA			
Primers designed and kindly provided by Assoc. Prof. Dr. Özgür Şahin:					
CDH1 F	RT	CCCGGGACAACGTTTATTAC	58	72	92
CDH1 R		GCTGGCTCAAGTCAAAGTCC			
CDH2 F	RT	ACAGTGGCCACCTACAAAGG	58	201	105
CDH2 R		CCGAGATGGGGTTGATAATG			
FN F	RT	CTGGCCGAAAATACATTGTAAA	58	114	91
FN R		CCACAGTCGGGTCAGGAG			
KRT18 F	RT	TGATGACACCAATATCACACGA	58	112	98
KRT18 R		GGCTTGTAGGCCTTTTACTTCC			
MMP9 F	RT	GAACCAATCTCACCGACAGG	58	67	103
MMP9 R		GCCACCCGAGTGTAACCATA			
SNAI2 F	RT	TGGTTGCTTCAAGGACACAT	58	66	93
SNAI2 R		GTTGCAGTGAGGGCAAGAA			
VIM F	RT	GGTGGACCAGCTAACCAACGA	58	183	N/A
VIM R		TCAAGGTCAAGACGTGCCAGA			
ZEB1 F	RT	GGGAGGAGCAGTGAAAGAGA	58	70	96
ZEB1 R		TTTCTTGCCCTTCCTTTCTG			
ZEB2 F	RT	AAGCCAGGGACAGATCAGC	58	74	100
ZEB2 R		CCACACTCTGTGCATTTGAACT			
ZO1 F	RT	CAGAGCCTTCTGATCATTCCA	58	69	98
ZO1 R		CATCTCTACTCCGGAGACTGC			

*[a] Purpose of PCR, RT: qRT-PCR. [b] Annealing temperature. [c]Amplicon size. [d]Eff: Efficiency values calculated by Dr. Nilüfer Sayar (**3**).*

2.1.6. Equipment

Equipment which are used for the experiments are listed in **Table 2.8**.

Table 2.8: Equipment used for the experiments.	
Name of the instrument	**Company**
PCR Thermal cycler	Applied Biosystems (USA)
AutoFlow NU-8500 Water Jacket CO_2 Incubator	NuAire (USA)
AxioCam MRc5 image capture device	Carl Zeiss (Germany)
Centrifuges 5810 and 5810 R	Eppendorf (Germany)
Stratagene Mx3005P Real-Time PCR System	Agilent (USA)
Bond Max equipment	Leica Microsystems (Germany)
Amersham Imager 600	Dharmacon (USA)

2.2. Solutions and Media

2.2.1. Routinely Used Laboratory Solutions

Buffers and solutions which are commonly used in the laboratory are listed in **Table 2.9**.

Table 2.9: Routinely used buffers and solutions.	
Buffer	**For 100 ml aqueous solution (if not otherwise stated)**
30% Acryl:Bis-acryl Solution	29 g acrylamide; 1 g bis-acrylamide (in dark)
0.5 M EDTA	18.61 g disodium EDTA.2H2O (pH=8.0)
50X TAE	24.2 g Tris-base; 5.71 ml glacial acetic acid; 10 mL 0.5 M EDTA (pH=8.0)
10X PBS	8.0 g NaCl; 0.2 g KCl; 1.44 g Na2HPO4.2H2O; 0.24 g KH_2PO_4 (pH=7.4)
10X TBS	2.4 g Tris-base; 8.8 g NaCl. pH = 8.0
10X Running Buffer	3.03 g Tris-base; 14.41 g glycine; 1 g SDS

Table 2.9: Routinely used buffers and solutions.	
Buffer	**For 100 ml aqueous solution (if not otherwise stated)**
Lysis Buffer (1ml)	67.5 µl 2M NaCl; 45 µl 1M Tris-HCL (pH = 8.0); 10 µl Triton X-100, 100 µl 10X Proteinase inhibitor.
5x Protein Loading Buffer	0.001g Bromophenol blue; 2g SDS; 62.5 mM Tris-HCL (pH:6.8); 15% glycerol. 5% β-mercaptoethanol is added prior to loading.
Wet Transfer Buffer	0.6 g Tris-Base; 2.88 g glycine; 15% Methanol
Crystal Violet Solution	0.5% crystal violet; 25% MetOH in 1X PBS
Fixation Solution for IF	4% Formaldehyde in 1X PBS
Permeabilization Solution for IF	0.25 % Triton X-100 in 1X PBS
LB	1 g Tryptone; 1 g NaCl; 0.5 g Yeast Extract

2.2.2. Cell Culture Solutions and Media

Cell lines and their growth media are listed in **Table 2.10**.

Table 2.10: Cell lines and their growth media.

*Cell line kindly provided by Assoc. Prof. Dr. Ali Osmay Güre, Bilkent University, Ankara. ** Cell line kindly provided by Assoc. Prof. Dr. Elif Erson Bensan, METU, Ankara. Other cell lines were purchased from ATCC. Basic Media consist of the given medium; 10% FBS; 1% Penicillin/Streptomycin; 1% Non-essential amino acids; 1% L-glutamine.*

Cell Line	**Medium**
BT474	Basic DMEM; 10µg/ml insulin
MCF10A**	Basic DMEM/Ham's F12; 10µg/ml insulin; 20 ng/ml EGF; 0.5 mg/ml hydrocortisone
MDA-MB-157	Basic DMEM; 1mM Sodium pyruvate
MDA-MB-231	Basic DMEM
MDA-MB-361	Basic DMEM; 1mM Sodium pyruvate

General characteristics of breast carcinoma cell lines used in this study is given in **Table 2.11**.

Table 2.11. Characteristics of breast carcinoma cell lines.

*Characteristics of each cell line is given. ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal Growth Factor Receptor 2; BRCA1: Breast Cancer 1; WT: Wild Type; MU: Mutated; LA: Luminal A; LB: Luminal B; H: HER2 positive; TNA: Triple Negative A; TNB: Triple Negative B; AC: Adenocarcinoma; DC: Ductal Carcinoma; IDC: Invasive Ductal Carcinoma; MC: Medullary Carcinoma; NT: Non-tumorigenic and N/A: Not Available (Table adapted from (***27***).*

Cell Lines	ER	PR	HER2	BRCA1 Mutation	Subtype	Tumor	Morphology
BT20	-	-	-	WT	TNA	IDC	Epithelial
BT474	+	+	+	WT	LB	IDC	Epithelial
HCC1937	-	-	-	MU	TNA	DC	Epithelial
MCF10A	-	-	-	N/A	NT		Epithelial
MCF12A	-	-	-	N/A	NT		Epithelial
MCF7	+	+	-	WT	LA	IDC	Epithelial
MDAMB157	-	-	-	WT	TNB	MC	Mesenchymal
MDAMB231	-	-	-	WT	TNB	AC	Mesenchymal
MDAMB436	-	-	-	MU	TNA	AC	Mesenchymal
MDAMB453	-	-	+	WT	H	AC	Epithelial
MDAMB468	-	-	-	WT	TNA	AC	Epithelial
SKBR3	-	-	+	WT	H	AC	Epithelial
ZR751	+	+/-	-	WT	LA	IDC	Epithelial

2.3. Methods

2.3.1. Plasmid Isolation from Bacterial Glycerol Stocks

Bacteria containing pCMV6 Entry and pCMV6 TAGLN overexpression vectors and bacteria containing GIPZ Empty shRNA and GIPZ TAGLN shRNA vectors glycerol stocks were stored at -80°C. Bacteria were initially grown in a smaller vessel to be a starter culture and after 2-3 hours, then were transferred to a larger vessel and grown for overnight. Plasmid DNAs were isolated from bacteria by using Qiagen Plasmid MidiPrep Kit according to the manufacturer's instructions. Concentrations and A260/A280 and A260/A230 of DNA were measured by using NanoDrop.

2.3.2. General Maintenance and Handling of Human Cell Lines

All cell culture practices were performed in sterile environment by using sterile techniques under cell culture laminar flow hoods. Cells were preserved in sterile screw capped cryo-vials in liquid nitrogen tanks for long term storage. Before using them, the cells were thawed. The cryo-vial was heated until almost completely melted and the mixture was transferred to 37°C growth medium which was followed by centrifugation at 1500 rpm for 5 minutes at room temperature. After the supernatant was removed, the cell pellet was dissolved in growth medium and then transferred to T25 tissue culture flask. All cells were maintained in CO2 incubators with 5% CO2 and 37°C temperature. Cells were split or passaged to a larger tissue culture flask once they reached confluency. Cells were detached from the flasks by washing cells with 1X PBS and then treating them with 0.25% Trypsin/EDTA with volumes adequate to cover the entire surface. To freeze the cells, the detached cells were mixed with the freshly prepared freezing medium including 90% FBS and 10% DMSO. Later, cells were kept in -20°C freezer for 1 hour, in -80°C freezer for overnight and then transferred to liquid nitrogen tanks.

2.3.3. siRNA Transfection of MDA-MB-157 and MDA-MB-231 Cells

TAGLN gene was silenced in MDA-MB-157 and MDA-MB-231 cells with siGENOME Human TAGLN siRNA (Thermo Scientific) by using Lipofectamine

2000 Transfection Reagent. For control, siGENOME Non-Targeting siRNA #2 (Thermo Scientific) was used for the transfection of the cells. Cells were transfected by using reverse transfection protocol. For this protocol, 100 pmol of siRNA was diluted in 250 µl of Opti-MEM Reduced Serum Medium in wells of 6 well plate. 5 µl of Lipofectamine 2000 Transfection Reagent was diluted in 250 µl of Opti-MEM Reduced Serum Medium. After 5 minutes, diluted Lipofectamine 2000 Transfection Reagent was added to diluted siRNA in wells. The mixture was incubated at room temperature for 20 minutes. Later, 150,000 cells were added to the mixture in the wells. The silencing of *TAGLN* gene was assessed with qRT-PCR and western blot experiments.

2.3.4. Kill Curve Assay and Determination of Selective Antibiotic Concentrations

In order to determine the selective antibiotic concentration for stable cell line generation, cells were treated with varying antibiotic concentrations and then SRB assay was performed to calculate the survival percentage of the cells. For kill curve assay, 50,000 cells per well were seeded to each well of 24 well plate. One day later, the medium was aspirated from wells and fresh medium without antibiotics and with antibiotics at varying concentrations were given to the cells. Cells were treated for one week by changing media every three days. After one week, SRB assay was performed. For SRB assay, medium was aspirated from cells. Cells were washed with 1X PBS and then they were fixed with 10% ice-cold TCA for 1 hour at 4°C in dark. After one hour, cells were washed with ddH_2O for 5 times and they were left to air dry. 0.04 g of SRB dye was dissolved in 10 mL of 1% acetic acid to prepare the SRB solution which was added to the cells. Plates were incubated for 10 minutes at room temperature in dark. After 10 minutes, cells were washed with 1% acetic acid for 5 times and they were left for air dry. Finally, 300 µl of 0.5X TBS was added to the wells and then the absorbance values were read at 562 nm. The minimum concentration at which all the cells were killed were determined to be used for the stable clone generation.

2.3.5. Overexpression and shRNA Plasmid Transfection of Breast Cancer Cells

TrueORF-Gold pCMV6 TAGLN (Origene) vector was used for overexpression of *TAGLN* gene in MDA-MB-157, MDA-MB-231 and MDA-MB-361 cells and GIPZ Lentiviral TAGLN shRNA (Dharmacon) vectors were used for knockdown of *TAGLN* gene in MDA-MB-157 and MDA-MB-231 cells. TrueORF-Gold pCMV6 Entry (Origene) and GIPZ Lentiviral Empty shRNA (Dharmacon) vectors were used as control for overexpression of *TAGLN* gene and knockdown of *TAGLN* gene, respectively. For plasmid transfection, 250,000 cells were seeded per well to 6 well plates one day before. Next day, 1.5 µg of vector was diluted in 250 µl of Opti-MEM Reduced Serum Medium and 3 µl of Lipofectamine 2000 Transfection Reagent was diluted in 250 µl of Opti-MEM Reduced Serum Medium. 5 minutes later, they were mixed and the mixtures were incubated at room temperature for 20 minutes. Then, the mixtures were added to cells in 6 well plates. Medium was refreshed 6 hours after transfection. 3 days later, the cells were transferred to 100 mm cell culture plates in growth medium with selective antibiotics at the pre-determined concentrations. Single cells were allowed to form colonies by changing antibiotic containing media every three days. After colonies were formed, they were collected by colony rings and then they were grown and the cells were harvested for the validation of successful transfection. The cell clones were frozen for further use.

2.3.6. RNA Isolation and Determination of Quantity

The cells were trypsinized and collected with ice-cold PBS and centrifuged to collect the cell pellets. RNA isolation were performed from cell pellets by using MN Nucleospin RNA Kit as described in the kits's user protocol. Concentrations and A260/A280 and A260/A230 of RNA samples were measured by using NanoDrop. Isolated RNAs were stored at -80°C for later use.

2.3.7. cDNA Preparation of RNA Samples

cDNA was prepared from 100 ng of RNA from each sample by using RevertAid 1st Strand cDNA Synthesis Kit (Thermo Scientific) and by using oligo-dT primers as described in the kit's user protocol. The cDNAs were kept at -20°C for later use.

2.3.8. qRT-PCR and Expression Analysis

qRT-PCRs were performed in duplicates by using DyNAmo™ HS SYBER® Green qPCR Kit. The reaction volume was 10 µl which includes 2 µl of cDNA, 0.4 µl of the indicated primers (10 µM), 5 µl of master mix and 2.2 µl of nuclease free water. The reaction conditions were as follows: Initial denaturation at 95°C for 15 minutes, 40 cycles denaturation at 94°C for 20 seconds, annealing (Tms as in **Table 2.7**) for 20 seconds and elongation at 72°C for 15 seconds. Melting curve was performed at 95°C for 1 minute, at 55°C for 10 seconds and at 95°C for 30 seconds. The Ct values (cycle number at which the fluorescence exceeds the fixed threshold) of the duplicates were averaged and the expression values of the target gene were normalized to the housekeeping control gene *GAPDH*. The relative expression values were compared using the equation below. The qRT-PCR experiments were performed with Stratagene Mx3005P Real-Time PCR System.

$$Relative\ expression = \frac{Eff(target)^{-Ct(target)}}{Eff(reference\ gene)^{-Ct(reference\ gene)}}$$

2.3.9. Protein Isolation from Cell Pellets

The cells were trypsinized and collected with ice-cold PBS and centrifuged to collect the cell pellets. Depending on the size of the pellets, 30-100 µl cell lysis buffer (**Table 2.9**) was added to the cells on ice and they were mixed by pipetting. The mixtures were incubated on ice for 30 minutes with occasional vortex and then they were centrifuged at 13,000 rpm for 30 minutes at 4°C. Supernatant, the cell lysate containing proteins, was collected and stored at -80°C to be used in western blot experiments.

2.3.10. SDS-PAGE

10-12% gels were used for proteins, as shown in **Table 2.12**.

Table 2.12. Constituents of SDS-Polyacrylamide Gel.					
SDS-Polyacrylamide Gel Preparation					
Resolving Gel				**Stacking Gel**	
Constituent	**10%**	**12%**		**Constituent**	**5%**
ddH_20	3.9 mL	3.3 mL		ddH_20	3.4 mL
30% Acrylamide-bisacrylamide Mix	3.4 mL	4.0 mL		30% Acrylamide-bisacrylamide Mix	830 µl
1M Tris HCl (pH 8.8)	2.5 mL	2.5 mL		1 M Tris HCl (pH 6.8)	630 µl
10% SDS	100 µl	100 µl		10% SDS	50 µl
15% APS	100 µl	100 µl		15% APS	50 µl
TEMED	4 µl	4 µl		TEMED	5 µl

Gels were run in 1X running buffer (**Table 2.9**) at 80V for 15 minutes and at 120V until the loading dye reaches the end of the gel.

2.3.11. Western Blot

The proteins which were run in SDS-PAGE were transferred to nitrocellulose membranes by using wet transfer system (Bio-Rad) and wet transfer buffer (**Table 2.9**). Proteins were transferred at 150A for 2 hours. After the transfer, the membrane was incubated in blocking solution, including 5% skim milk powder in 1X TBS with 0.2% Tween-20 (0.2 % TBS-T) for 1 hour at room temperature on shaker (slow). After 1 hour, the membrane was transferred to primary antibody solution, including 5% BSA in 0.2% TBS-T and primary antibody (**Table 2.4** and **Table 2.5**) for overnight at +4°C on shaker (slow). After the incubation, the membrane was washed three times with 0.2% TBS-T for 10 minutes each on shaker (fast). The membrane was transferred to the secondary antibody solution, including 5% skim milk powder in 0.2% TBS-T and secondary antibody (**Table 2.4** and **Table 2.6**) for 1 hour at room temperature on shaker (slow). After the incubation, the membrane was washed again as before, and was developed using ECL prime system as described, visualization was performed in Amersham Imager 600.

2.3.12. Viability Assays

After 72 hours of siRNA transfections, the cells were seeded as 10,000 cells per well in 24-well plates in triplicates. Cells were left to grow for different time periods and then SRB assay was performed as described previously. Absorbance values were used as estimations for cell viability. The same experiments were also performed for stable cells containing TAGLN overexpression and control vectors. Statistical significance of differences between the absorbance values were determined with two-tailed t-test in GraphPad Prism 6.01.

2.3.13. 2D Colony Formation Assays

After 72 hours of siRNA transfections, the cells were collected and 2000 cells seeded per well in 6-well plates in triplicates. The medium was changed every three days. Once the colonies were visible, the cells were fixed with ice-cold 100% Methanol for 10 minutes in freezer and they were stained with 0.5% crystal violet solution for 15 minutes at room temperature in dark. The wells were washed with ddH_2O at least 5 times and left to air dry. Then, the photos of the wells were taken and the colonies were counted. Image J program was used to determine the number of the colonies. Statistical significance of differences between the numbers of the colonies were determined with two-tailed t-test in GraphPad Prism 6.01.

2.3.14. Co-immunofluorescence Staining of Breast Cancer and NT Breast Cells

Cells were plated onto cover-slips in 6-well plates, and allowed to settle and grow for two days to reach 50% confluency. Then, cells were fixed with 4% Formaldehyde in 1X PBS for 15 minutes under the hood, followed by 3 times wash with 1X PBS. Then the cell membranes were permeabilized by using 0.25% Triton X-100 in 1X PBS for 10 minutes at room temperature. After washing 2 times with 1X PBS, plates were incubated with blocking solution, including 5% BSA and 0.1% Tween-20, for one hour at room temperature. Plates were incubated with primary antibody solutions, including 1% BSA with 0.1% Tween-20 and primary antibody (**Table 2.4** and **Table 2.5**), for overnight at +4°C, in humidified atmosphere. Following day, the plates were washed 3 times with 0.1% PBS-T, and were incubated with secondary antibody solution,

including 1% BSA and 0.1% Tween-20 and secondary antibody (**Table 2.4** and **Table 2.6**), for 1 hour at room temperature. The washing steps were repeated as before, and finally the slides were washed with ddH_2O, removed from the plates and they were mounted using 10 µl of mounting medium (Santa-Cruz).

2.3.15. FACS Analyses

200,000 cells were plated in wells of 6-well plates and were grown in normal growth conditions for 24 hr. After 24 hr, cells were trypsinized and collected in PBS, followed by centrifugation at 13000 rpm for 10 minutes at +4°C. After the PBS was discarded, 300 µl of 1X PBS was added onto the pellets. The tubes were vortexed vigorously, while at the same time 700 µl of ice-cold 100% Ethanol was added drop-wise. The tubes were incubated on ice for 30 minutes with occasional vortex. Cells were centrifuged at 13000 rpm for 10 minutes to remove supernatant which was followed two times wash with 1X PBS and centrifugation. Then, the cell pellet was dissolved in 1X PBS and then transferred to +4°C for storage or were directly stained with propidium iodide (PI). For staining, RNase A at the final concentration of 20 µg/mL was added and the mixture was incubated at 37°C for 15 minutes. Then, the mixture was centrifuged at 13000 rpm for 10 minutes and dissolved in 1X PBS. PI solution (0.5 µg/mL) at the final concentration of 40 µg/mL was added and the cells were incubated at room temperature for 30 minutes in dark. After the incubation was completed, the cells were used to perform FACS analyses.

2.3.16. Statistical Analyses

All statistical analyses were performed in GraphPad Prism 6.01 software. Comparison of data for 2D colony formation and viability were performed with two-tailed t test or one-way ANOVA or two-way ANOVA where applicable.

2.3.17. Experimental Approach

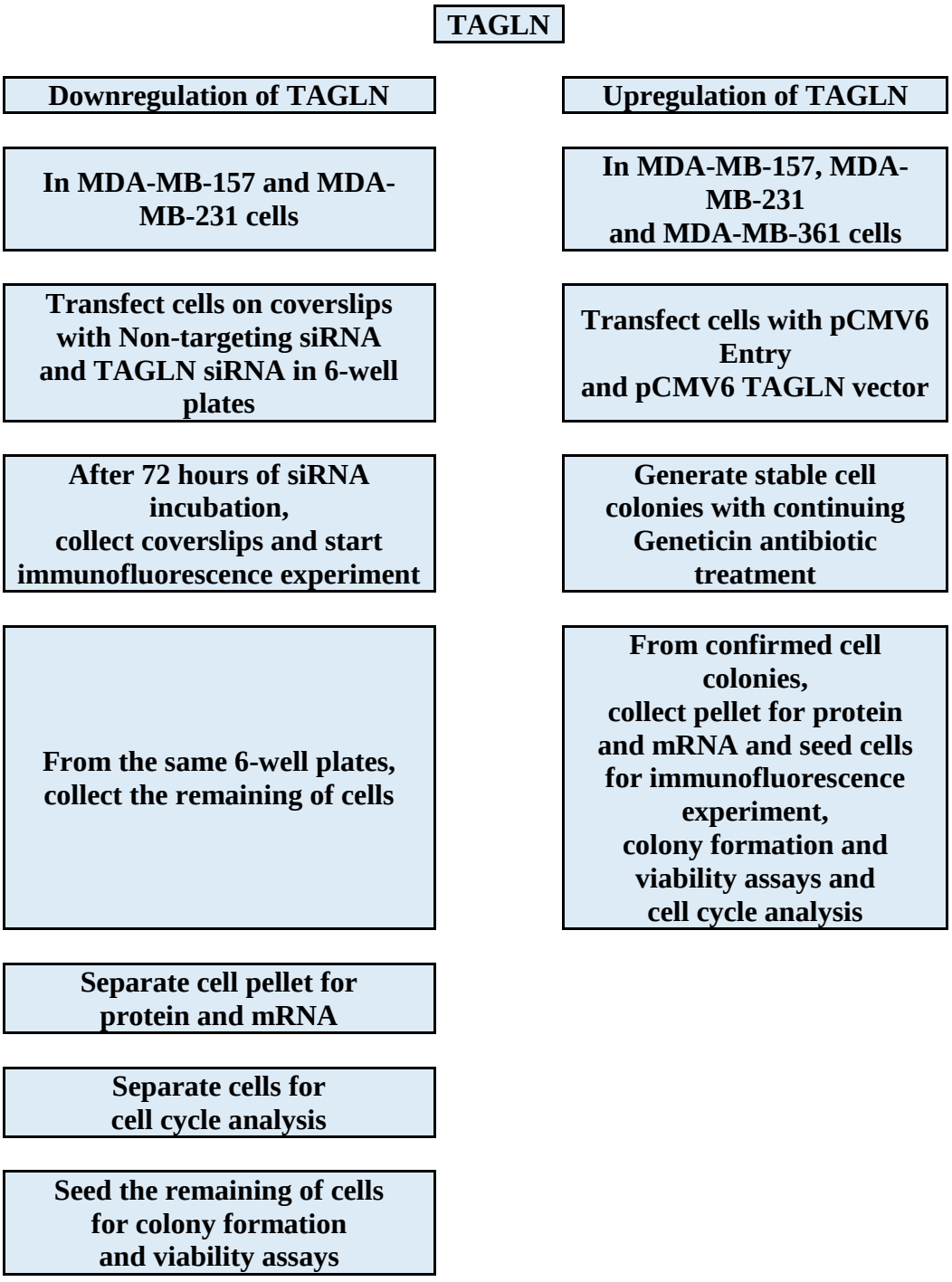


Figure 2.1. Experimental approach which was followed for siRNA and overexpression transfection.

Left panel is the approach for siRNA transfection. Right panel is the approach for overexpression transfection. Order is from top to bottom.

Chapter 3. Results

3.1. Immunofluorescence Staining of Breast Cancer Cell Lines

Various breast cancer cell lines and non-tumorigenic cell line were stained with Anti-TAGLN antibody and different Epithelial to Mesenchymal Transition (EMT) markers. The characteristics of the breast carcinoma cell lines used are given in **Table 3.1**. In this study, E-Cadherin and Occludin were used as epithelial cell markers whereas N-Cadherin and Vimentin were used as mesenchymal cell markers.

Table 3.1. Summary table of staining with TAGLN and EMT markers in breast carcinoma cell lines.
-: Non-stained due to low expression; NS: No staining with the indicated antibody and + or ++ or +++: Staining degrees from low to high.

Cell Line	TAGLN	E-Cadherin	N-Cadherin	Occludin	Vimentin
BT-474	++	+++	NS	NS	-
MCF-12A	++	+	NS	NS	++
MDA-MB-157	+++	-	NS	NS	+++
MDA-MB-231	++	-	NS	NS	++
MDA-MB-361	+	+	NS	NS	-

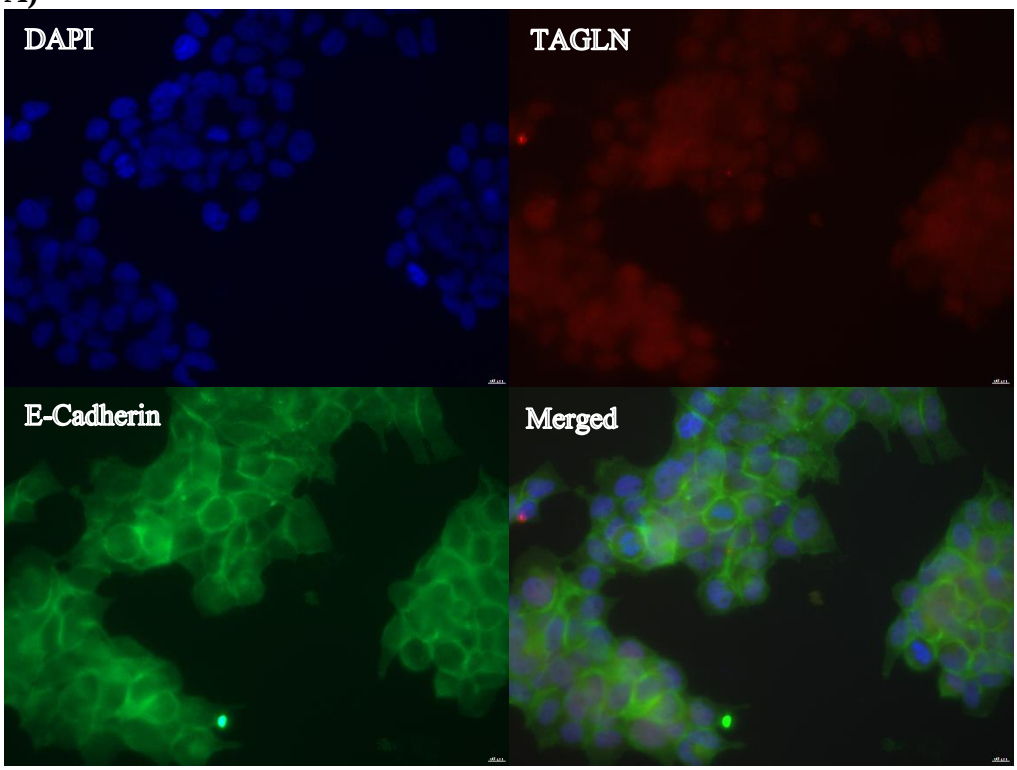
Initially, epithelial BT-474 cells and epithelial MCF-12A cells were co-stained with EMT antibodies to assess the efficiency of EMT antibodies.

3.1.1. Co-immunofluorescence Staining of BT-474 Breast Carcinoma Cell Line

Co-immunofluorescence staining of BT-474 cells showed that *TAGLN* expression was more intense in nucleus where it co-localized with DAPI staining and it was also observed throughout the cell body. BT-474 cells displayed high E-Cadherin expression that supported the epithelial characteristics of BT-474 cells. Mesenchymal marker

Vimentin expression was not present in the same cells. Occludin and N-Cadherin were undetectable in the same BT-474 cells (**Figure 3.1**).

A)



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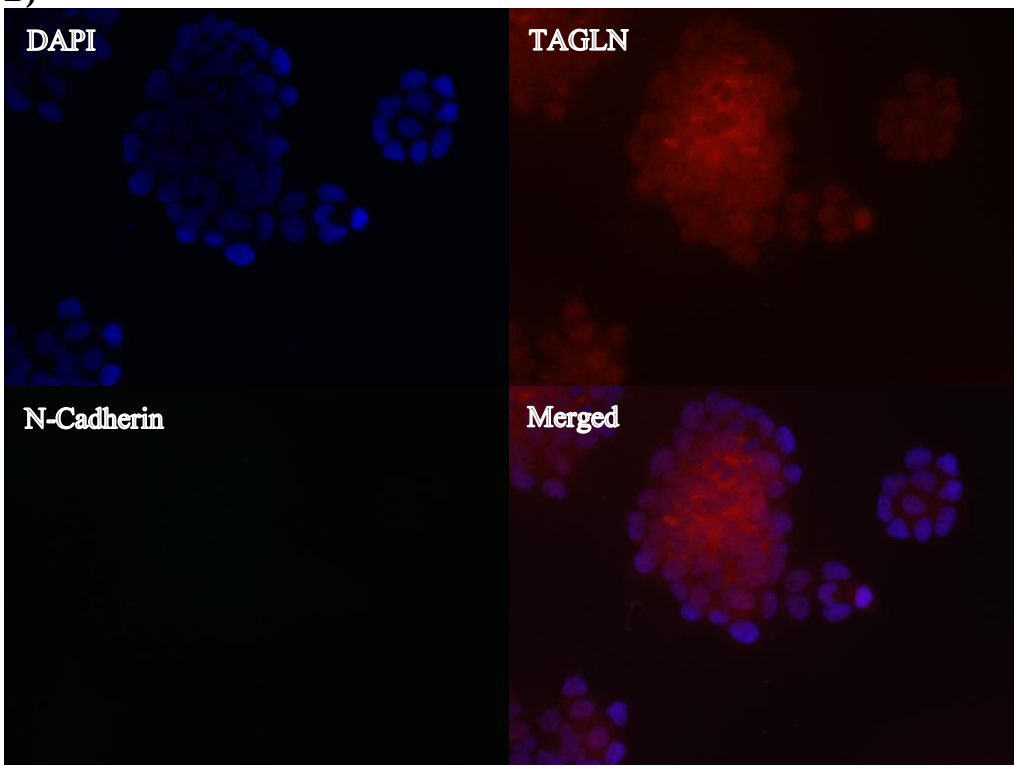

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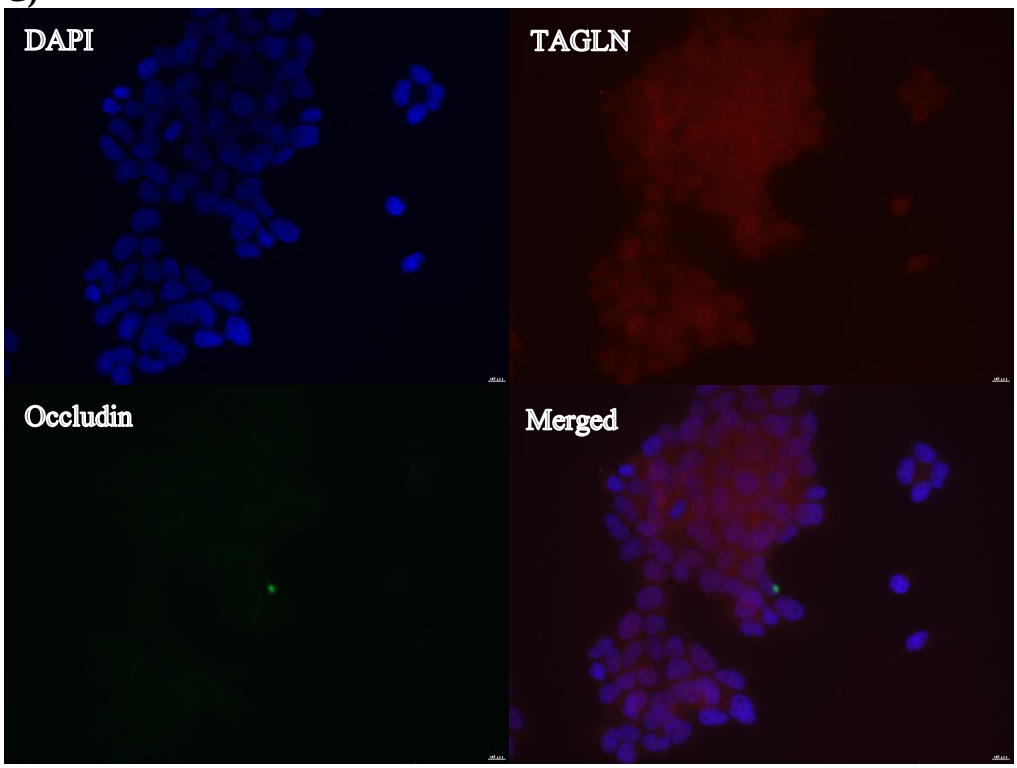

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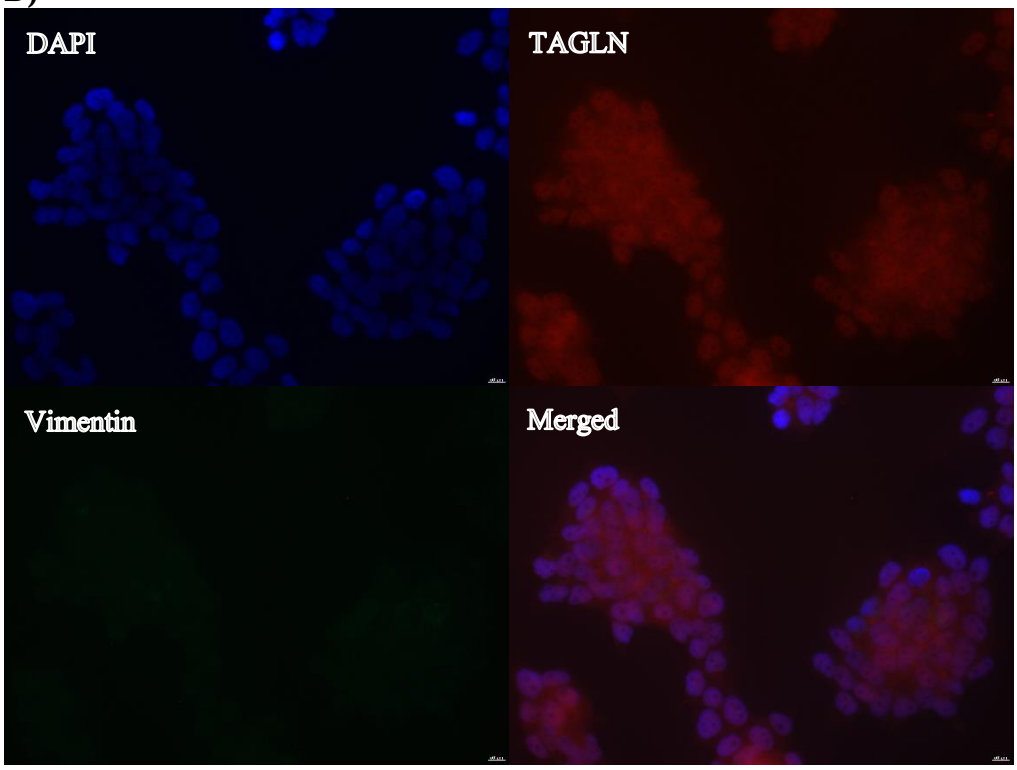


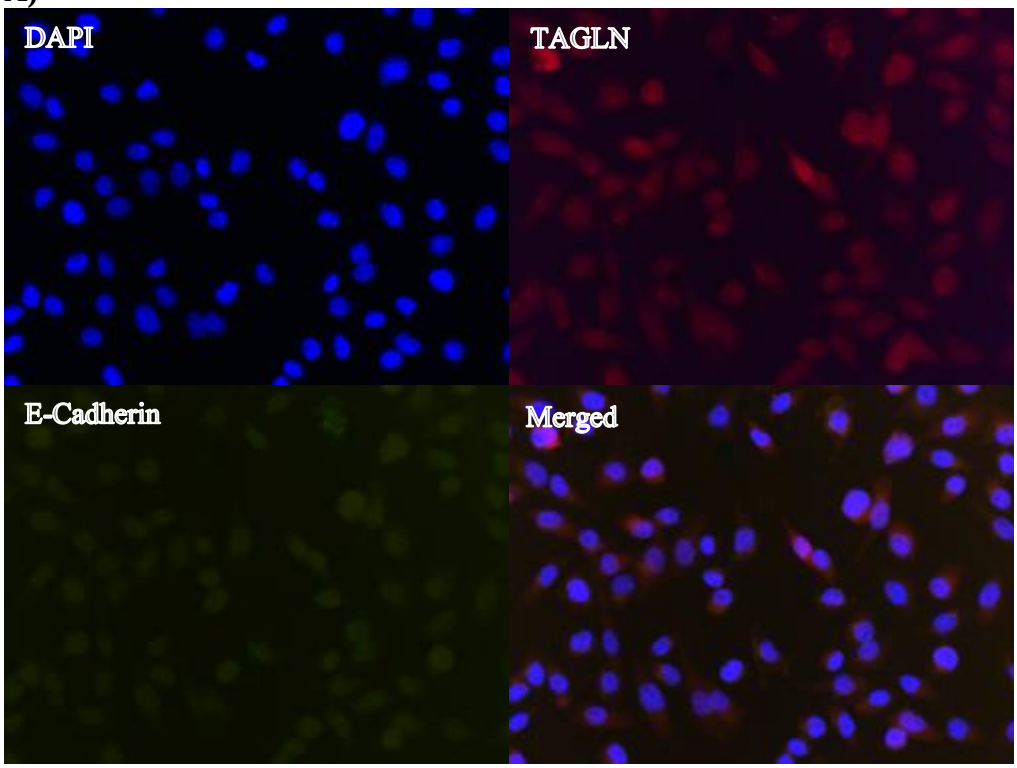
Figure 3.1. Co-immunofluorescence staining of BT-474 cells.

Co-staining of Transgelin with epithelial markers A) E-Cadherin and B) Occludin and mesenchymal markers C) N-Cadherin and D) Vimentin antibodies. Images were taken at 40X. The scale bar is 20 μm. The staining was performed twice.

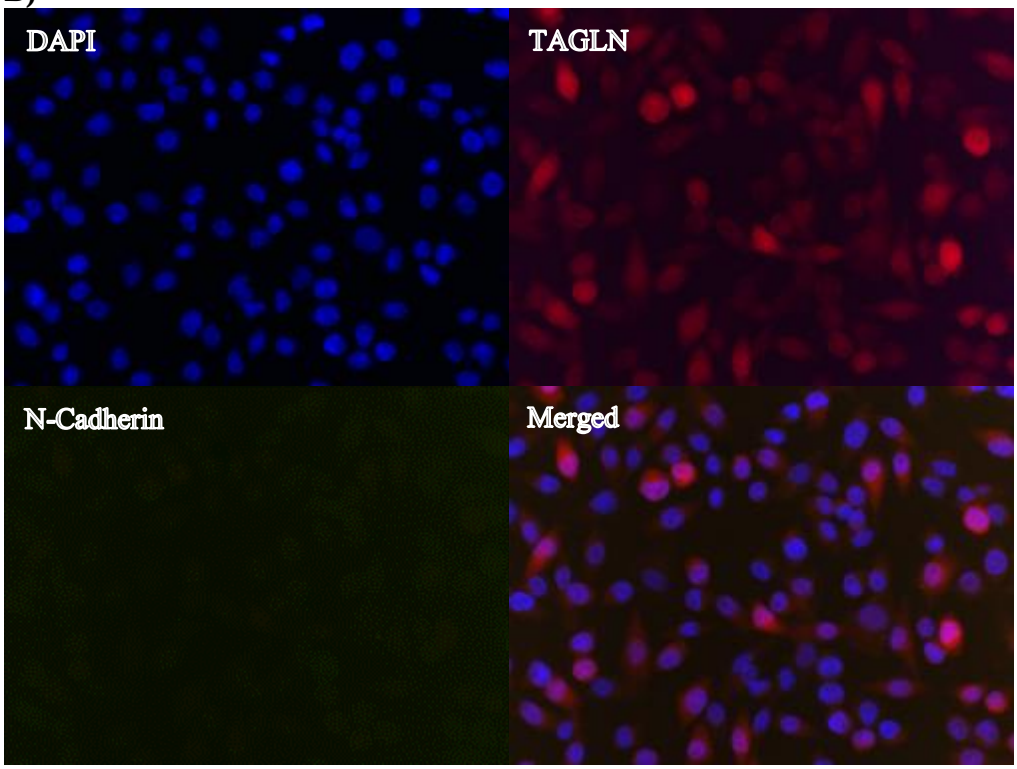
3.1.2. Co-immunofluorescence Staining of MCF-12A NT Breast Cell Line

One non-tumorigenic epithelial breast cell line, MCF-12A, was also included to test the quality of EMT antibodies. Co-immunofluorescence staining of MCF-12A cells showed that *TAGLN* expression was observed throughout the cell body. MCF-12A cells displayed low E-Cadherin and high Vimentin expression contrary to the fact that it was reported as epithelial cell (24). Occludin and N-Cadherin were undetectable in the same MCF-12A cells (**Figure 3.2**).

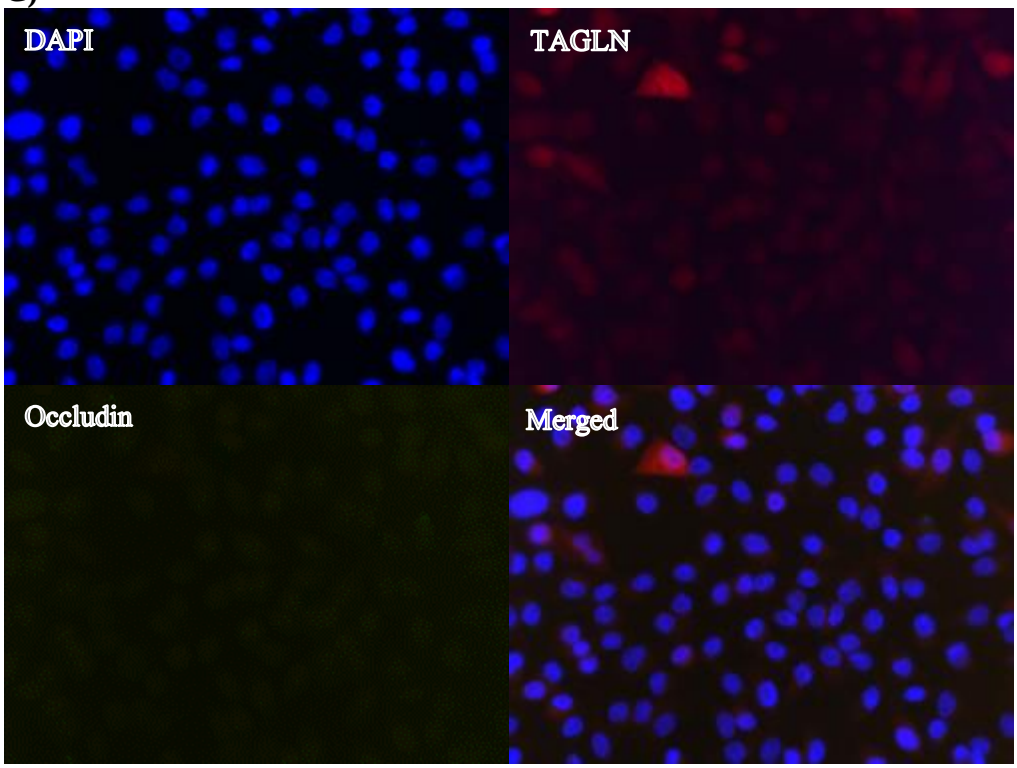
A)



B)



C)



D)

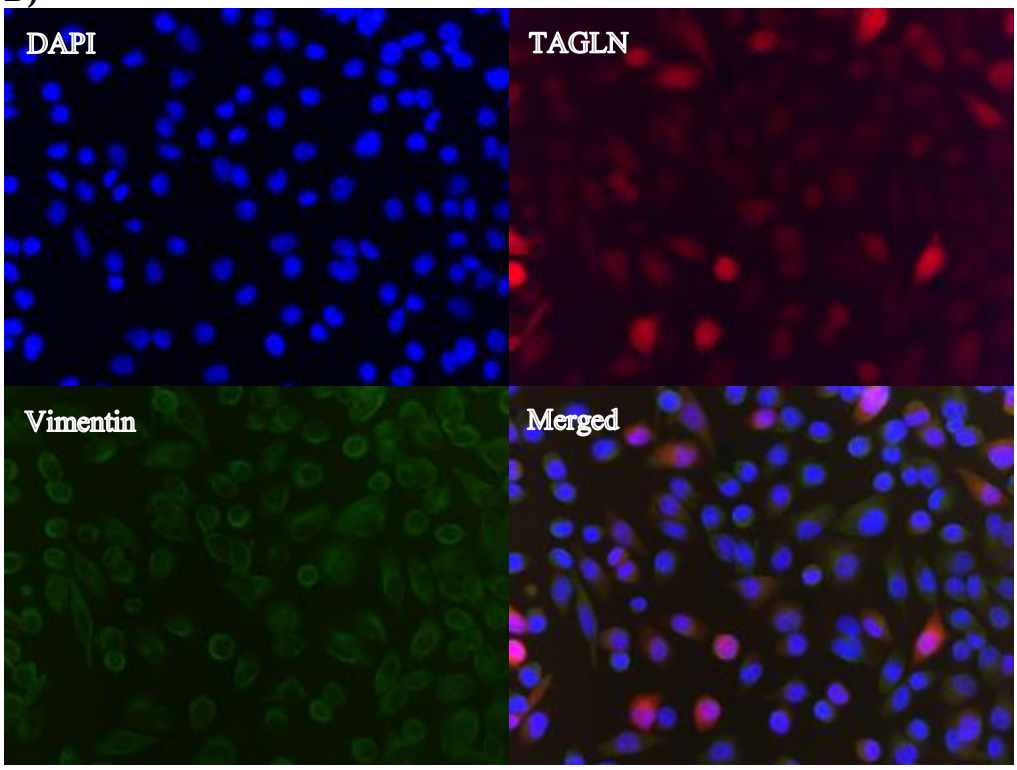
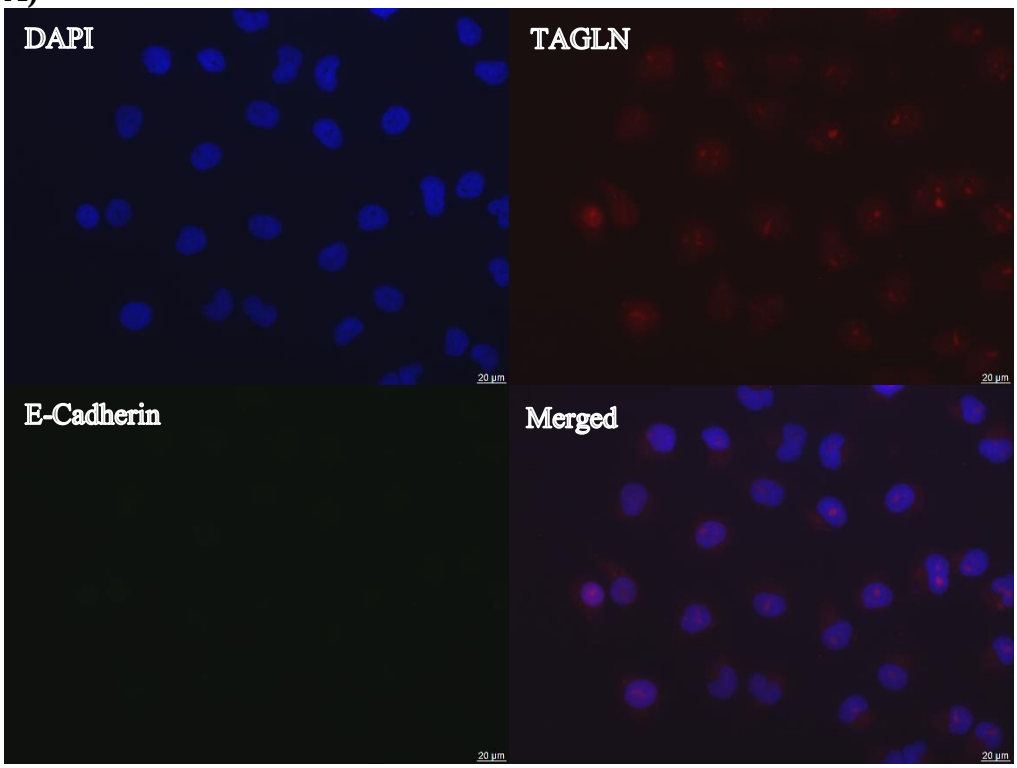


Figure 3.2. Co-immunofluorescence staining of MCF-12A cells.
Co-staining of Transgelin with epithelial markers A) E-Cadherin and B) Occludin and mesenchymal markers C) N-Cadherin and D) Vimentin antibodies. Images were taken at 40X. The scale bar is 20 μm. The staining was performed once.

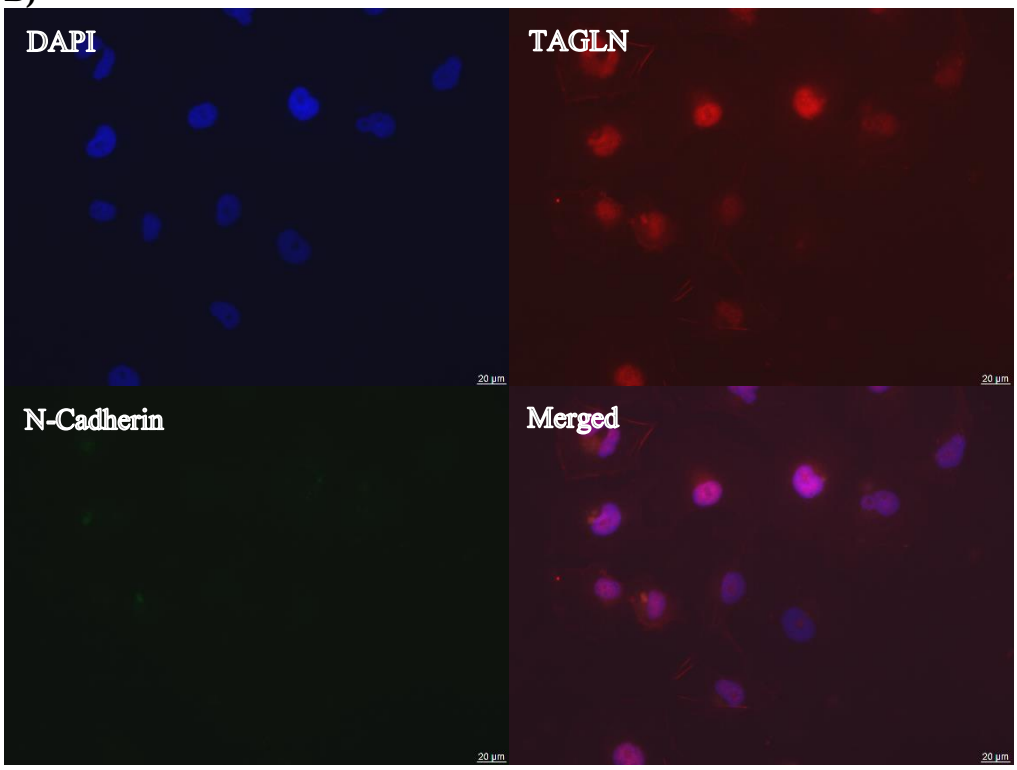
3.1.3. Co-immunofluorescence Staining of MDA-MB-157 Breast Carcinoma Cell Line

Co-immunofluorescence staining of MDA-MB-157 cells showed that *TAGLN* expression was more intense in nucleus where it co-localized with DAPI staining and it was also observed throughout the cell body. MDA-MB-157 cells displayed high Vimentin expression which supported the mesenchymal characteristics of MDA-MB-157 cells. Epithelial marker E-Cadherin was not present in the same cells. Occludin and N-Cadherin were undetectable in the same MDA-MB-157 cells (**Figure 3.3**).

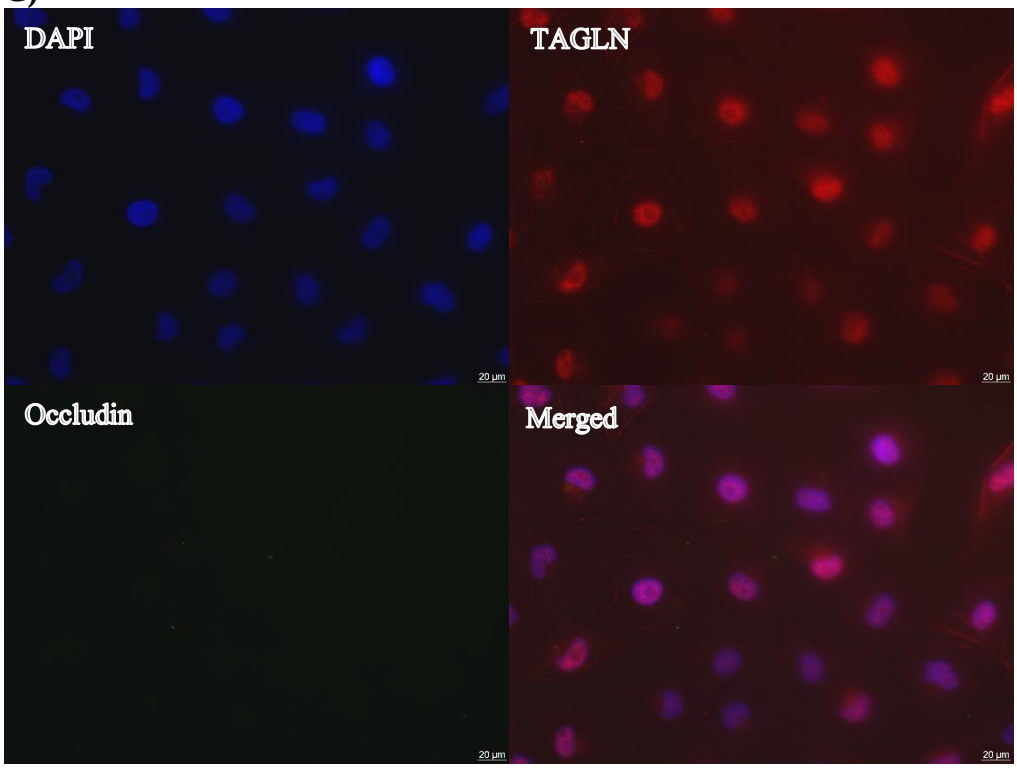
A)


DAPI
TAGLN
E-Cadherin
Merged
20 µm

B)


DAPI
TAGLN
N-Cadherin
Merged
20 µm

C)



D)

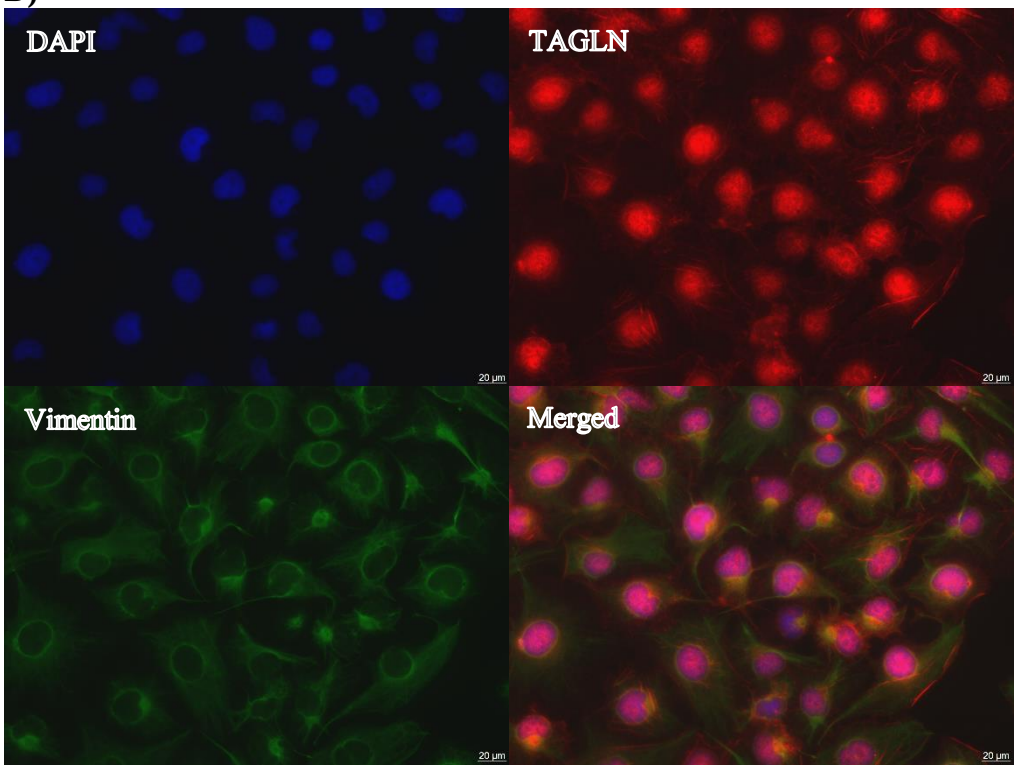
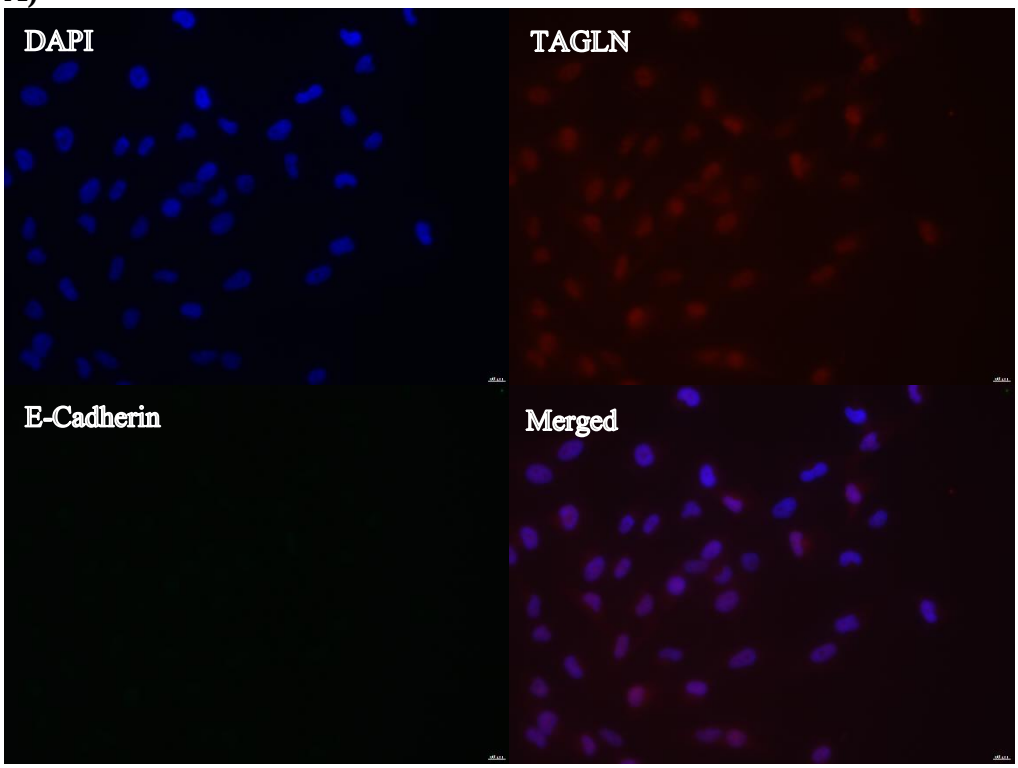


Figure 3.3. Co-immunofluorescence staining of MDA-MB-157 cells.
Co-staining of Transgelin with epithelial markers A) E-Cadherin and B) Occludin and mesenchymal markers C) N-Cadherin and D) Vimentin antibodies. Images were taken at 40X. The scale bar is 20 µm. The staining was performed twice.

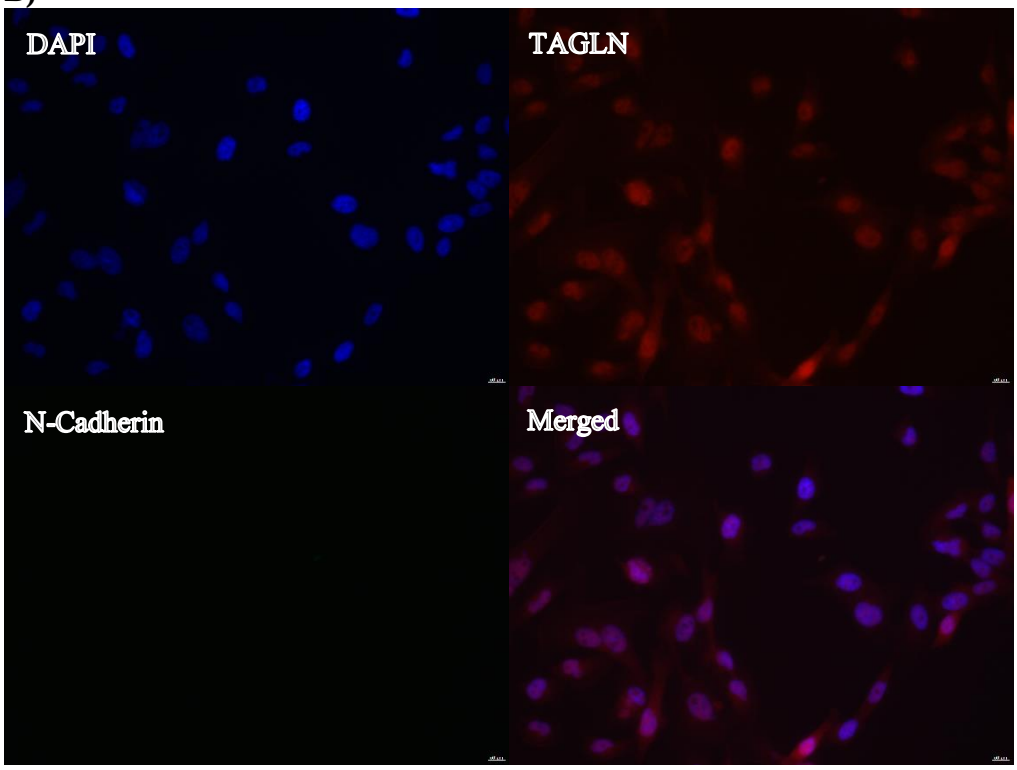
3.1.4. Co-immunofluorescence Staining of MDA-MB-231 Breast Carcinoma Cell Line

Co-immunofluorescence staining of MDA-MB-231 cells showed that *TAGLN* expression was more intense in nucleus where it co-localized with DAPI staining and it was also observed throughout the cell body. MDA-MB-231 cells displayed high Vimentin expression which supported the mesenchymal characteristics of MDA-MB-231 cells. Epithelial marker E-Cadherin was not present in the same cells. Occludin and N-Cadherin were undetectable in the same MDA-MB-231 cells (**Figure *3.4***).

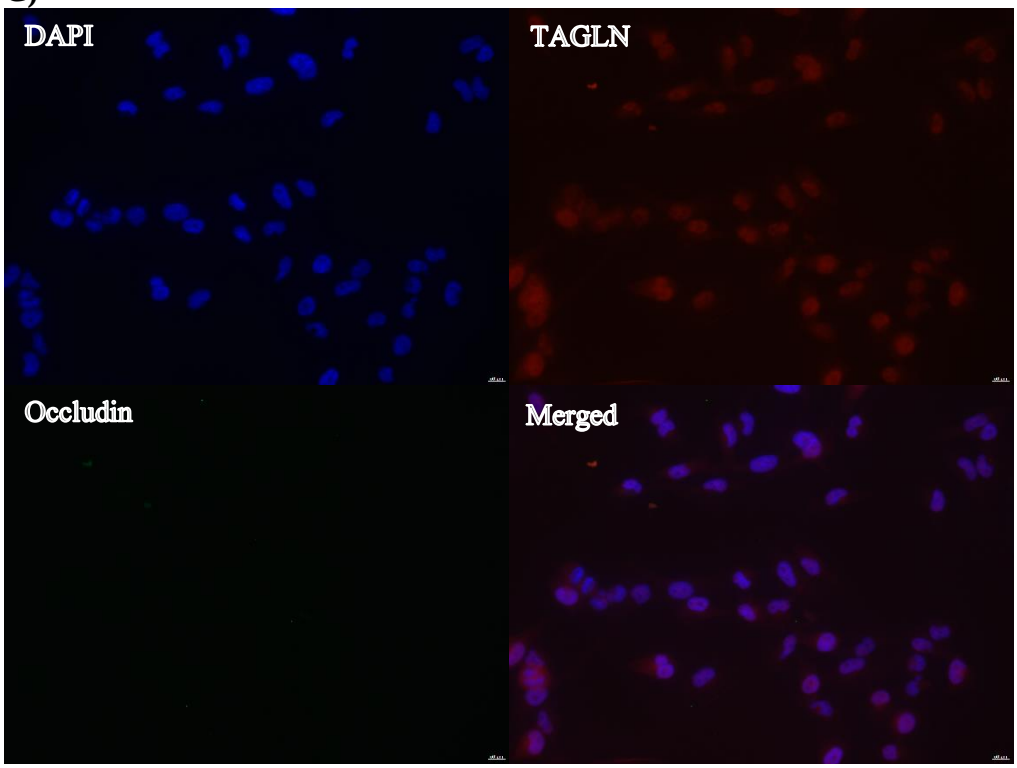
A)



B)



C)



D)

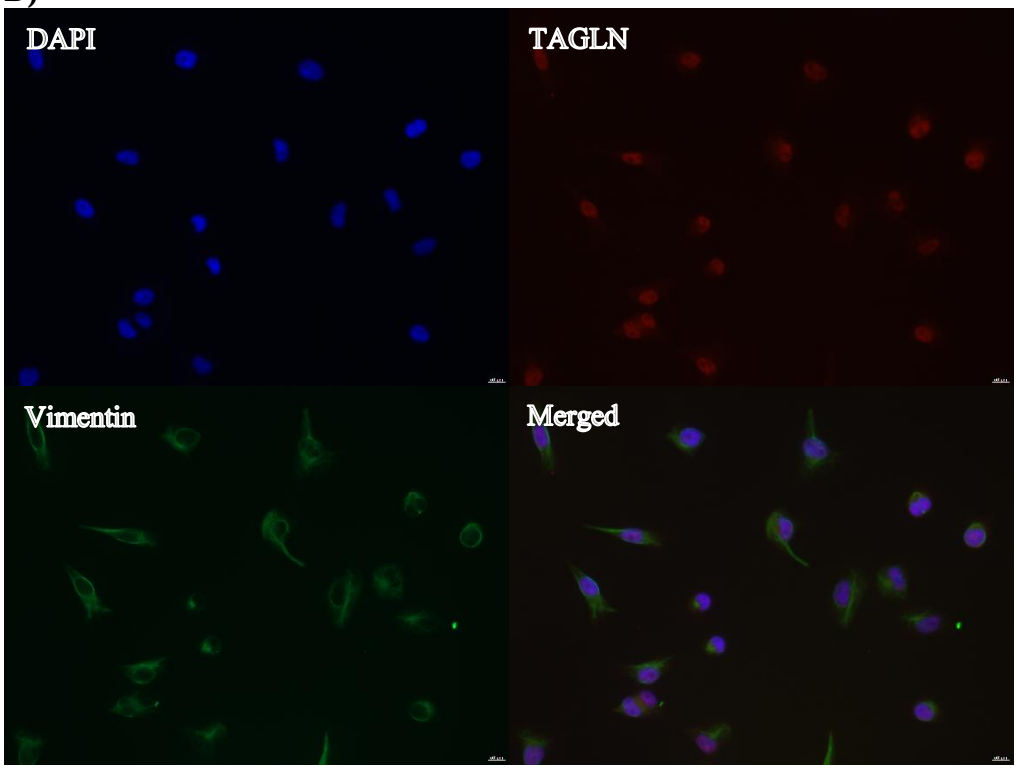
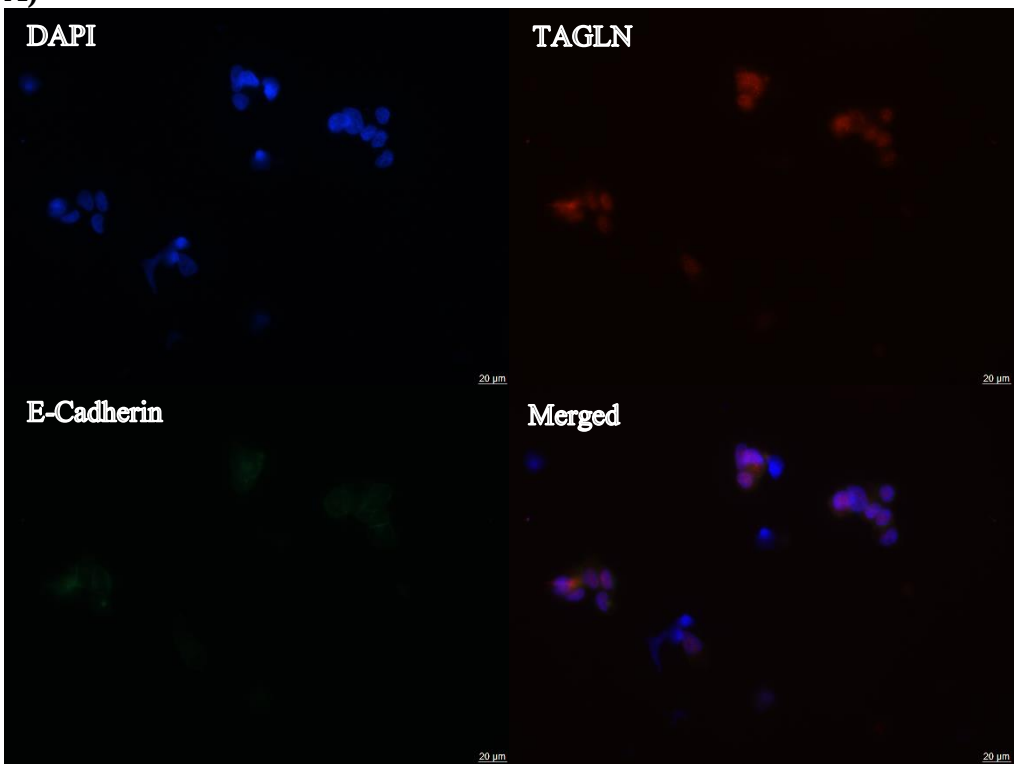


Figure 3.4. Co-immunofluorescence staining of MDA-MB-231 cells.
Co-staining of Transgelin with epithelial markers A) E-Cadherin and B) Occludin and mesenchymal markers C) N-Cadherin and D) Vimentin antibodies. Images were taken at 40X. The scale bar is 20 μm. The staining was performed twice.

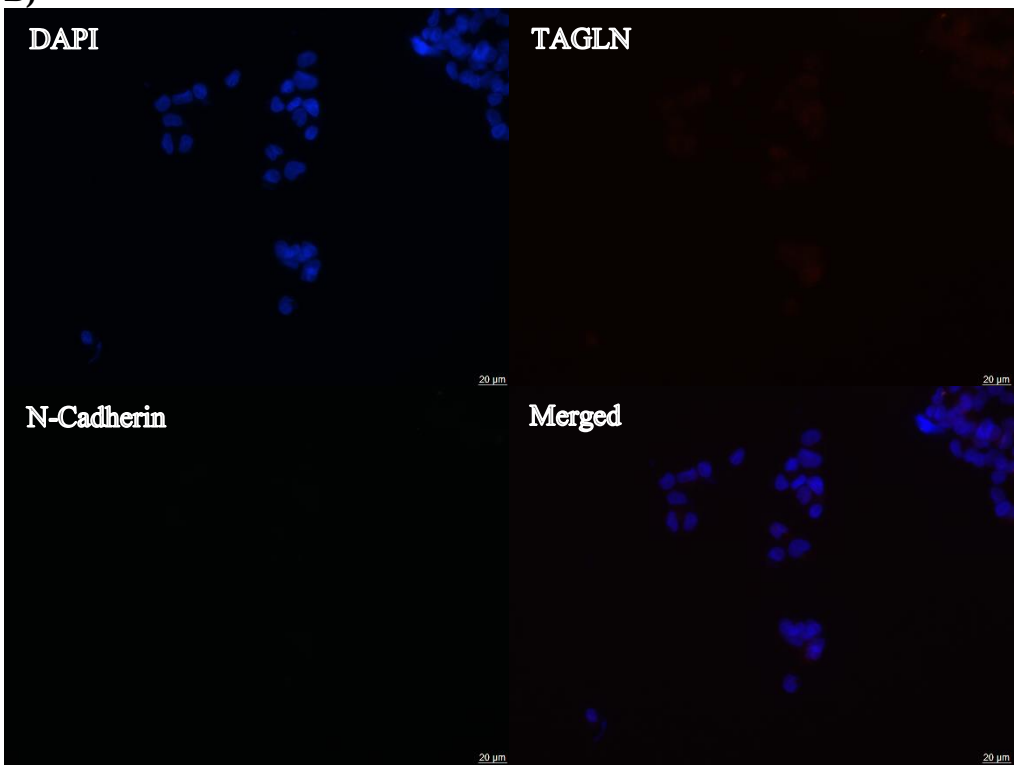
3.1.5. Co-immunofluorescence Staining of MDA-MB-361 Breast Carcinoma Cell Line

Co-immunofluorescence staining of MDA-MB-361 cells showed that *TAGLN* expression was low and it was observed throughout the cell body. MDA-MB-361 cells displayed medium E-Cadherin expression that supported the epithelial characteristics of MDA-MB-361 cells. Mesenchymal marker Vimentin expression was not present in the same cells. Occludin and N-Cadherin were undetectable in the same MDA-MB-361 cells (**Figure 3.5**).

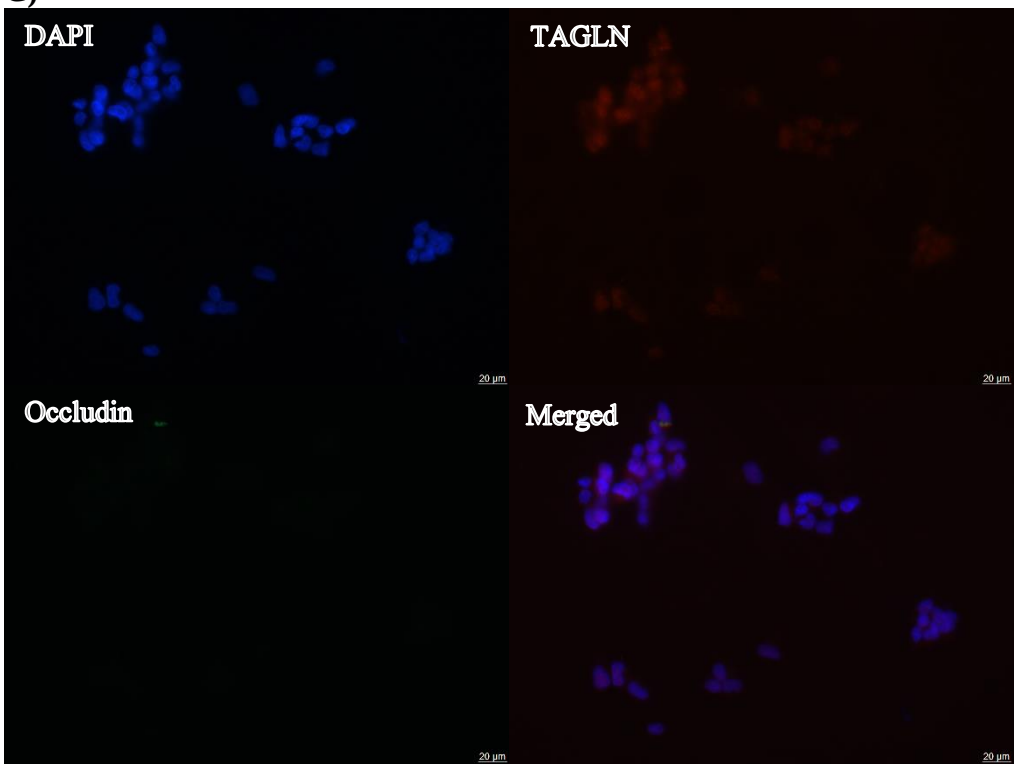
A)


DAPI
TAGLN
E-Cadherin
Merged
20 µm

B)


DAPI
TAGLN
N-Cadherin
Merged
20 µm

C)



D)

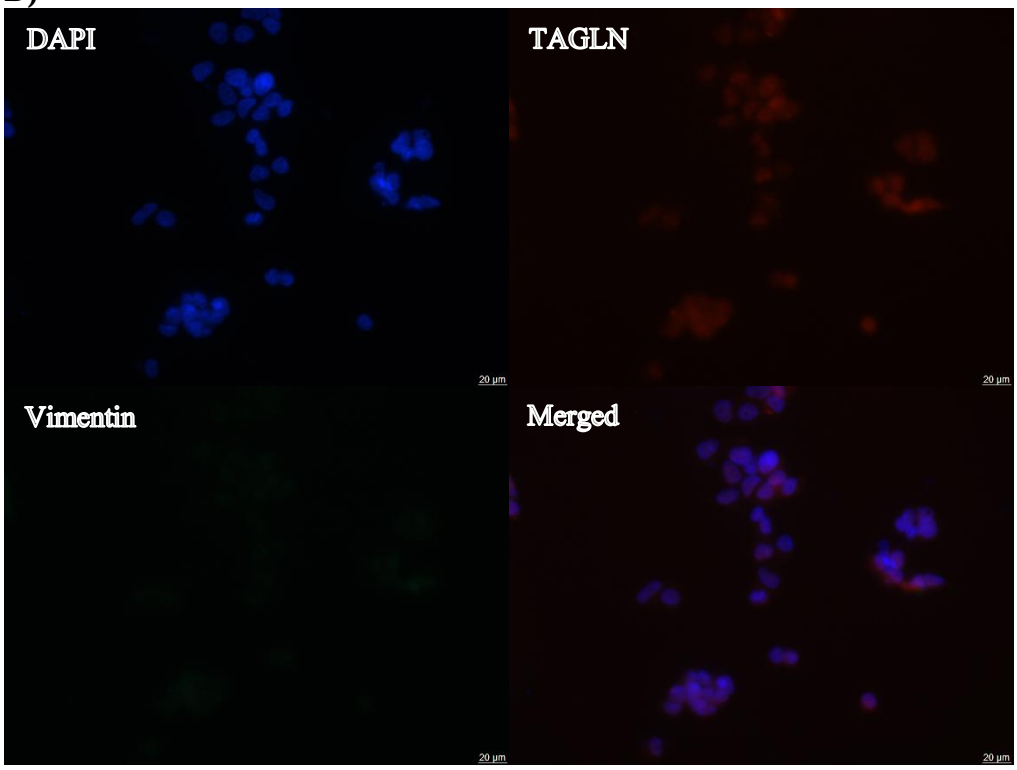


Figure 3.5. Co-immunofluorescence staining of MDA-MB-361 cells.
Co-staining of Transgelin with epithelial markers A) E-Cadherin and B) Occludin and mesenchymal markers C) N-Cadherin and D) Vimentin antibodies. Images were taken at 40X. The scale bar is 20 µm. The staining was performed twice.

3.2. Analysis of TAGLN Silencing in Breast Cancer Cell Lines

TAGLN gene was aimed to be silenced in MDA-MB-157 and MDA-MB-231 cells by stable transfection by using GIPZ-TAGLN vectors. Although the cells were transfected successfully and showed green fluorescence signal, TAGLN gene silencing could not be maintained in the cells in the long run with selection medium. It was also observed that cells which were transfected with Empty shRNA vector showed more decrease in TAGLN expression compared to the cells which transfected with TAGLN shRNA vectors (Data not shown).

Therefore, it was decided to use siRNA transfection for gene silencing.

TAGLN gene was transiently silenced in MDA-MB-157 and MDA-MB-231 cells by siRNA transfection. The cells were collected after 48 and 72 hours of siRNA treatment and analyzed with Western Blot. At 48 hours, TAGLN expression was very low in both Non-targeting siRNA transfected and TAGLN siRNA transfected cells. Long exposure did not reveal any better quality. It was concluded that at 72 hours TAGLN siRNA treatment is better in decreasing TAGLN expression and used to perform further experiments (**Figure 3.6**). TAGLN protein expression reduced by 35% in MDA-MB-157 cells and by 51% in MDA-MB-231 cells when compared to Non-targeting siRNA treated cells (**Figure 3.7**).

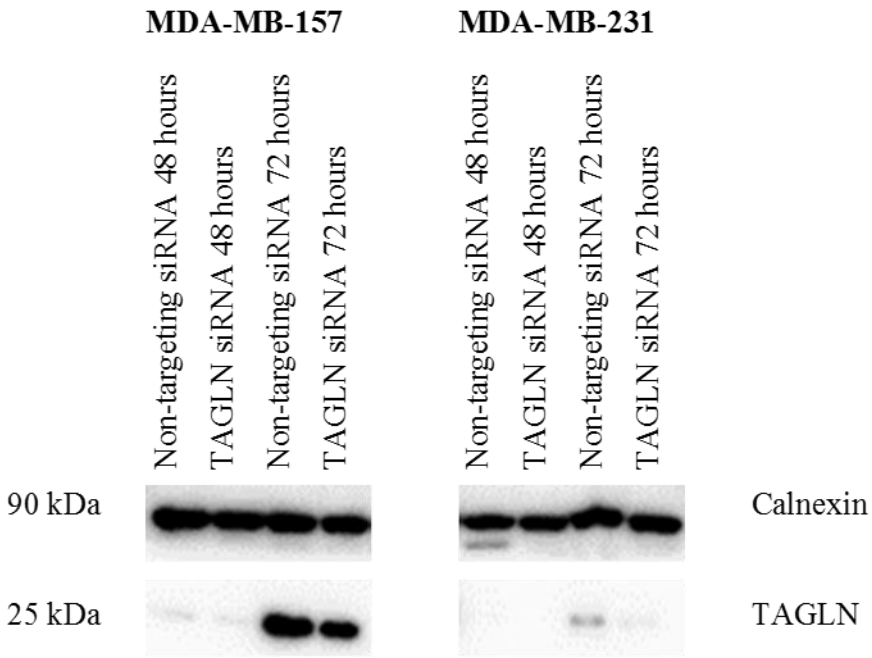


Figure 3.6. TAGLN gene was silenced in MDA-MB-157 and MDA-MB-231 cells with TAGLN siRNA after 72 hours of treatment.

MDA-MB-157 and MDA-MB-231 cells were transfected with Non-targeting siRNA or TAGLN siRNA. Proteins were collected at 48 hours and at 72 hours. The experiment was performed once.

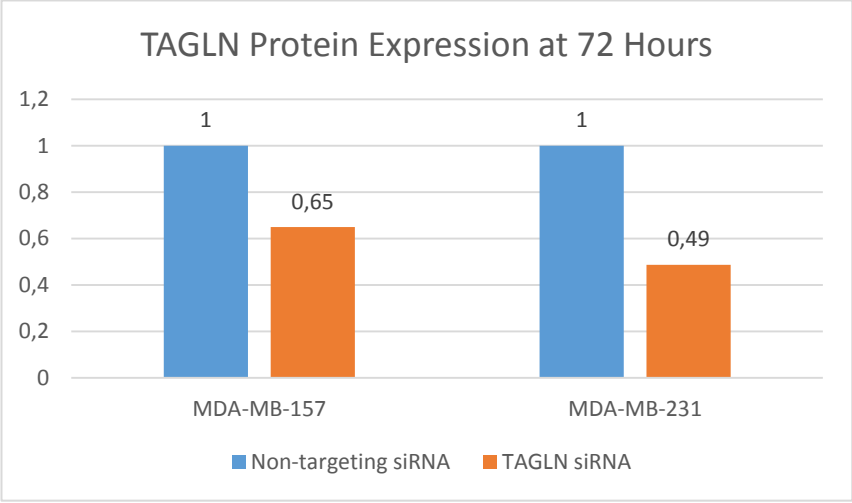


Figure 3.7. TAGLN gene silencing was quantified in MDA-MB-157 and MDA-MB-231 cells with TAGLN siRNA after 72 hours of treatment.

Quantification was performed only for samples at 72 hours since at 48 hours, the bands were not clear. Protein expressions were normalized to Calnexin protein levels. Image J was used for quantification.

3.2.1. Silencing TAGLN Gene in MDA-MB-157 and MDA-MB-231 Cells at 72 Hours Affects Mesenchymal Marker Vimentin Expression.

Both cells lines were transfected with Non-targeting siRNA and TAGLN siRNA and after 72 hours, cell pellets were collected to isolate protein and RNA and the remaining of cells were used in the other functional analysis. Western blot analysis showed that TAGLN siRNA transfected MDA-MB-157 cells displayed 45% decrease in *TAGLN* expression compared to Non-targeting siRNA transfected cells after 72 hours of treatment while TAGLN siRNA transfected MDA-MB-231 cells displayed 86% decrease in TAGLN expression. By silencing *TAGLN* expression, Vimentin protein expression had reduced by 25% in MDA-MB-157 cells and by 27% in MDA-MB-231 cells (**Figure 3.8** and **Figure 3.9**).

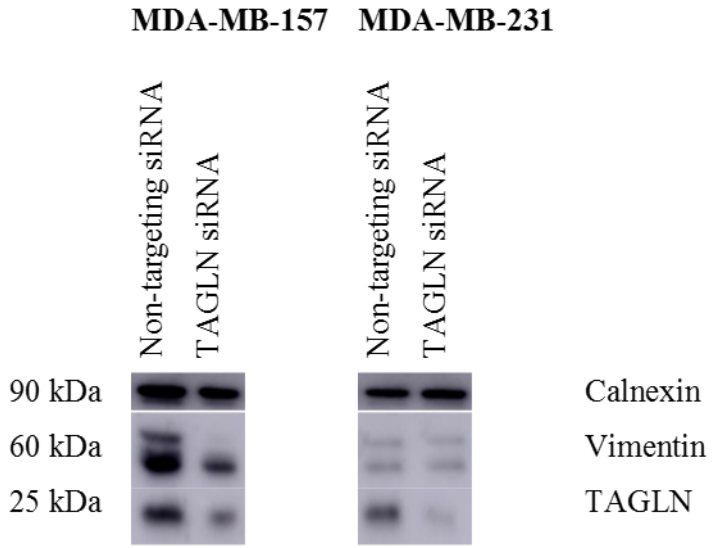


Figure 3.8. TAGLN gene was silenced in MDA-MB-157 and MDA-MB-231 cells with TAGLN siRNA.

By silencing TAGLN gene, Vimentin levels decreased. Calnexin was used as an equal loading control. Data was normalized to Calnexin protein levels. The experiment was performed twice.

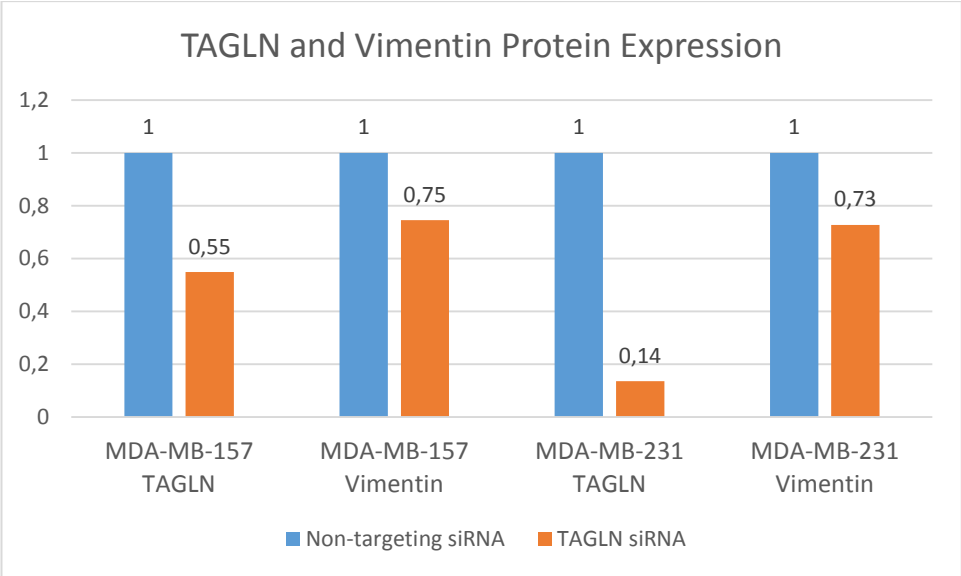


Figure 3.9. TAGLN gene silencing was quantified in MDA-MB-157 and MDA-MB-231 cells with TAGLN siRNA after 72 hours of treatment.
Quantification was performed for samples at 72 hours. Protein expressions were normalized to Calnexin protein levels. Image J was used for quantification.

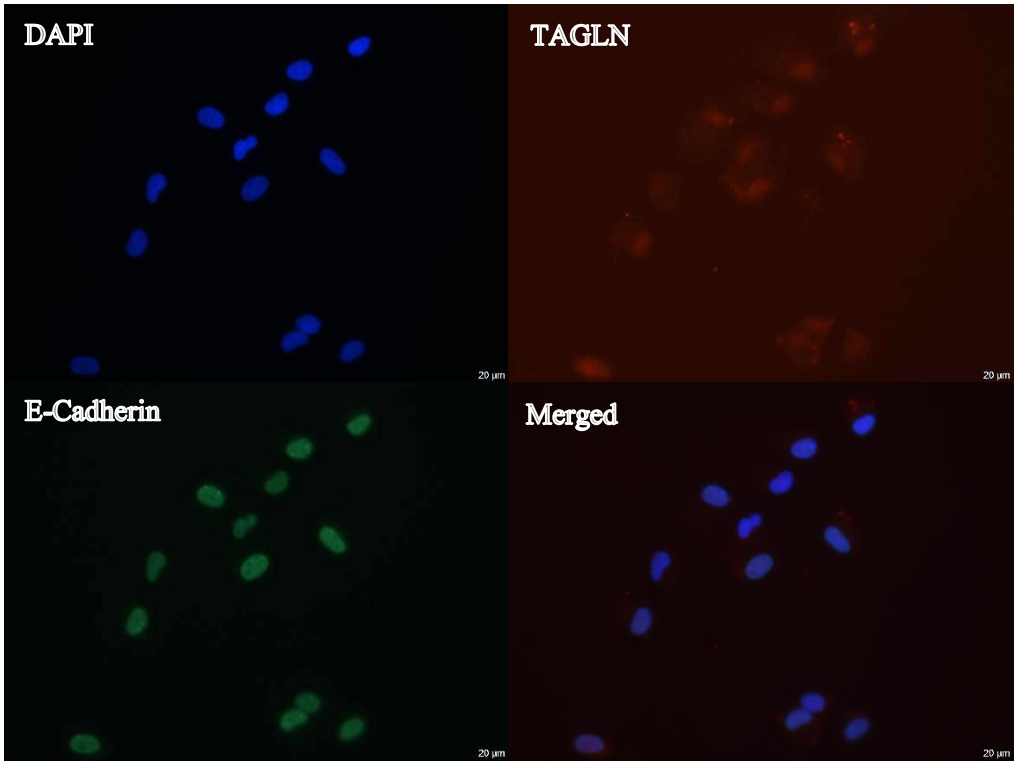
3.2.2. Immunofluorescence Analysis of EMT markers in MDA-MB-157 and MDA-MB-231 cells transfected with Non-targeting siRNA and TAGLN siRNA

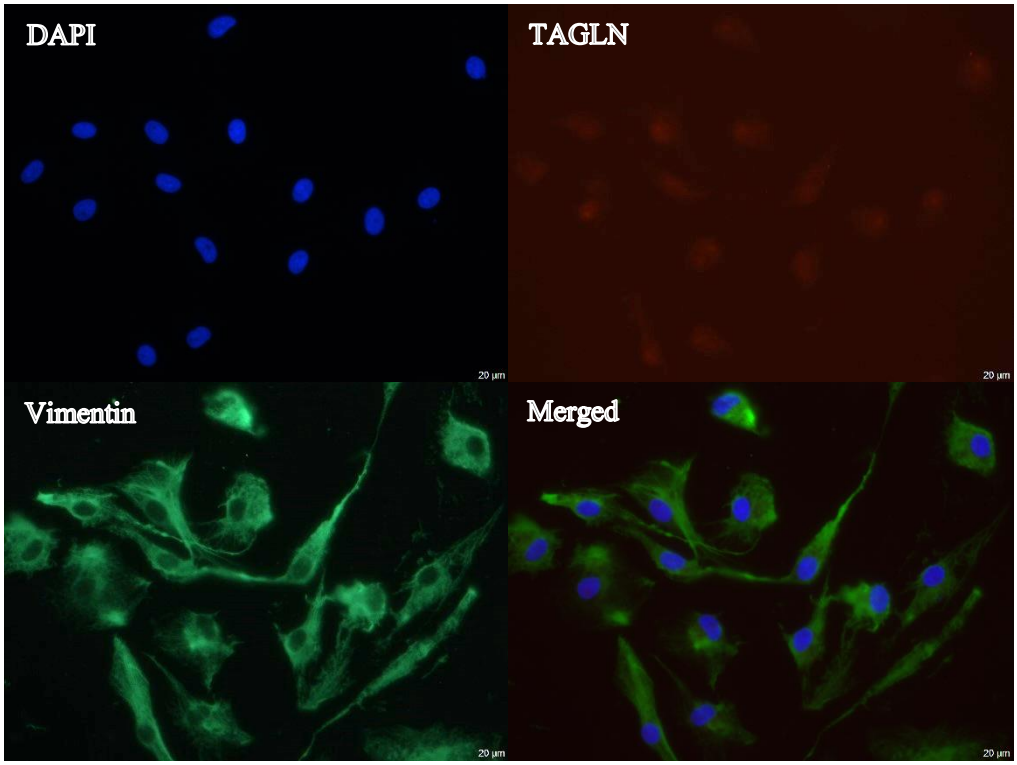
The Vimentin expression was analyzed in both siRNA transfected cell lines to assess the effect of TAGN expression in EMT process. TAGLN silencing had reduced mesenchymal marker Vimentin expression at the protein level in both cell lines (**Figure 3.8***).*

With immunofluorescence staining upon TAGLN silencing, in MDA-MB-157 cells, while there was no difference in E-Cadherin expression, Vimentin protein expression is reduced at the cytoplasm. In MDA-MB-231 cells upon TAGLN silencing, while there was no difference in E-Cadherin expression, Vimentin protein seems to localize to the nucleus (**Figure 3.10**).

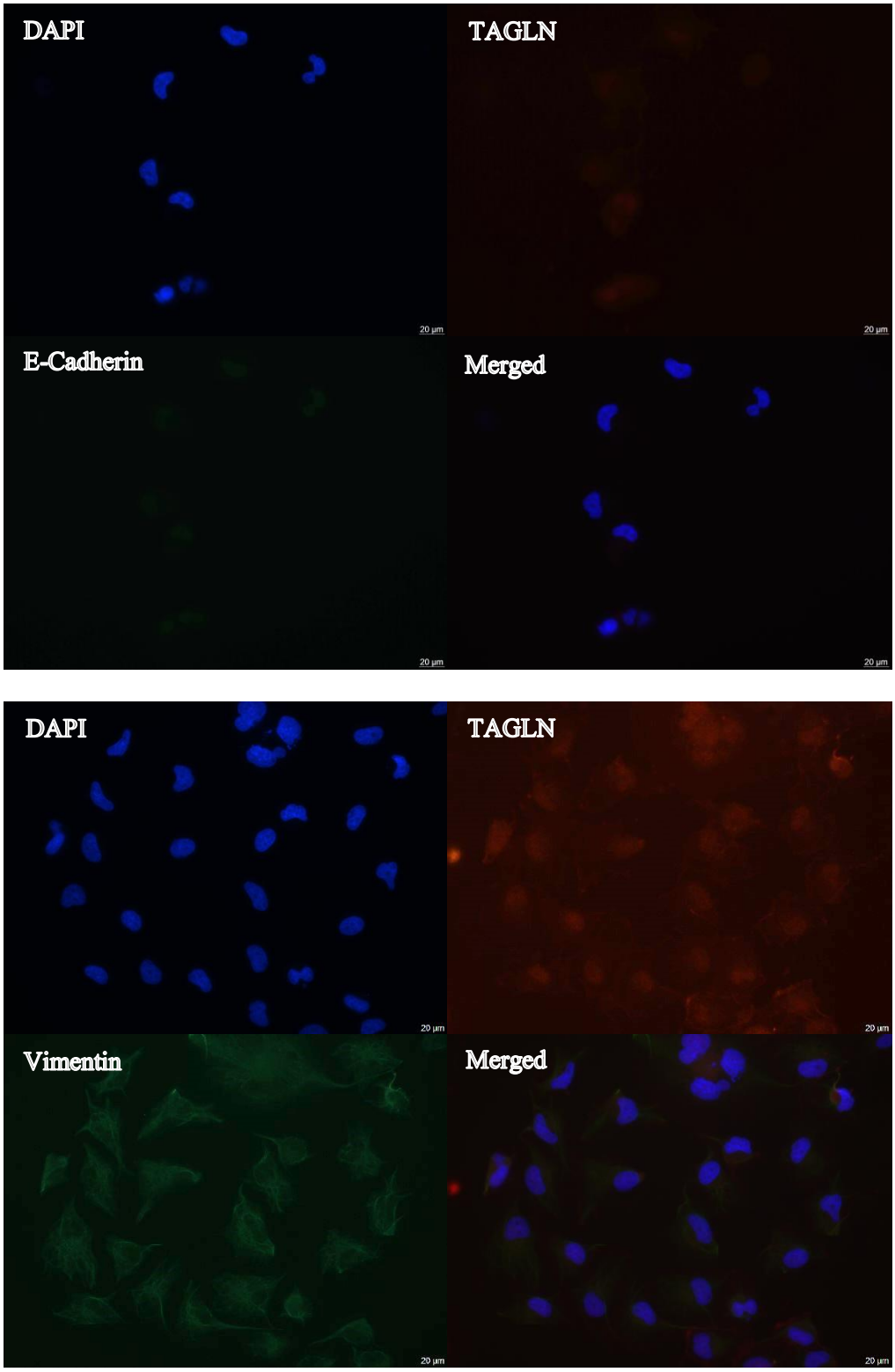
A) MDA-MB-157

MDA-MB-157 Non-targeting siRNA



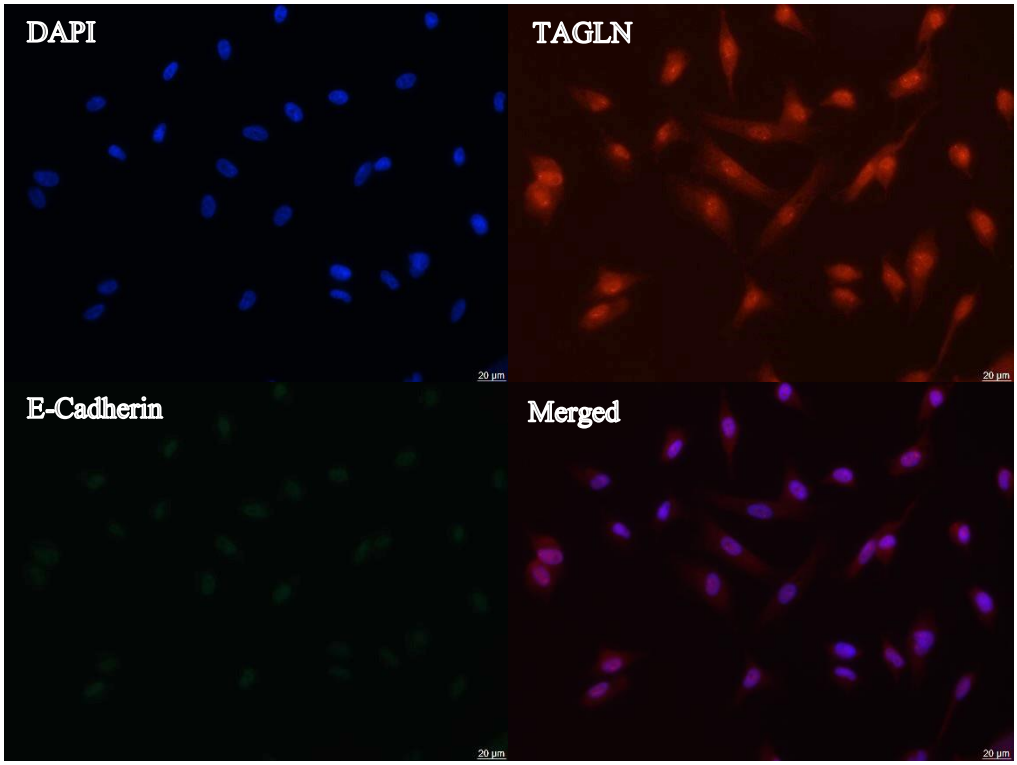


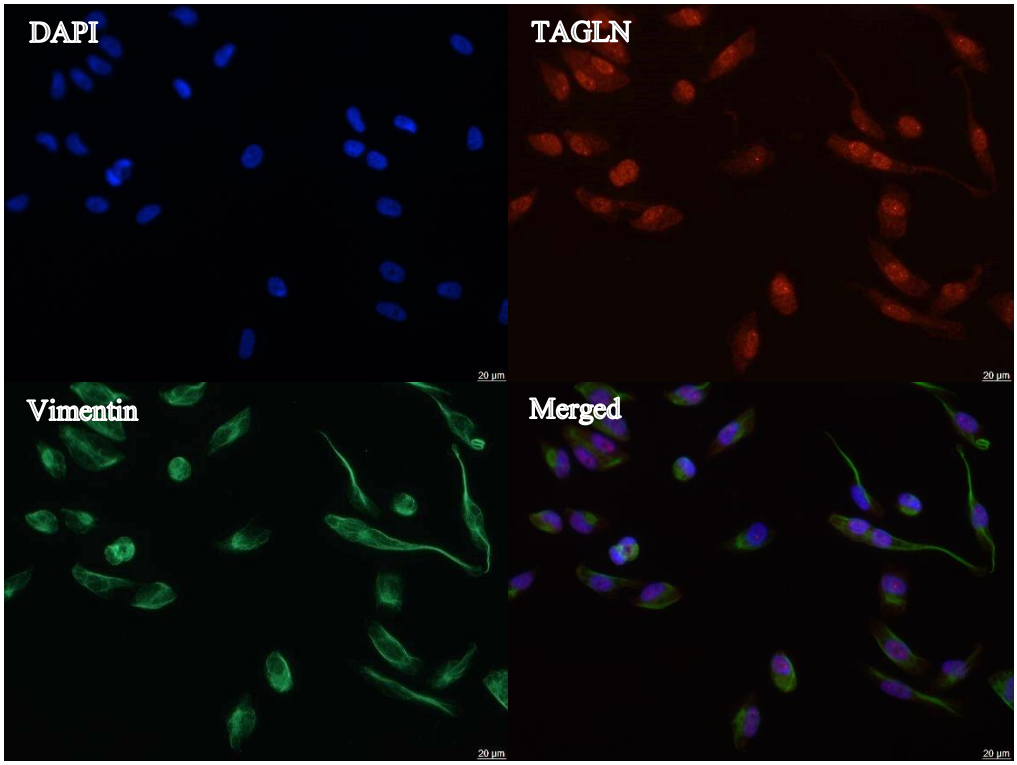
MDA-MB-157 TAGLN siRNA



B) MDA-MB-231

MDA-MB-231 Non-targeting siRNA





MDA-MB-231 TAGLN siRNA

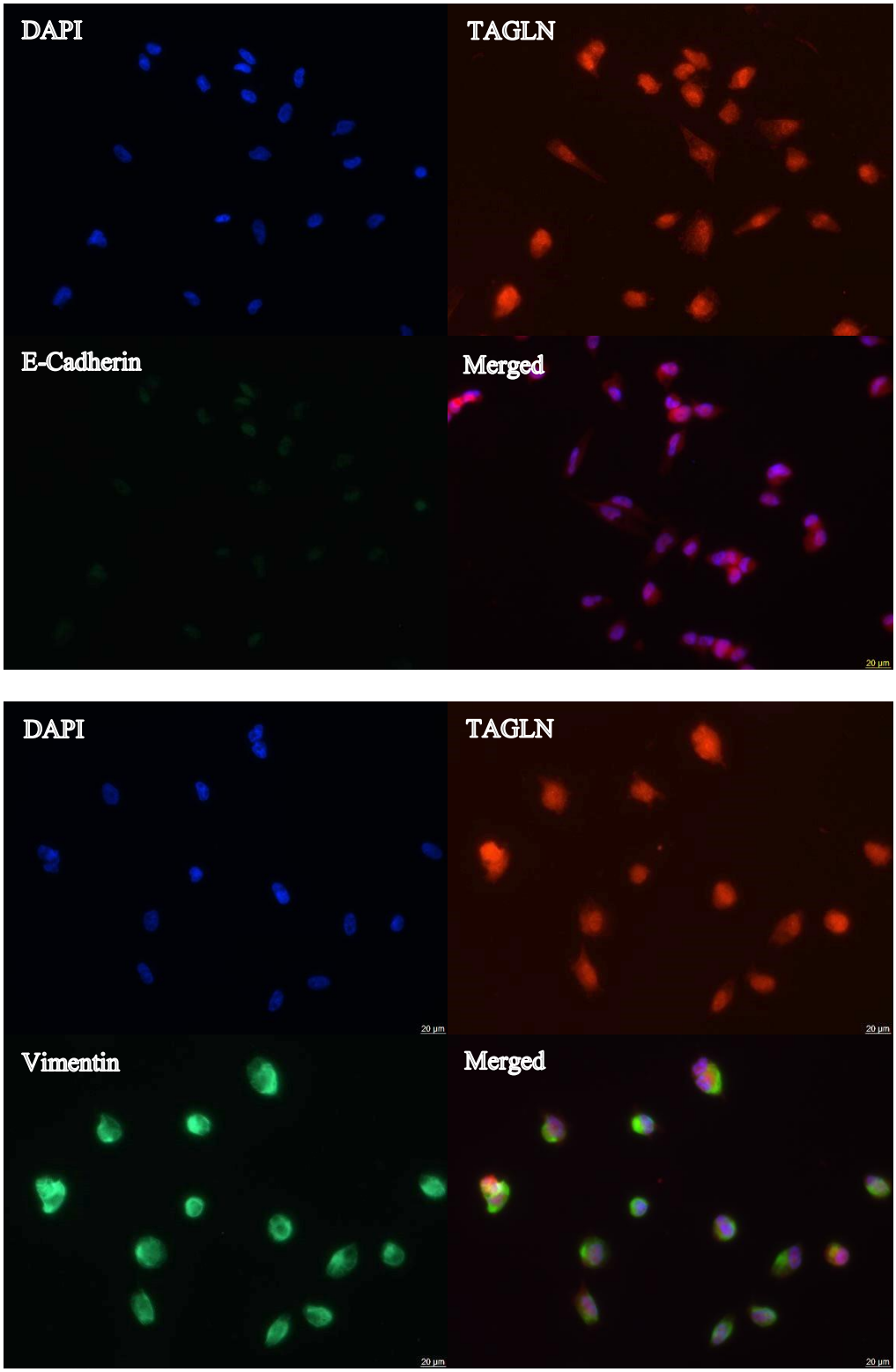


Figure 3.10. Silencing TAGLN gene in MDA-MB-157 and MDA-MB-231 cells effected the expression and localization of mesenchymal marker Vimentin.
Co-immunofluorescence staining, with TAGLN silencing, in A) MDA-MB-157 cells and B) MDA-MB-231 cells were performed after 72 hours of siRNA transfection. Images were taken at 40x. *The scale bar is 20 µm. The experiment was performed once.*

3.2.3. Colony Formation Assay of MDA-MB-157 and MDA-MB-231 Cells Transfected with Non-targeting siRNA and TAGLN siRNA

With TAGLN silencing, there was increase in colony numbers in MDA-MB-157 cells while there was decrease in colony numbers in MDA-MB-231 cells when compared to Non-targeting siRNA transfected cells. Statistical significance was determined by using unpaired two-tailed t-test ($P < 0.05$ in both cell lines) (**Figure 3.11**, **Figure 3.12** and **Table 3.2**).

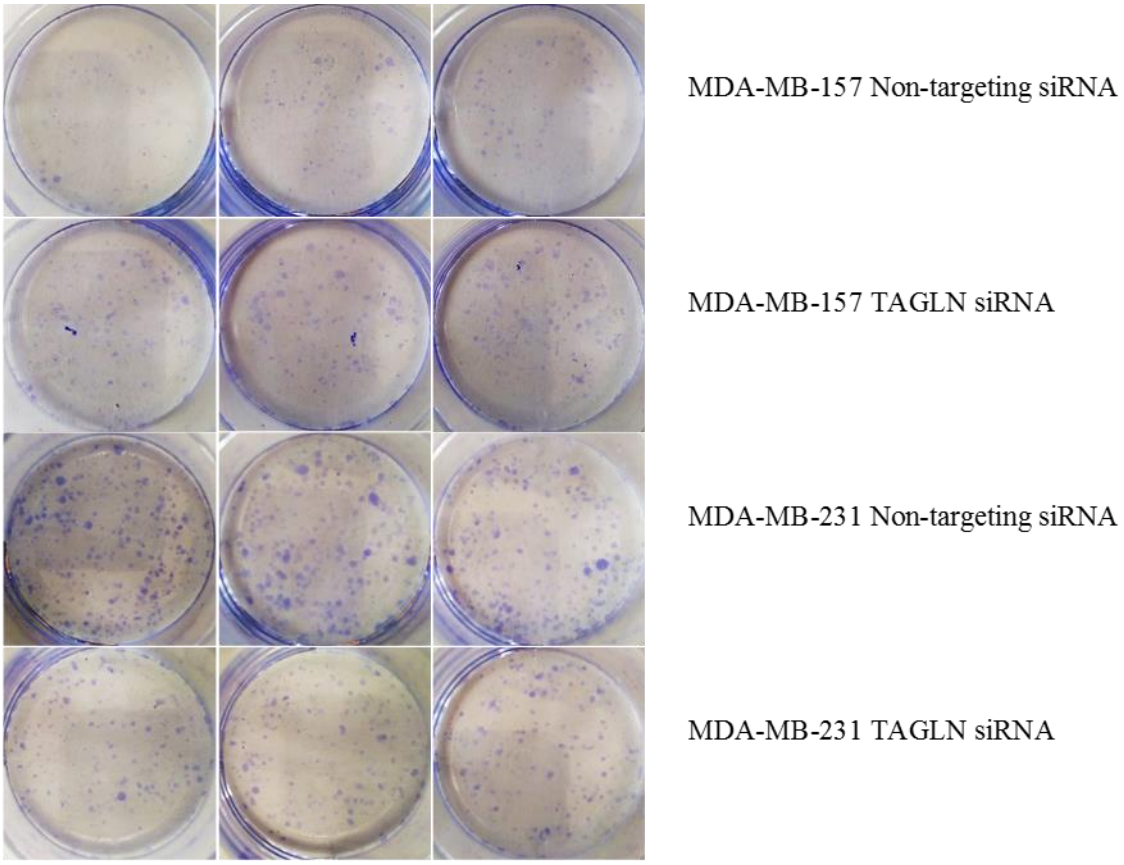


Figure 3.11. Silencing TAGLN gene has increased colony formation capacity of MDA-MB-157 cells but has reduced it in MDA-MB-231 cells.

Cells were seeded in equal numbers in each well after 72 hours of siRNA treatment. They were cultured in normal growth medium until colonies became visible. After 1 or 2 weeks, cells were fixed with ice-cold methanol and then stained with crystal violet solution and colonies were counted. The experiment had three replicates per sample and was performed once.

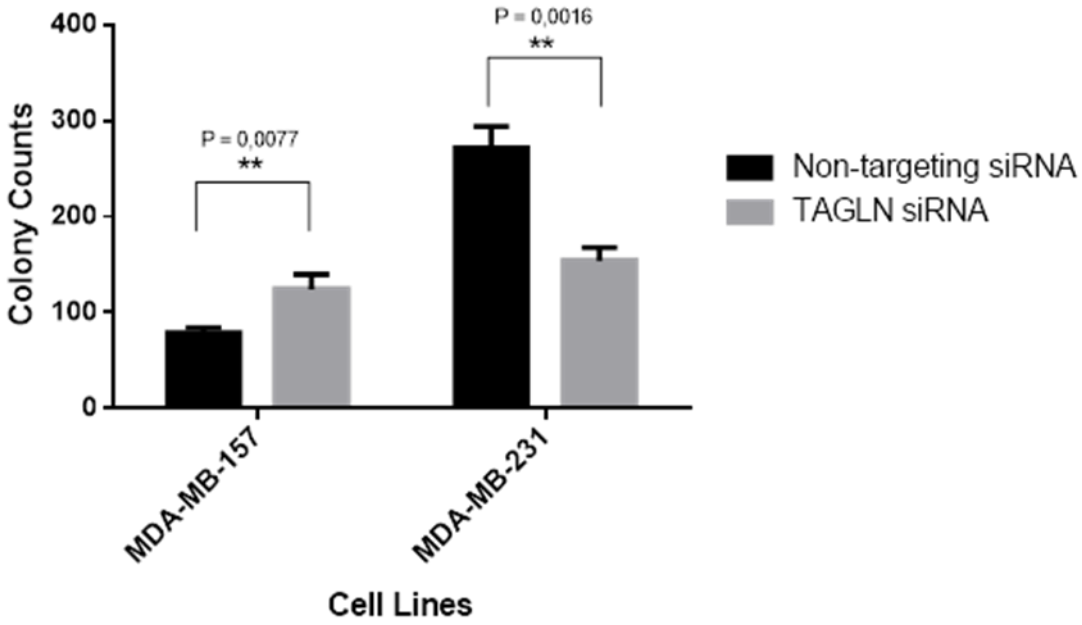


Figure 3.12. Silencing TAGLN gene caused the formation of more colonies in MDA-MB-157 cells but less colonies in MDA-MB-231 cells.
Cells were seeded in equal numbers in each well after 72 hours of siRNA treatment. Unpaired two-tailed t-test was used for statistical analysis.

Table 3.2. Statistics of colony formation of MDA-MB-157 and MDA-MB-231 cells transfected with Non-targeting siRNA and TAGLN siRNA.
Unpaired two-tailed t-test was used for statistical analysis.

MDA-MB-157	
Unpaired t test	
P value	0.0077
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.970 df=4
MDA-MB-231	
Unpaired t test	
P value	0.0016
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=7.634 df=4

3.2.4. Cell Viability Assay of MDA-MB-157 and MDA-MB-231 Cells Transfected with Non-targeting siRNA and TAGLN siRNA

For MDA-MB-157 cells, with decreased TAGLN expression (45% decrease compare to control), the cell viability increased at both days significantly. For MDA-MB-231 cells, with decreased TAGLN expression (86% decrease compare to the control), the viability decreased at Day 4. Statistical significance was determined by using unpaired two-tailed t-test ($P < 0.05$ in both cell lines) (**Figure 3.13** and **Table 3.3**).

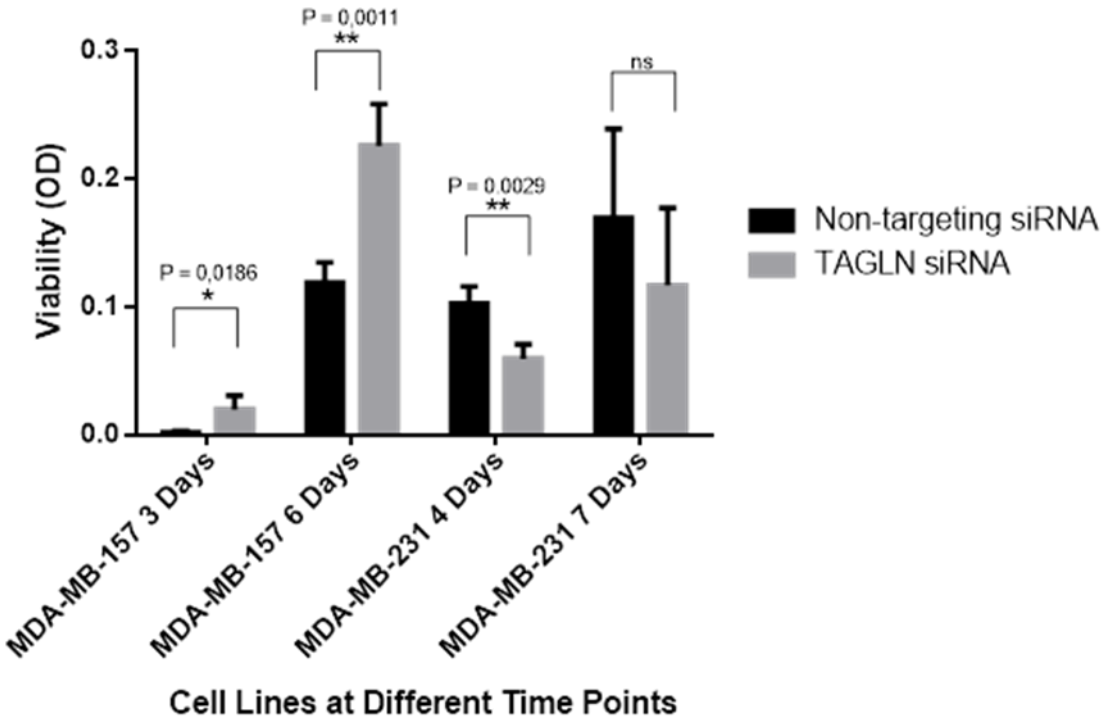


Figure 3.13. Silencing TAGLN gene increased the viability of MDA-MB-157 cells but decreased the viability of MDA-MB-231 cells.

Cells were seeded in equal numbers in each well after 72 hours of siRNA treatment and cells were fixed and stained with SRB dye at the specified days. It is calculated with relative optical density. 2 tailed t-test was used for statistical analysis. The experiment had four replicates per sample and was performed once.

Table 3.3. Statistics of viability of MDA-MB-157 and MDA-MB-231 cells transfected with Non-targeting siRNA and TAGLN siRNA.
Unpaired two-tailed t-test was used for statistical analysis.

MDA-MB-157 3 Days	
Unpaired t test	
P value	0.0186
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=3.201 df=6
MDA-MB-157 6 Days	
Unpaired t test	
P value	0.0011
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.861 df=6
MDA-MB-231 4 Days	
Unpaired t test	
P value	0.0029
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=4.833 df=6
MDA-MB-231 8 Days	
Unpaired t test	
P value	0.2968
P value summary	Ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.142 df=6

3.2.5. qRT-PCR Analysis of EMT Marker Panel in MDA-MB-157 and MDA-MB-231 Cells Transfected with Non-targeting siRNA and TAGLN siRNA.

With TAGLN silencing, only the changes at the mRNA level which are greater than +1 or less than -1 were selected. Silencing TAGLN gene in MDA-MB-157 cells resulted in increase in the expression of mesenchymal markers FN and MMP9. Silencing TAGLN gene in MDA-MB-231 cells resulted in increase in the expression of epithelial marker CDH1, decrease in the expression of mesenchymal marker CDH2 and increase in the expression of mesenchymal markers FN and MMP9. The PCR reactions were performed in duplicates (**Figure 3.14** and **Table 3.4**).

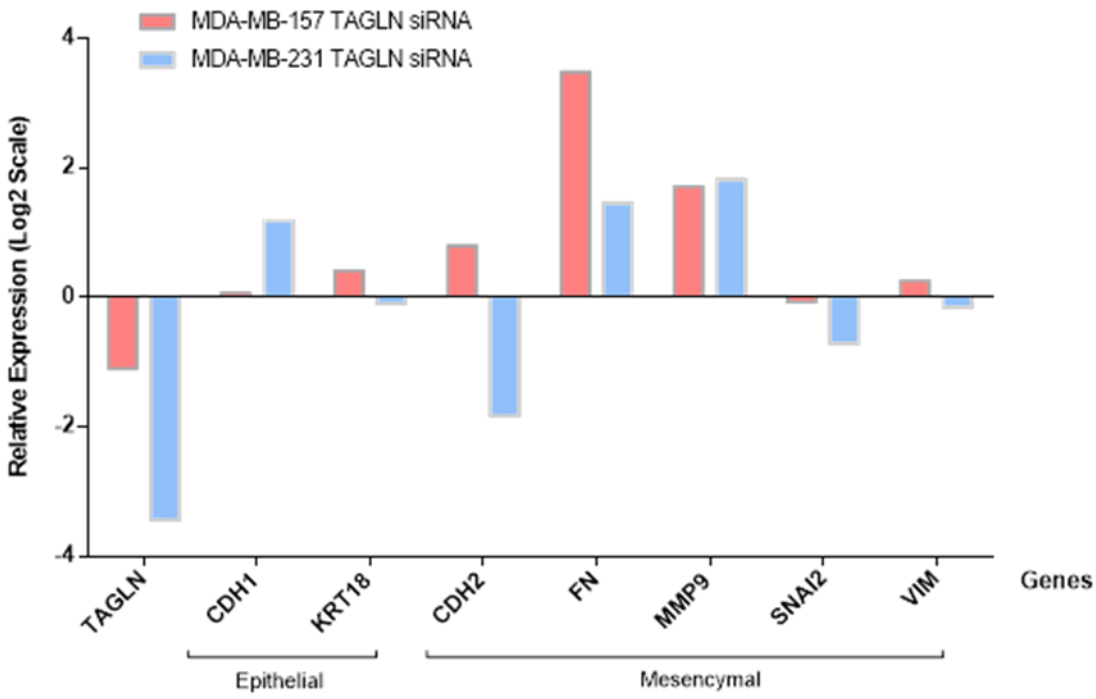


Figure 3.14. qRT-PCR analysis of TAGLN and EMT markers in MDA-MB-157 and MDA-MB-231 cell lines with TAGLN silencing.

All data was normalized to GAPDH expression and then to Non-targeting siRNA treated samples while drawing the graph. All data are in Log2 scale. The experiment had duplicates per sample and was performed once.

Table 3.4. Expression values that were used in Figure 3.14.

	MDA-MB-157 TAGLN siRNA	MDA-MB-231 TAGLN siRNA
TAGLN	-1.10	-3.43
CDH1	0.07	1.19
KRT18	0.41	-0.09
CDH2	0.80	-1.82
FN	3.48	1.45
MMP9	1.71	1.82
SNAI2	-0.07	-0.71
VIM	0.26	-0.15

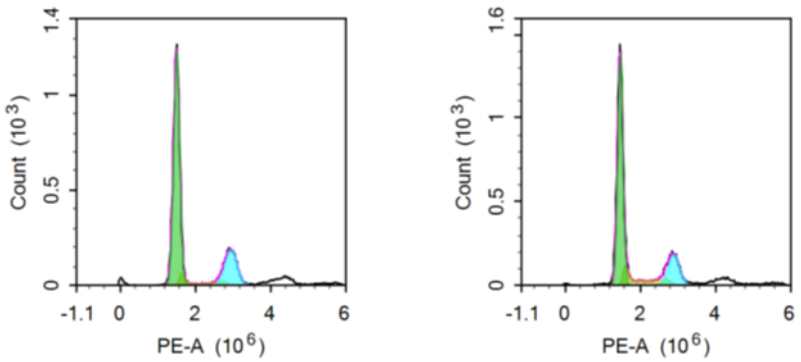
3.2.6. Cell Cycle Analysis of MDA-MB-157 and MDA-MB-231 Cells Transfected with Non-targeting siRNA and TAGLN siRNA

In MDA-MB-157 cells, TAGLN silencing leads to an increase in the percentages of cells at the S phase from 6.69 to 13.31 (**Figure 3.15-A**).

In MDA-MB-231 cells, TAGLN silencing leads to an increase in the percentages of cells at the G2/M phase from 0.65 to 4.20 (**Figure 3.15-B**).

A) Cell cycle analysis of MDA-MB-157 cells upon TAGLN silencing

Non-targeting siRNA **TAGLN siRNA**

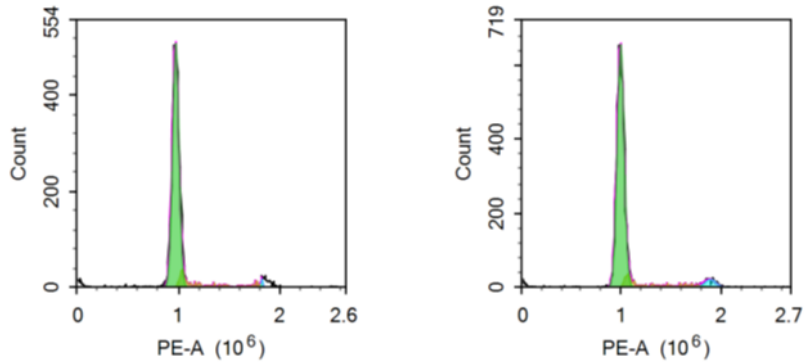


RMS	11.22 %
Freq G0/G1	62.52 %
Freq S	6.69 %
Freq G2/M	18.77 %

RMS	15.02 %
Freq G0/G1	57.98 %
Freq S	13.31 %
Freq G2/M	16.64 %

B) Cell cycle analysis of MDA-MB-231 cells upon TAGLN silencing

Non-targeting siRNA **TAGLN siRNA**



RMS	9.41 %
Freq G0/G1	82.48 %
Freq S	11.54 %
Freq G2/M	0.65 %

RMS	9.53 %
Freq G0/G1	84.09 %
Freq S	9.76 %
Freq G2/M	4.20 %

Figure 3.15. Cell cycle analysis of A) MDA-MB-157 and B) MDA-MB-231 cells upon TAGLN silencing.

The cells were *collected, fixed and stained with* Propidium *Iodide after 72 hours of siRNA treatments were completed. The experiment was performed once.*

3.3. Effect of TAGLN Overexpression in Breast Cancer Cell Lines

TAGLN gene was overexpressed in MDA-MB-157, MDA-MB-231 and MDA-MB-361 cells by transfecting with pCMV6 TAGLN vector and then treating with Geneticin Antibiotic to select for non-transfected cells and then finally single colony selection to obtain the best transfected and highest TAGLN expressing colony.

3.3.1. Overexpression of TAGLN in MDA-MB-157 Cells

3.3.1.1. Overexpressing *TAGLN* Gene in MDA-MB-157 Cells Affects Mesenchymal Marker Vimentin Expression.

MDA-MB-157 cells which were transfected with pCMV6 TAGLN vector had the larger second band above the intrinsic TAGLN band. With the transfection of MDA-MB-157 cells with pCMV6 vector, TAGLN expression has increased by 46% and Vimentin expression has increased by 53% when compared to pCMV6 Entry vector transfected cells (**Figure 3.16** and **Figure 3.17**).

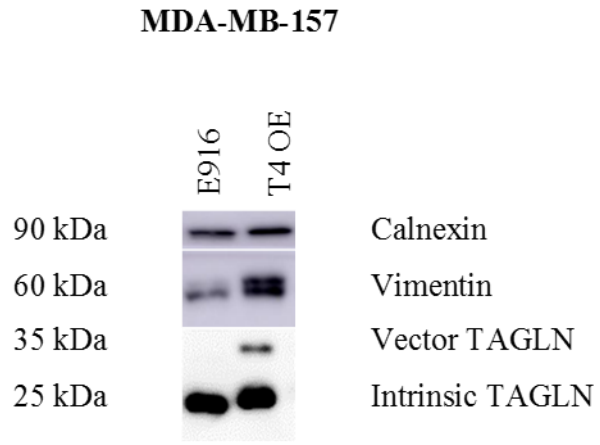


Figure 3.16. *TAGLN* gene was overexpressed in MDA-MB-157 cells with pCMV6 TAGLN vector.
By overexpressing TAGLN gene, Vimentin levels increased. Calnexin was used as an equal loading control. Data was normalized to Calnexin protein levels. The experiment was performed twice.

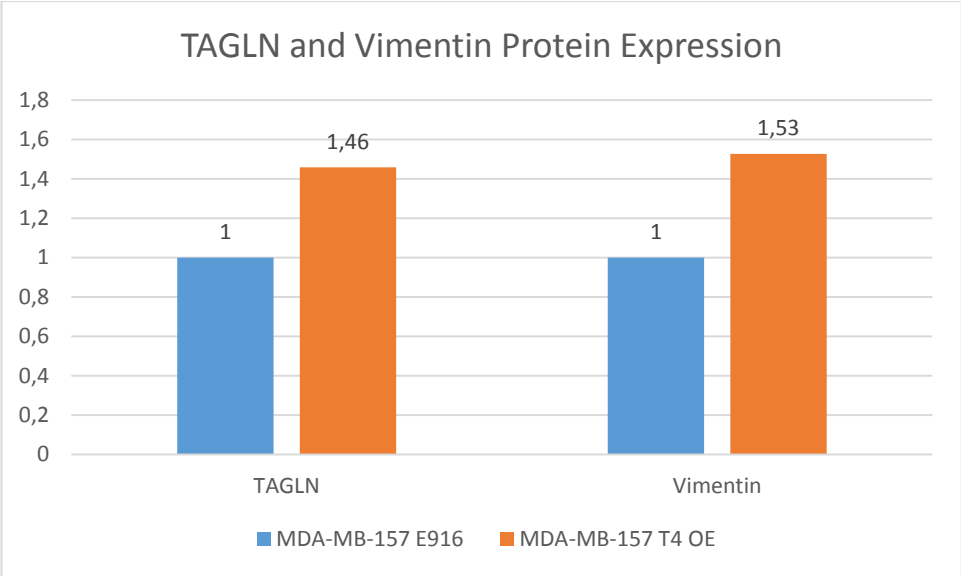


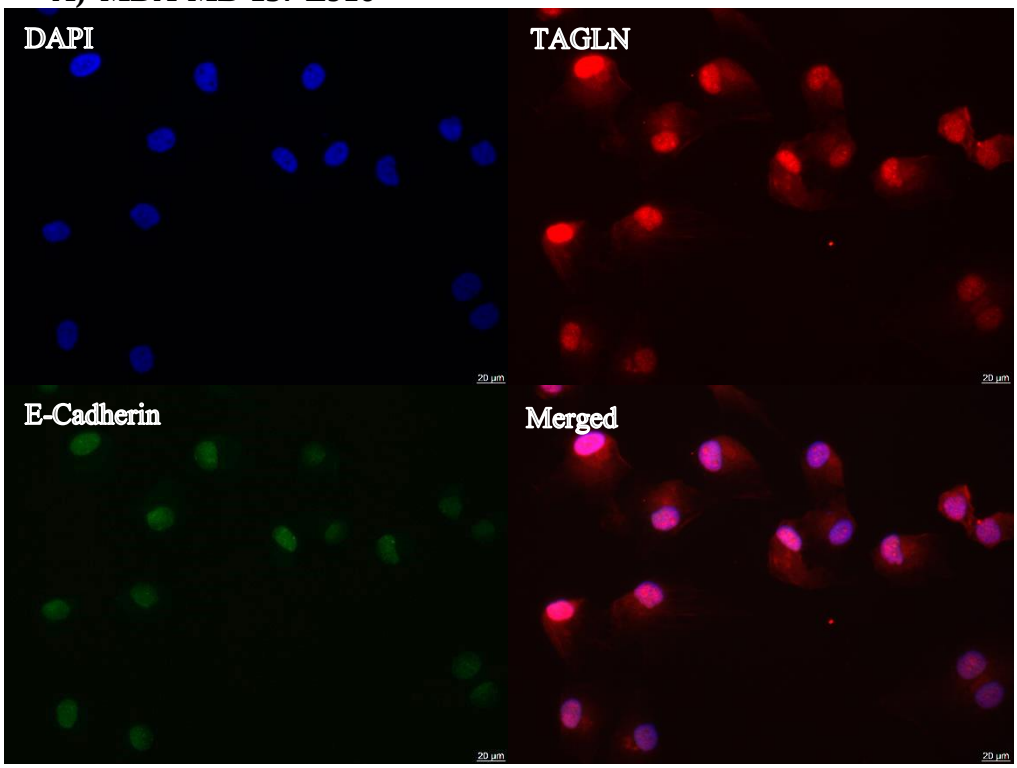
Figure 3.17. TAGLN gene overexpression was quantified in MDA-MB-157 cells.

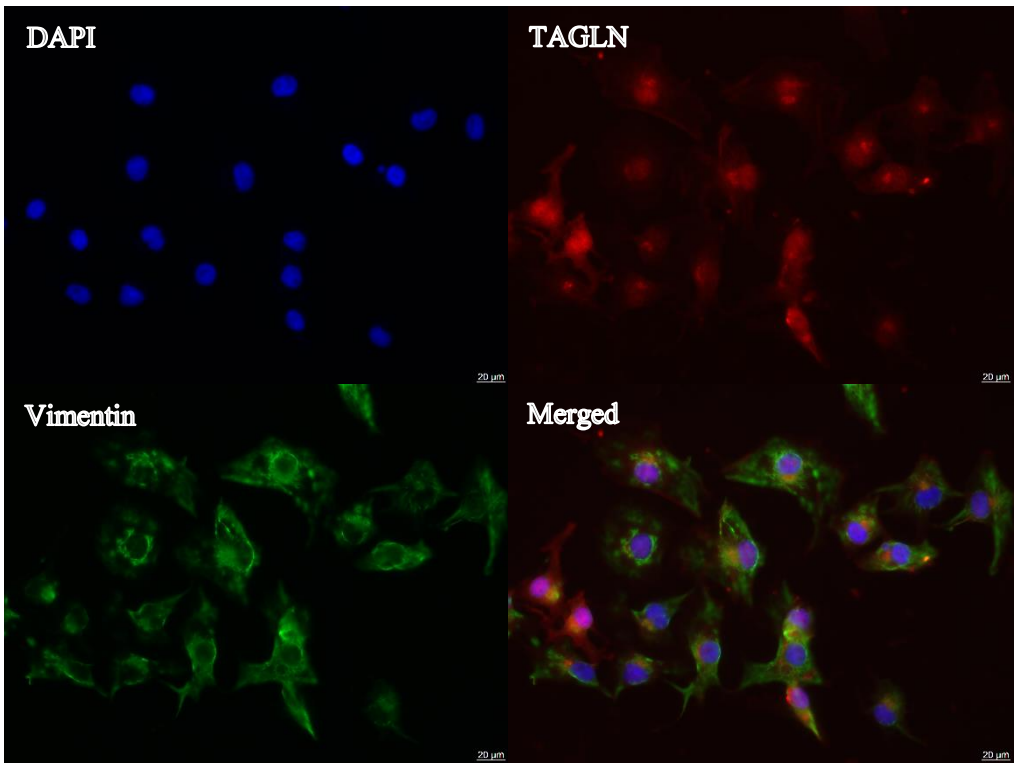
Quantification was performed for MDA-MB-157 overexpression colony and its control. Protein expressions were normalized to Calnexin protein levels. Image J was used for quantification.

3.3.1.2. Immunofluorescence Analysis of EMT Markers in MDA-MB-157 Cells Overexpressing TAGLN and Control

In immunofluorescence staining, with the overexpression of *TAGLN* gene, Vimentin expression increased at the cytoplasm of MDA-MB-157 cells (**Figure 3.18**).

A) MDA-MB-157 E916





B) MDA-MB-157 T4 OE

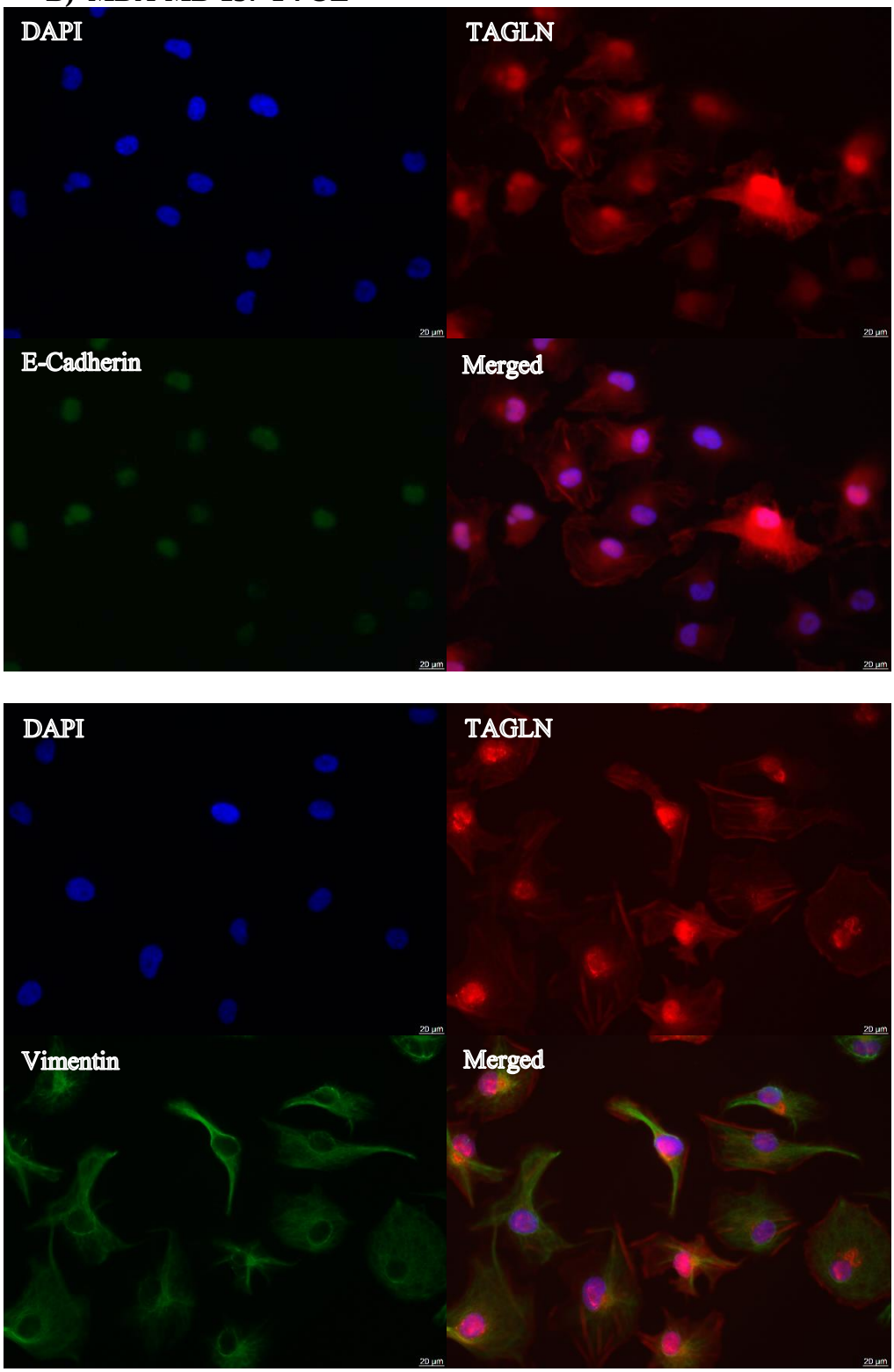


Figure 3.18. Overexpressing TAGLN gene in MDA-MB-157 cells effected the expression of mesenchymal marker Vimentin.

*With the overexpression of TAGLN, Vimentin expression increased in cytoplasm of MDA-MB-157 cells (**A** and **B**). Images were taken at 40X. The scale bar is 20 µm. The experiment was performed once.*

3.3.1.3. Colony Formation Assay in MDA-MB-157 Cells Overexpressing TAGLN and Control

With TAGLN overexpression, there was no significant change in colony numbers of MDA-MB-157 cells when compared to pCMV6 Entry vector transfected cells. Statistical significance was determined by using unpaired two-tailed t-test ($p<0.05$) (**Figure 3.19**, **Figure 3.20** and **Table 3.5**).

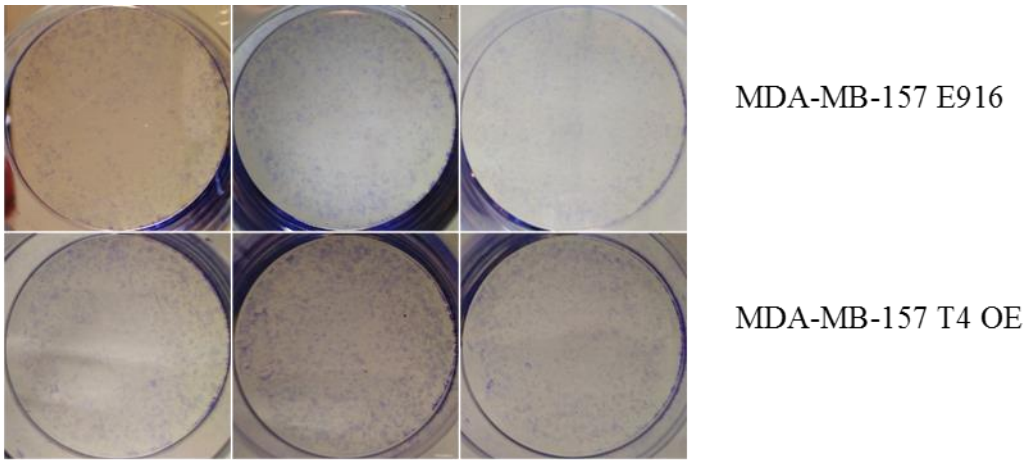


Figure 3.19. Overexpressing TAGLN gene has no effect on the colony formation capacity of MDA-MB-157 cells.

Cells were seeded in equal numbers in each well. They were cultured in normal growth medium with Geneticin Selective Antibiotic until colonies became visible to eye. After 1-2 weeks, cells were fixed with ice-cold methanol and then stained with crystal violet solution and colonies were counted. The experiment had three replicates per sample and was performed once.

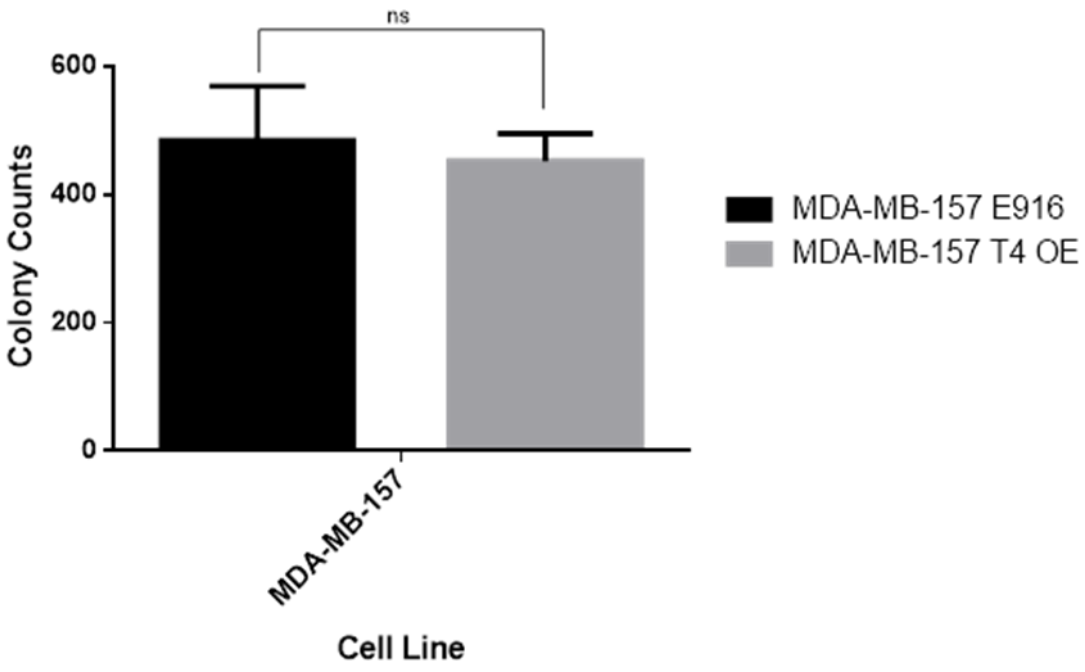


Figure 3.20. Overexpressing TAGLN gene has no effect on the formation of colonies in MDA-MB-157 cells.

Cells were seeded in equal numbers in each well. After colonies are formed, cells are fixed and colonies are counted. Unpaired two-tailed t-test was used for analysis (P < 0.05).

Table 3.5. Statistics of colony formation of MDA-MB-157 cells overexpressing TAGLN and control.

Unpaired two-tailed t-test was used for analysis (p<0.05).

MDA-MB-157	
Unpaired t test	
P value	0.5920
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5817 df=4

3.3.1.4. Cell Viability Assay in MDA-MB-157 Cells Overexpressing TAGLN and Control

Overexpressing TAGLN gene has no significant effect of the viability of MDA-MB-157 cells either at Day 3 or at Day 6 at the statistical level (**Figure 3.21** and **Table 3.6**).

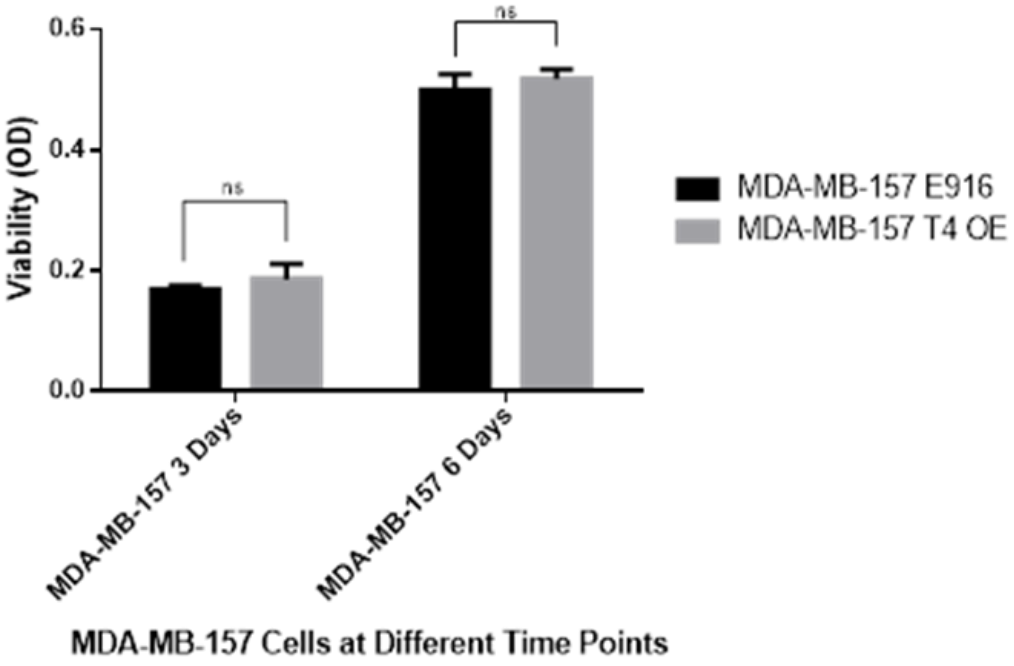


Figure 3.21. Overexpressing TAGLN gene has no effect on the viability of MDA-MB-157 cells.

Cells were seeded in equal numbers in each well and they were fixed and stained with SRB dye at the specified days. Two-tailed t-test was used for statistical analysis. The experiment had four replicates per sample and was performed once.

Table 3.6. Statistics of viability of MDA-MB-157 cells overexpressing TAGLN and control.

Two-tailed t-test was used for statistical analysis.

MDA-MB-157 3 Days	
Unpaired t test	
P value	0,2167
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1,380 df=6
MDA-MB-157 6 Days	
Unpaired t test	
P value	0,2842
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1,176 df=6

3.3.1.5. qRT-PCR Analysis of MDA-MB-157 Cells Overexpressing TAGLN and Control

While TAGLN overexpression was confirmed at the protein level, at the mRNA level TAGLN expression is reduced. mRNA levels of epithelial CDH1 and KRT18 markers appears to be increased compared to empty vector transfected cells (**Figure 3.22** and **Table 3.7**).

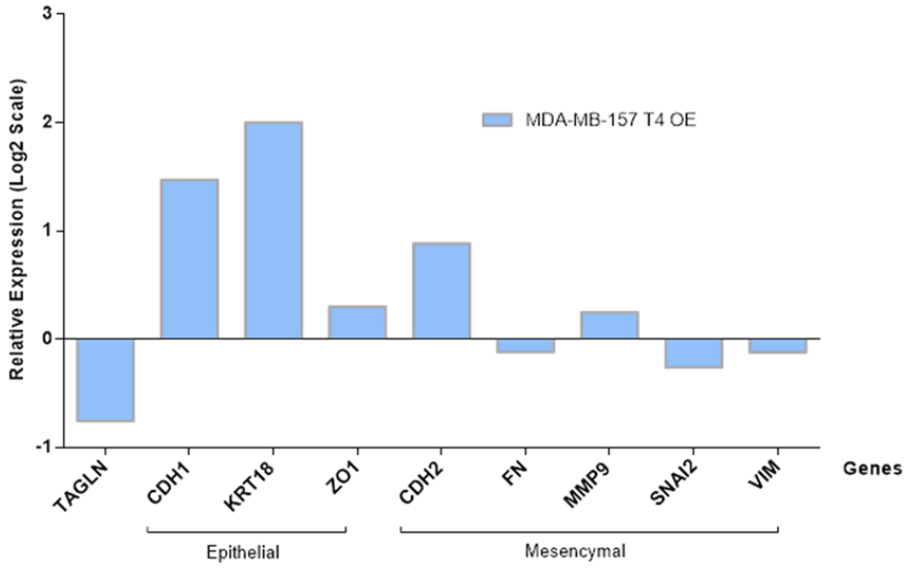


Figure 3.22. qRT-PCR analysis of TAGLN and EMT markers in MDA-MB-157 cells.

All data was normalized to GAPDH expression and then to empty vector transfected treated samples while drawing the graph. All data are in Log2 scale. The experiment had two replicates for each sample and was performed once.

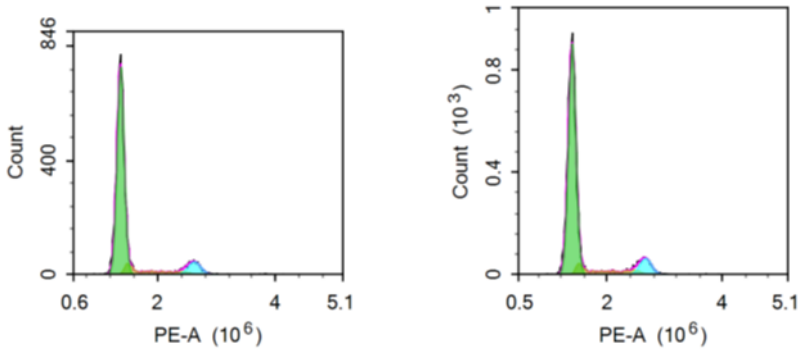
Table 3.7. Expression values that were used in Figure 3.22.

	MDA-MB-157 T4 OE
TAGLN	-0.75
CDH1	1.47
KRT18	2.00
ZO1	0.30
CDH2	0.88
FN	-0.12
MMP9	0.25
SNAI2	-0.26
VIM	-0,12

3.3.1.6. Cell Cycle Analysis of MDA-MB-157 Cells Overexpressing TAGLN and Control

The percentages of cell cycles phases did not vary significantly with TAGLN overexpression (**Figure 3.23**).

MDA-MB-157 E916 **MDA-MB-157 T4 OE**



RMS	7.95 %
Freq G0/G1	78.89 %
Freq S	10.78 %
Freq G2/M	8.61 %

RMS	7.59 %
Freq G0/G1	80.52 %
Freq S	10.32 %
Freq G2/M	8.75 %

Figure 3.23. Propidium iodide staining of MDA-MB-157 cells revealed no change in cell cycle phases with TAGLN overexpression.

Cells were seeded and collected after 3 days and then fixed. There is no significant change in cell cycle phase percentages with TAGLN overexpression. The experiment was performed once.

3.3.2. Overexpression of TAGLN in MDA-MB-231 Cells

3.3.2.1. Overexpressing TAGLN Gene in MDA-MB-231 Cells Increases Mesenchymal Marker Vimentin Expression

MDA-MB-231 cells which were transfected with pCMV6 TAGLN vector had the larger second band above the intrinsic TAGLN band. With the transfection of MDA-MB-231 cells with pCMV6 vector, TAGLN expression has increased by 52% in MDA-MB-231 T1 OE cells, by 157% in MDA-MB-231 T2 OE cells and by 140% in MDA-MB-231 T3 OE cells when compared to pCMV6 Entry vector transfected MDA-MB-231 E3 cells. Vimentin expression has increased by 23% in MDA-MB-231 T2 OE cells and by 35% in MDA-MB-231 OE cells when compared to pCMV6 Entry vector transfected MDA-MB-231 E3 cells (**Figure 3.24** and **Figure 3.25**).

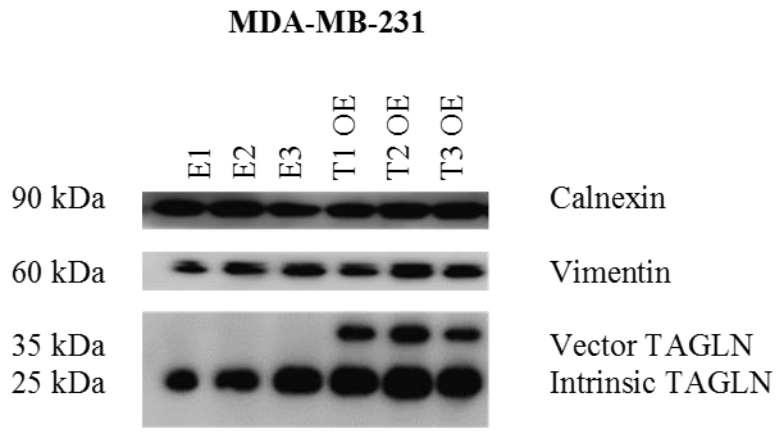


Figure 3.24. TAGLN gene was overexpressed in MDA-MB-231 cells with pCMV6 TAGLN vector.

By overexpressing TAGLN gene, Vimentin levels increased. Calnexin was used as an equal loading control. Data was normalized to Calnexin protein levels. The experiment was performed twice.

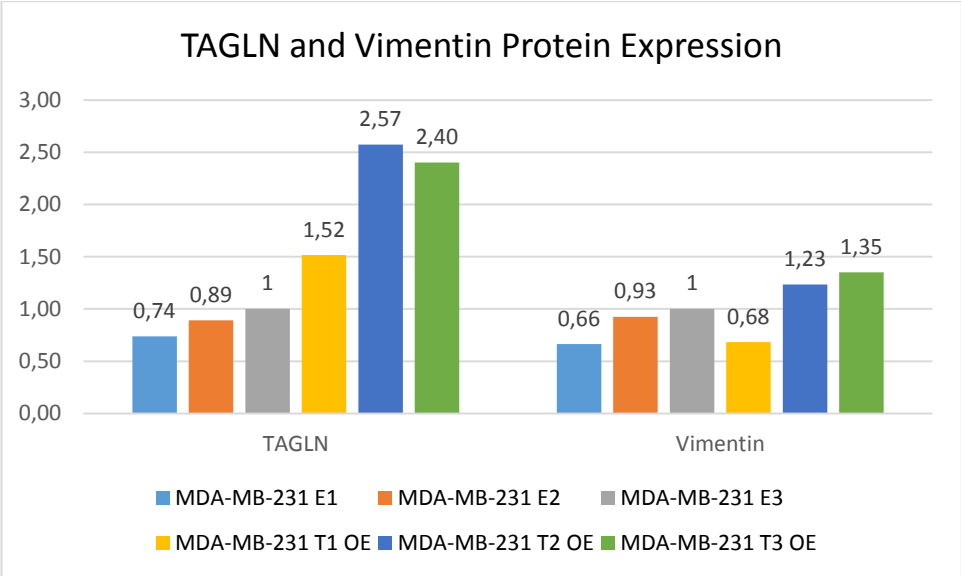


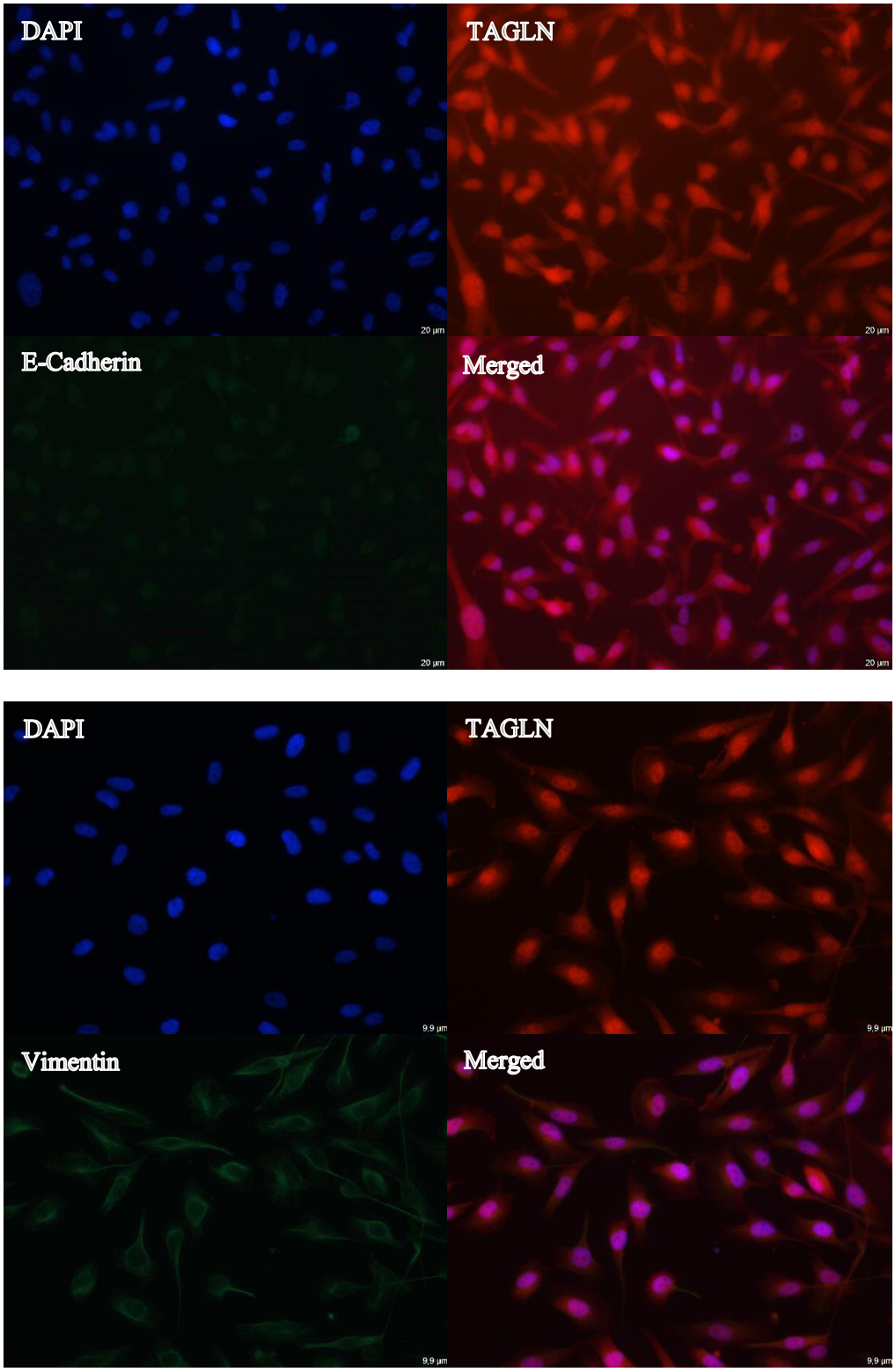
Figure 3.25. TAGLN gene overexpression was quantified in MDA-MB-231 cells.

Quantification was performed for MDA-MB-231 overexpression colony cells by comparing them to pCMV6 Entry vector transfected MDA-MB-231 E3 cells. Protein expressions were normalized to Calnexin protein levels. Image J was used for quantification.

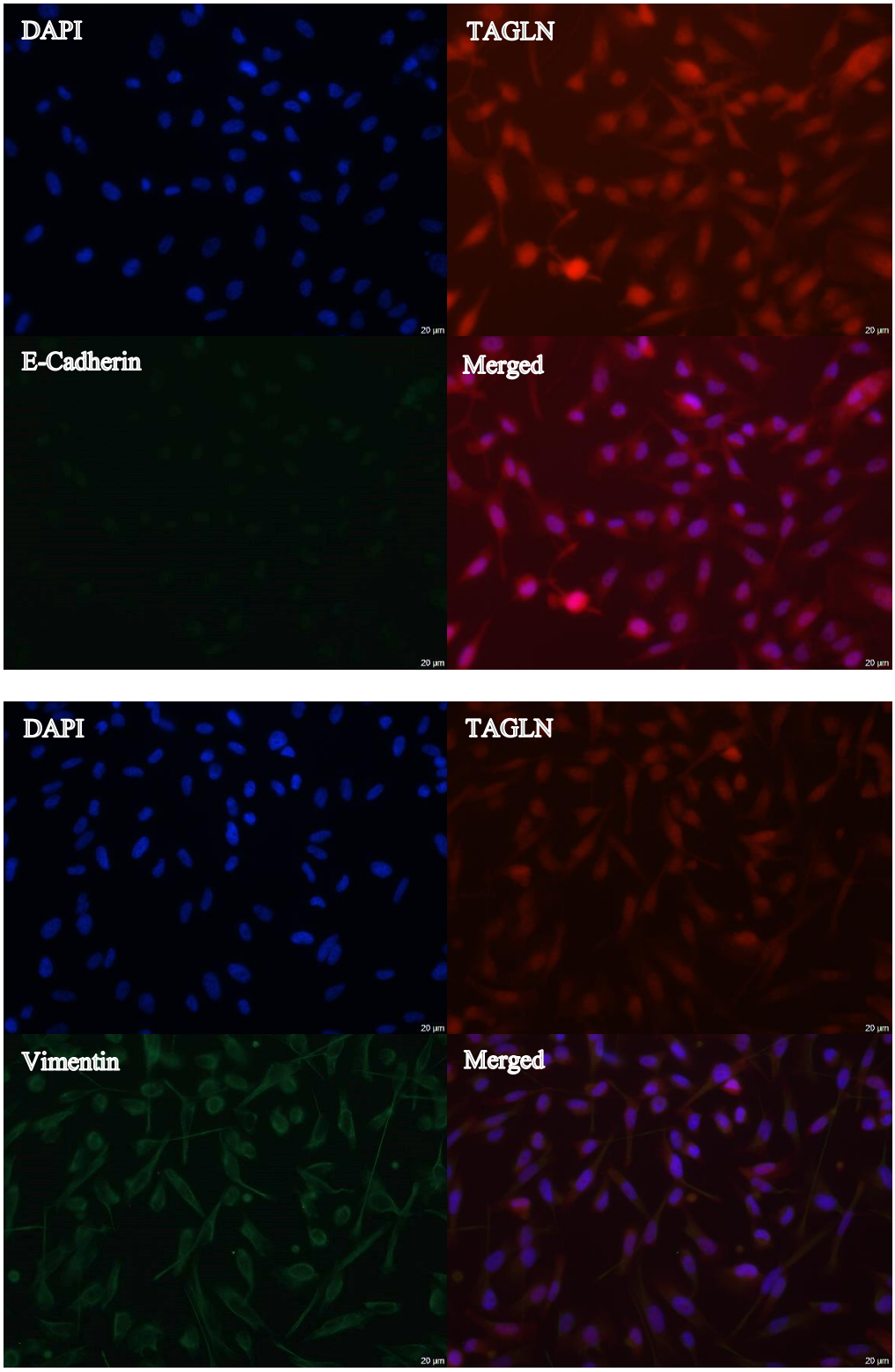
3.3.2.2. Immunofluorescence Analysis of EMT Markers in MDA-MB-231 Cells Overexpressing TAGLN and Control

With immunofluorescence staining, in MDA-MB-231 cells, while there was no difference in E-Cadherin expression, the expression of Vimentin has increased in colonies which were transfected with pCMV6 TAGLN vector when compared to colonies which were transfected with pCMV6 Entry vector (**Figure 3.26**).

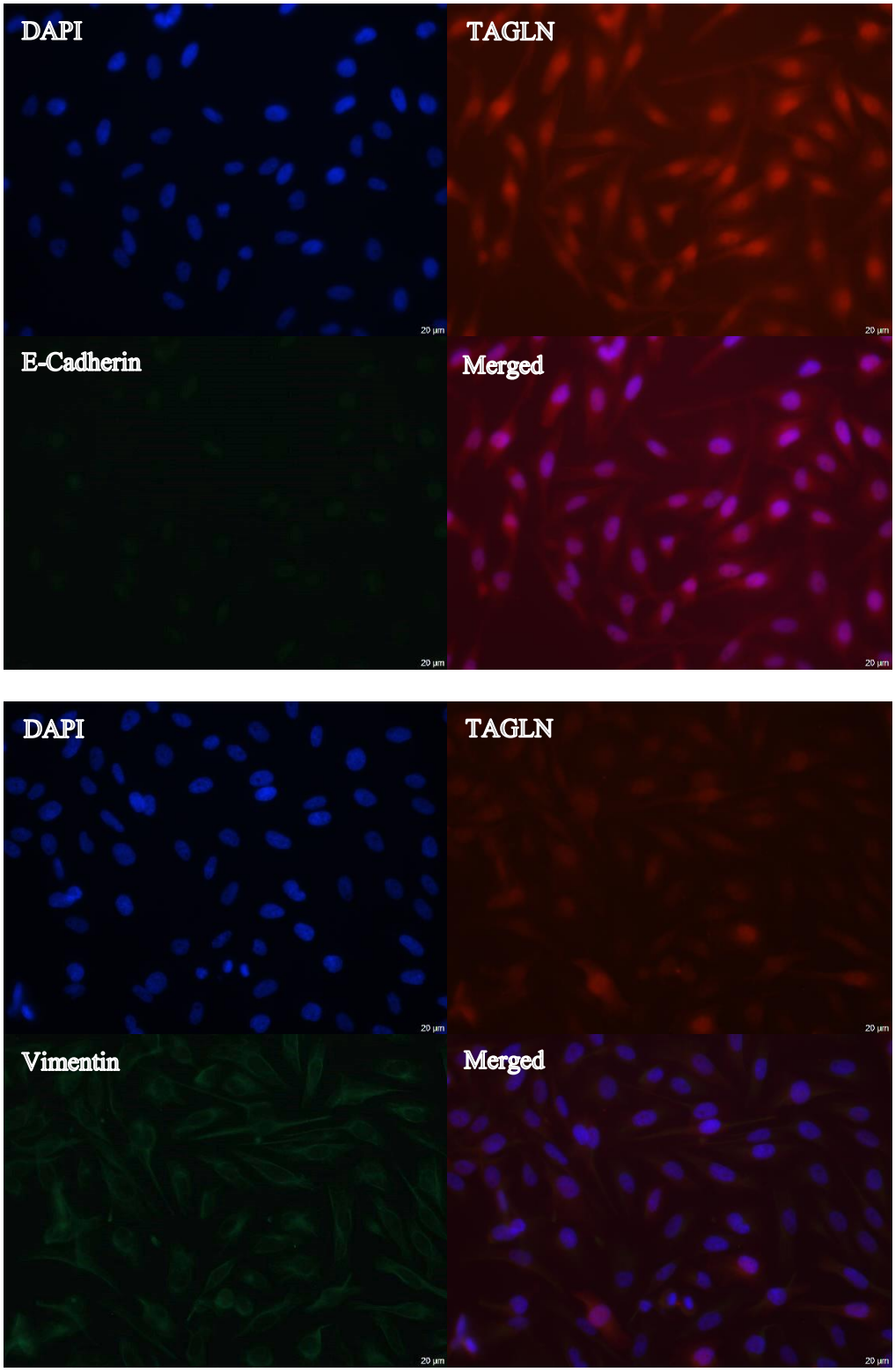
A) MDA-MB-231 E1



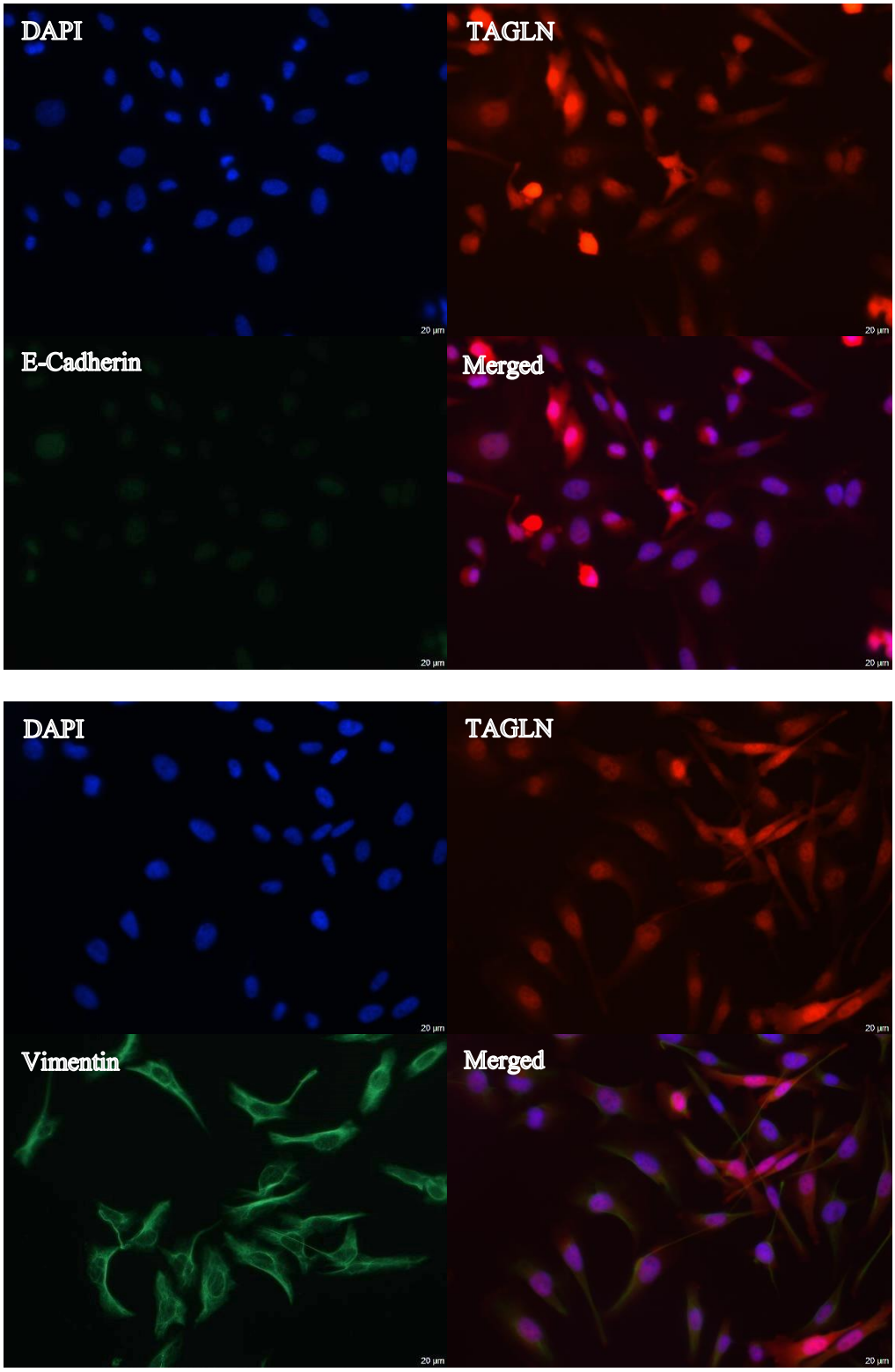
B) MDA-MB-231 E2



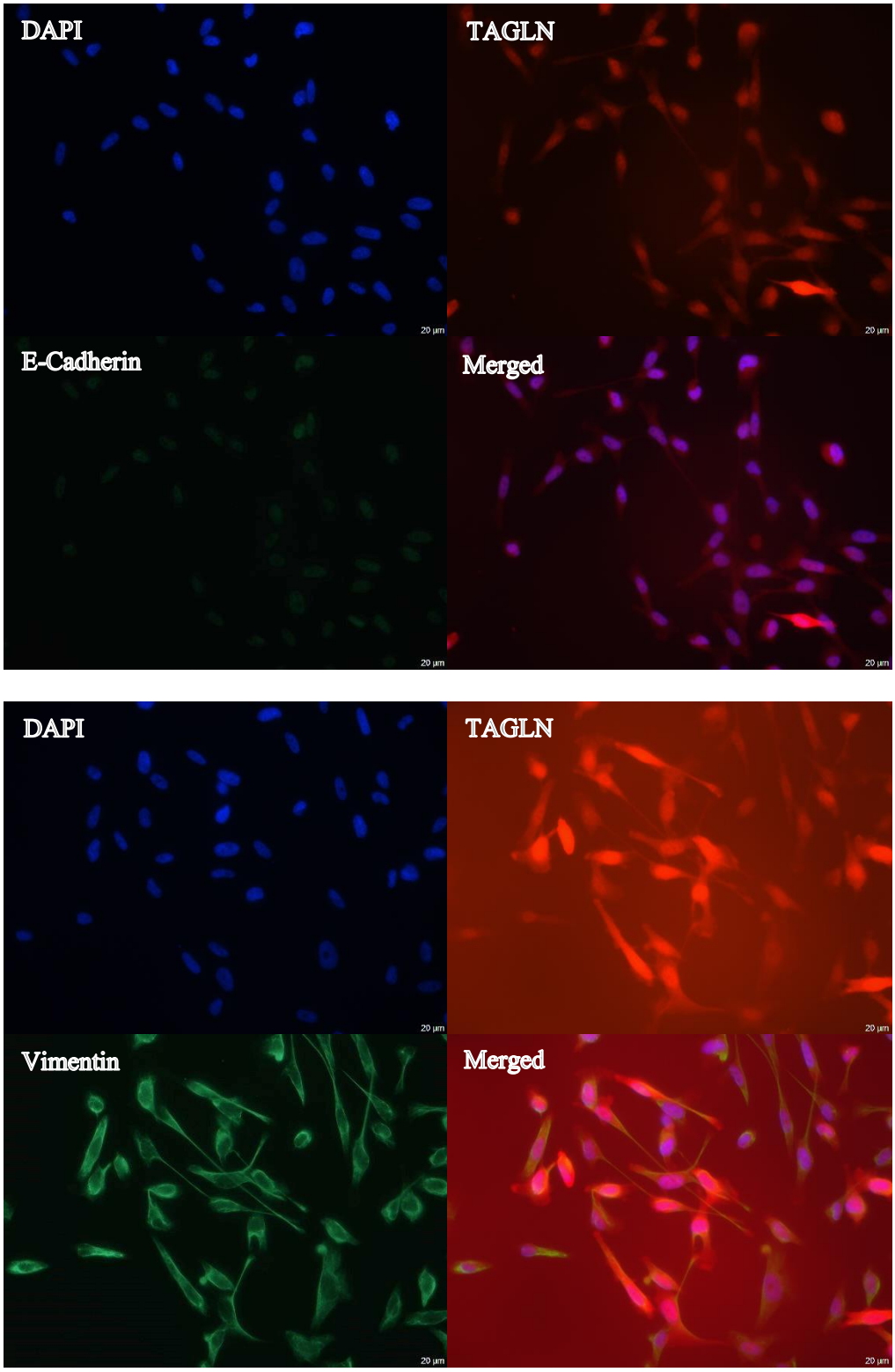
C) MDA-MB-231 E3



D) MDA-MB-231 T1 OE



E) MDA-MB-231 T2 OE



F) MDA-MB-231 T3 OE

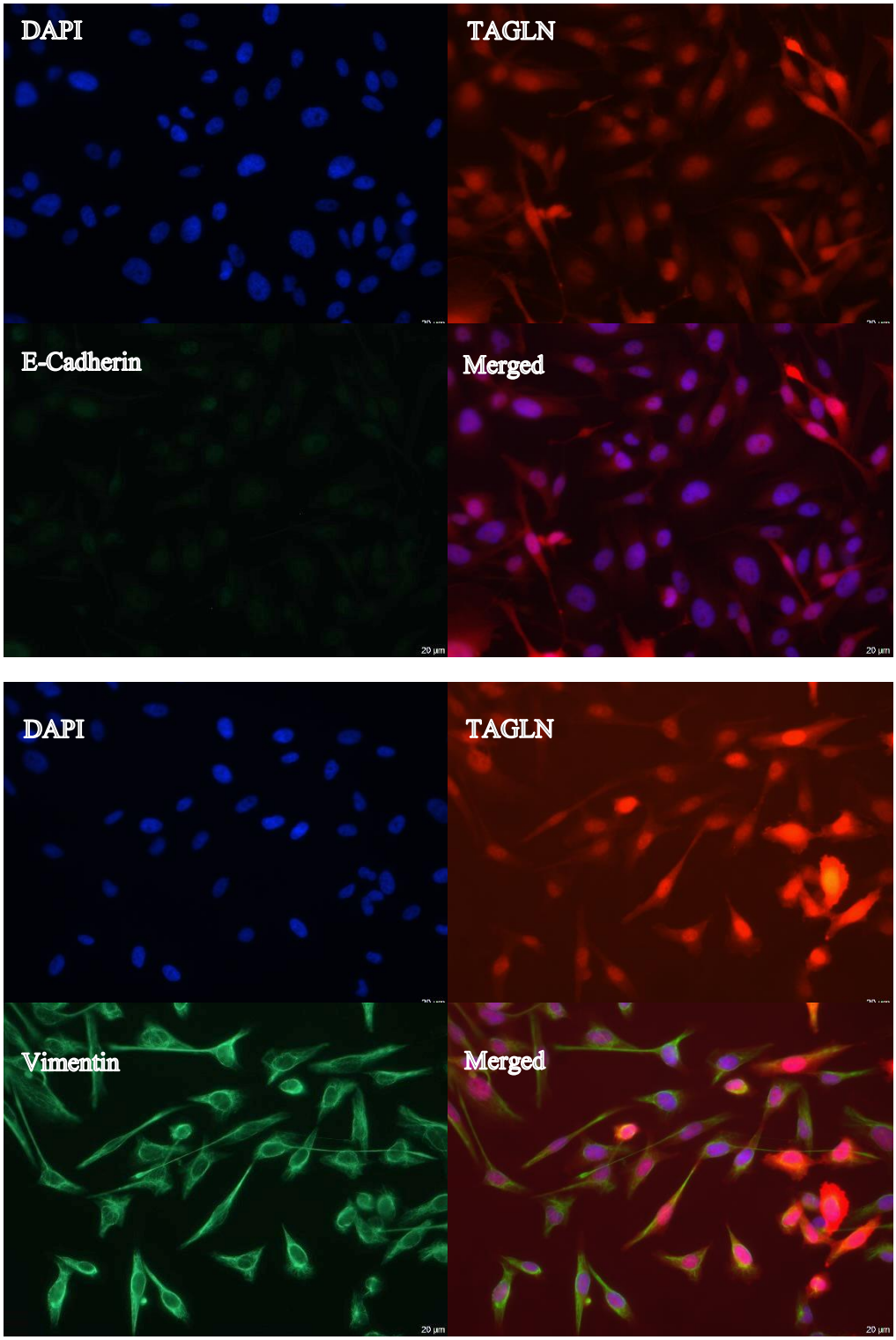


Figure 3.26. Overexpressing TAGLN gene in MDA-MB-231 cells effected the expression of mesenchymal marker Vimentin.

With the overexpression of TAGLN expression, while E-Cadherin expression did not change drastically, Vimentin expression increased in cytoplasm of MDA-MB-231 cells (A, B, C, D, E and F). Images were taken at 40X. The scale bar is 20 µm. The staining was performed once.

3.3.2.3. Colony Formation Assay in MDA-MB-231 Cells Overexpressing TAGLN and Control

With TAGLN overexpression, there was increase in colony numbers of MDA-MB-231 T2 OE cells when compared to pCMV6 Entry vector transfected MDA-MB-231 E1 and MDA-MB-231 E3 cells. Statistical significance was determined by using One-Way ANOVA ($p<0.05$) (**Figure 3.27**, **Figure 3.28** and **Table 3.8**).

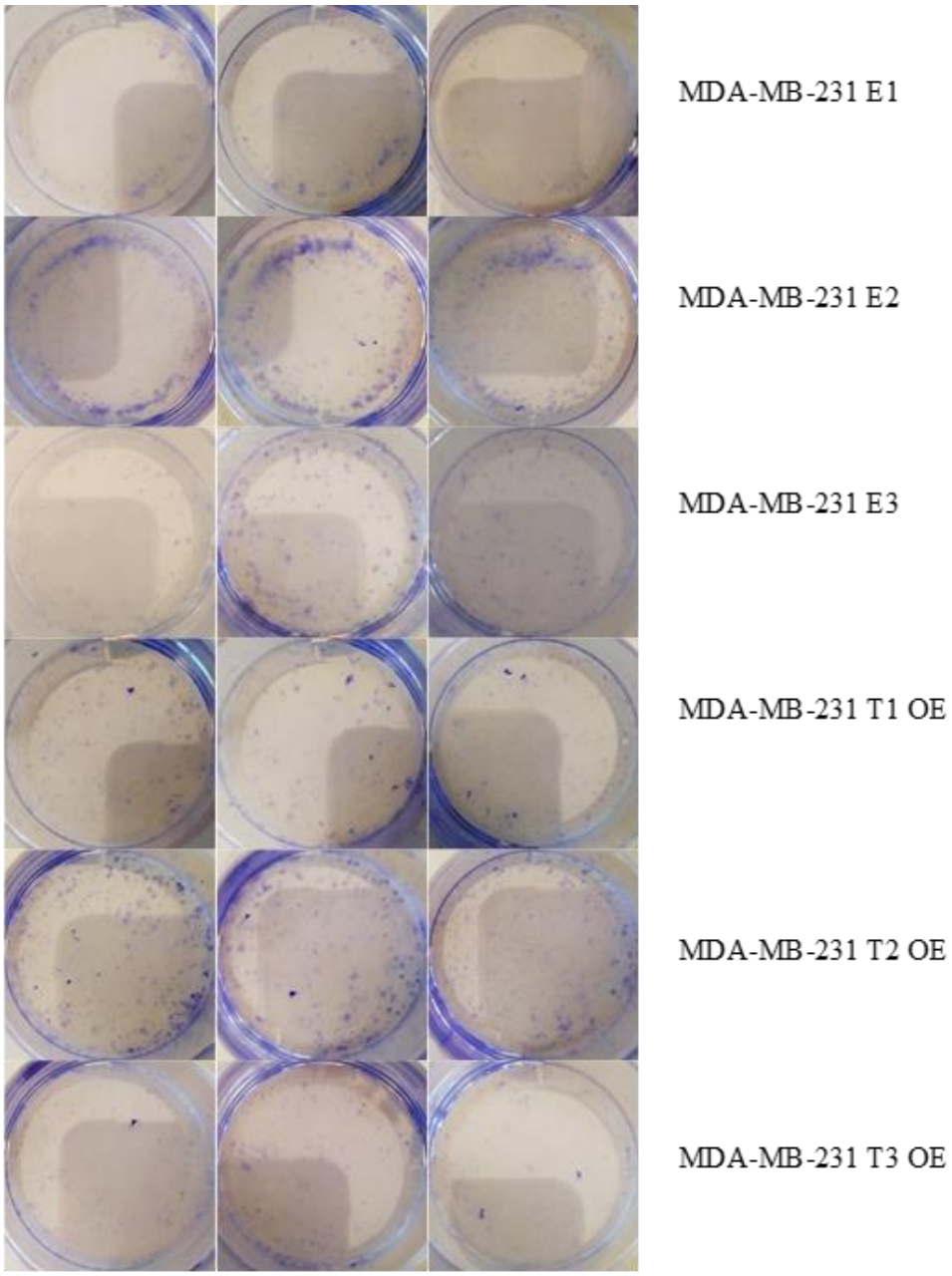


Figure 3.27. Overexpressing TAGLN gene has increased colony formation capacity of MDA-MB-231 T2 OE cells.

Cells were seeded in equal numbers in each well. They were cultured in normal growth medium with Geneticin Selective Antibiotic until colonies became visible to eye. After 1 or 2 weeks, cells were fixed with ice-cold methanol and then stained with crystal violet solution and colonies were counted. The experiment had three replicates per sample and was performed once.

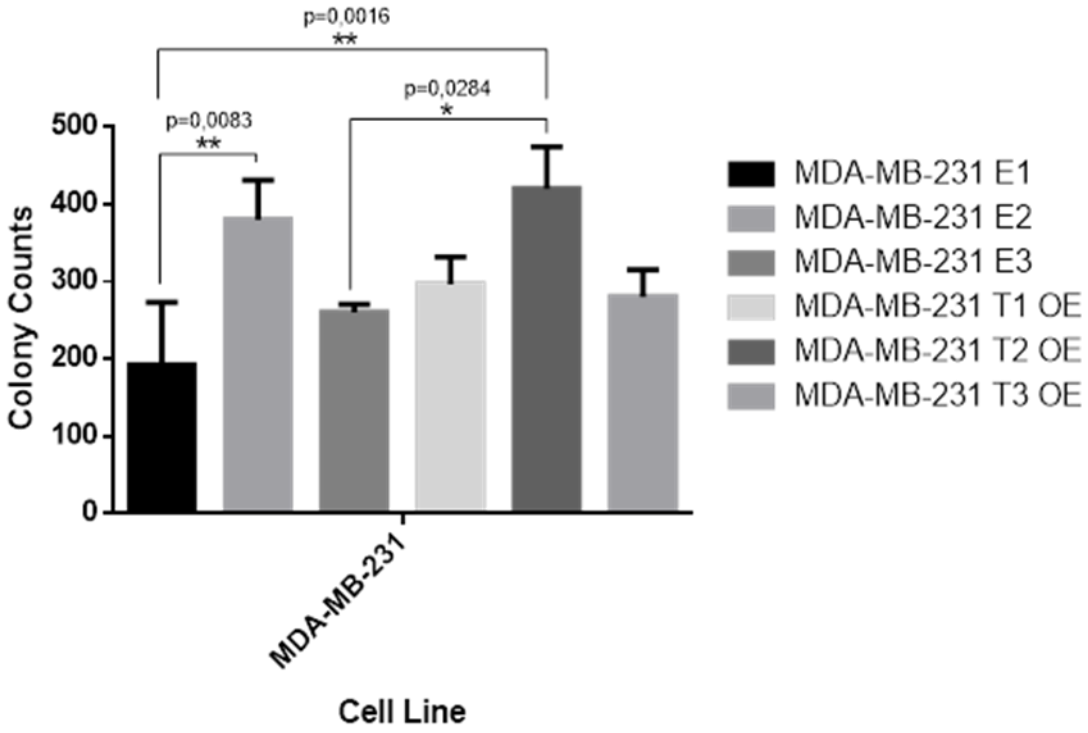


Figure 3.28. Overexpressing TAGLN gene has caused the formation of more colonies in MDA-MB-231 T2 OE cells.

pCMV6 TAGLN vector transfected MDA-MB-231 T2 OE cells had more colonies formed when compared to pCMV6 Entry vector transfected MDA-MB-231 E1 and MDA-MB-231 E3 cells. Statistical significance was determined by using One-Way ANOVA ($p<0.05$).

Table 3.8. Statistics of colony formation of MDA-MB-231 cells overexpressing TAGLN and control.

One-Way ANOVA was used for statistical analysis. Bonferroni multiple comparison test was used.

ANOVA summary			
F			8.414
P value			0.0013
P value summary			**
Are differences among means statistically significant? (P < 0.05)			Yes
R square			0.7781
Bonferroni's multiple comparisons test	Significant?	Summary	Adjusted P Value
E1 vs. E2	Yes	**	0.0083
E1 vs. E3	No	ns	> 0.9999
E1 vs. T1 OE	No	ns	0.3565
E1 vs. T2 OE	Yes	**	0.0016
E1 vs. T3 OE	No	ns	0.7343
E2 vs. E3	No	ns	0.1763
E2 vs. T1 OE	No	ns	0.9157
E2 vs. T2 OE	No	ns	> 0.9999
E2 vs. T3 OE	No	ns	0.4477
E3 vs. T1 OE	No	ns	> 0.9999
E3 vs. T2 OE	Yes	*	0.0284
E3 vs. T3 OE	No	ns	> 0.9999
T1 OE vs. T2 OE	No	ns	0.1489
T1 OE vs. T3 OE	No	ns	> 0.9999
T2 OE vs. T3 OE	No	ns	0.0714

3.3.2.4. Cell Viability Assay in MDA-MB-231 Cells Overexpressing TAGLN and Control

For MDA-MB-231 cells, with increased TAGLN expression, the viability increased at Day 8 when compared MDA-MB-231 T2 OE cells which express TAGLN gene at the

highest level according to the western blotting results and pCMV6 Entry vector transfected cells (MDA-MB-231 E1, MDA-MB-231 E2 and MDA-MB-231 E3) statistically. At Day 5, for MDA-MB-231 cells, there was no statistical difference between pCMV6 Entry and pCMV6 TAGLN vector transfected cells. Statistical significance was determined by using Two-Way ANOVA ($p<0.05$) (**Figure 3.29** and **Table 3.9**).

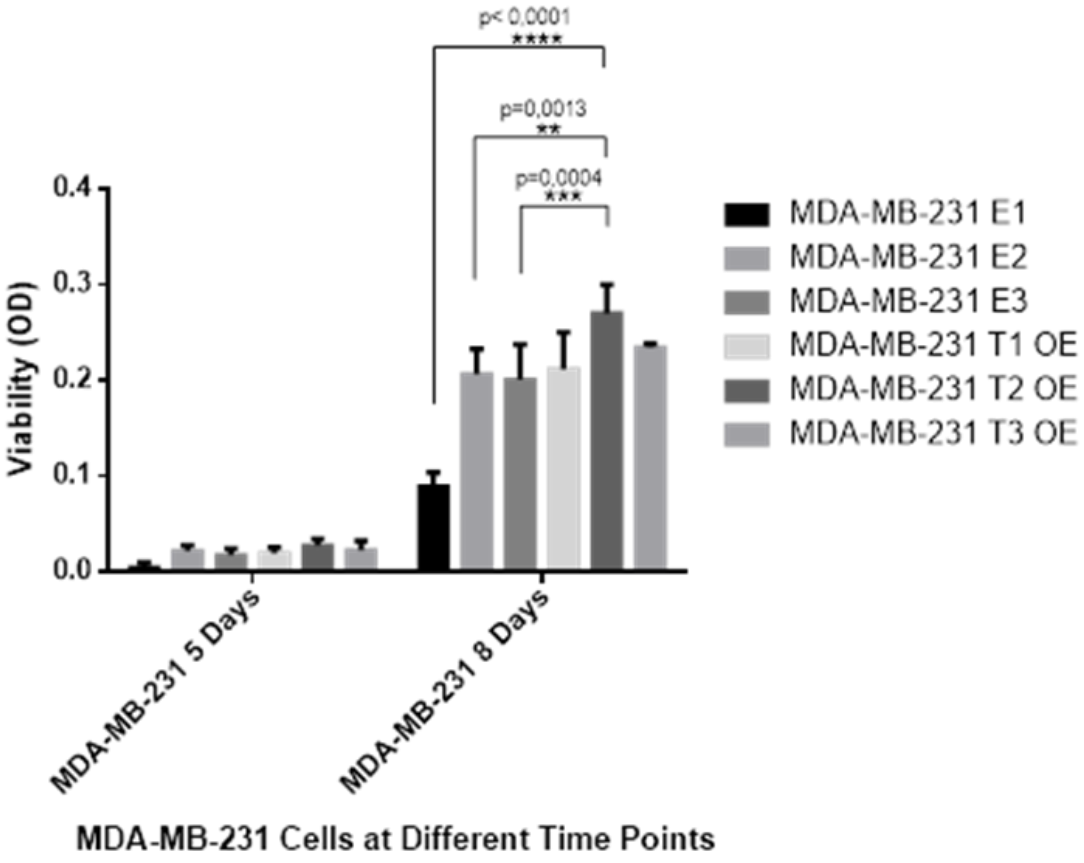


Figure 3.29. Overexpressing TAGLN Gene has increased the viability of MDA-MB-231 T2 OE Cells.

Cells were seeded in equal numbers in each well and they were fixed and stained with SRB dye at the specified days. Two-Way ANOVA was used for statistical analysis ($P < 0.05$). The experiment had four replicates per sample and was performed once.

Table 3.9 Statistics of viability of MDA-MB-231 cells overexpressing TAGLN and control.

Two-Way ANOVA was used for statistical analysis. Interaction accounts for 5.85% of the total variance and cell colonies affect the result with P < 0.0001. Bonferroni multiple comparison test was used with 95% confidence interval.

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.850	< 0.0001	****	Yes
Days	82.30	< 0.0001	****	Yes
Cell Colonies	9.685	< 0.0001	****	Yes

Bonferroni's multiple comparisons test	Significant?	Summary	Adjusted P Value
MDA-MB-231 8 Days			
MDA-MB-231 E1 vs. MDA-MB-231 E2	Yes	****	< 0.0001
MDA-MB-231 E1 vs. MDA-MB-231 E3	Yes	****	< 0.0001
MDA-MB-231 E1 vs. MDA-MB-231 T1 OE	Yes	****	< 0.0001
MDA-MB-231 E1 vs. MDA-MB-231 T2 OE	Yes	****	< 0.0001
MDA-MB-231 E1 vs. MDA-MB-231 T3 OE	Yes	****	< 0.0001
MDA-MB-231 E2 vs. MDA-MB-231 E3	No	ns	> 0.9999
MDA-MB-231 E2 vs. MDA-MB-231 T1 OE	No	ns	> 0.9999
MDA-MB-231 E2 vs. MDA-MB-231 T2 OE	Yes	**	0.0013
MDA-MB-231 E2 vs. MDA-MB-231 T3 OE	No	ns	> 0.9999
MDA-MB-231 E3 vs. MDA-MB-231 T1 OE	No	ns	> 0.9999
MDA-MB-231 E3 vs. MDA-MB-231 T2 OE	Yes	***	0.0004
MDA-MB-231 E3 vs. MDA-MB-231 T3 OE	No	ns	0.5958
MDA-MB-231 T1 OE vs. MDA-MB-231 T2 OE	Yes	**	0.0037
MDA-MB-231 T1 OE vs. MDA-MB-231 T3 OE	No	ns	> 0.9999
MDA-MB-231 T2 OE vs. MDA-MB-231 T3 OE	No	ns	0.3807

3.3.2.5. qRT-PCR Analysis of MDA-MB-231 Cells Overexpressing TAGLN and Control

While TAGLN overexpression was confirmed at the protein level, at the mRNA level TAGLN expression is reduced. mRNA levels of epithelial CDH1, KRT18 and ZO1 and mesenchymal FN appears to be reduced in MDA-MB-231 T1 OE cells compared to empty vector transfected MDA-MB-231 E3 cells (**Figure 3.30** and **Table 3.10**).

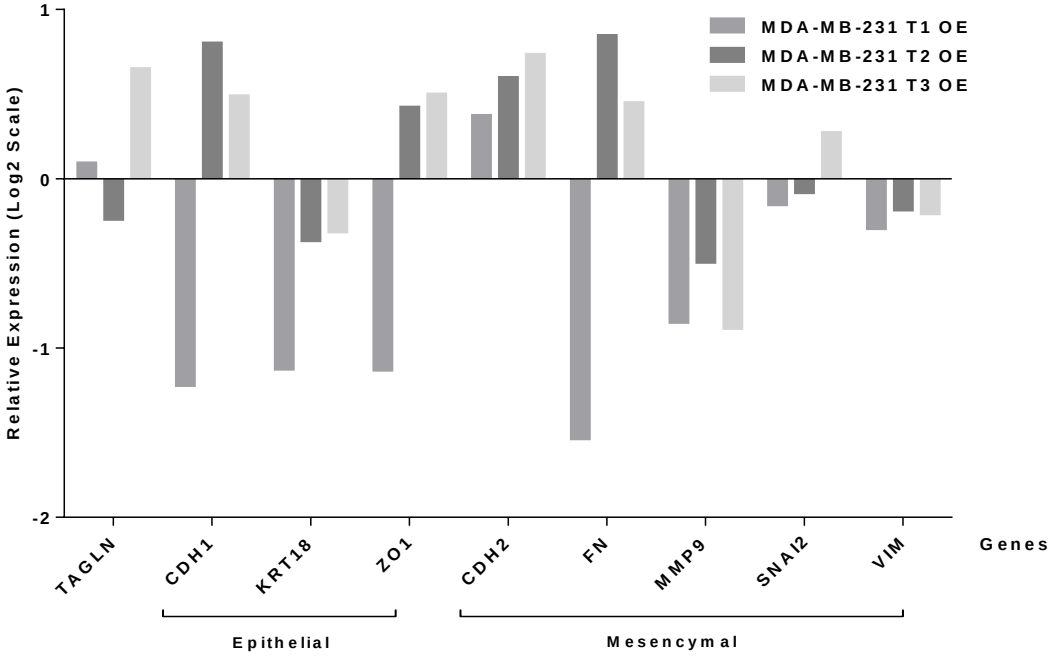


Figure 3.30. qRT-PCR analysis of TAGLN and EMT markers in MDA-MB-231 cells overexpressing TAGLN and control.

All data was normalized to GAPDH expression and then to empty vector transfected treated samples while drawing the graph. All data are in Log2 scale. The experiment was in duplicates and performed once.

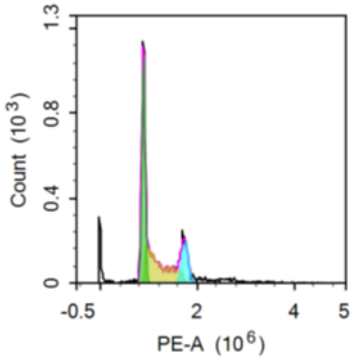
Table 3.10. Expression values that were used in Figure 3.30.

	MDA-MB-231 T1 OE	MDA-MB-231 T2 OE	MDA-MB-231 T3 OE
TAGLN	0.09	-0.24	0.65
CDH1	-1.22	0.80	0.49
KRT18	-1.12	-0.37	-0.31
ZO1	-1.13	0.42	0.50
CDH2	0.37	0.60	0.74
FN	-1.54	0.85	0.45
MMP9	-0.85	-0.49	-0.88
SNAIL	-0.15	-0.08	0.27
VIM	-0.29	-0.18	-0.21

3.3.2.6. Cell Cycle Analysis of MDA-MB-231 Cells Overexpressing TAGLN and Control

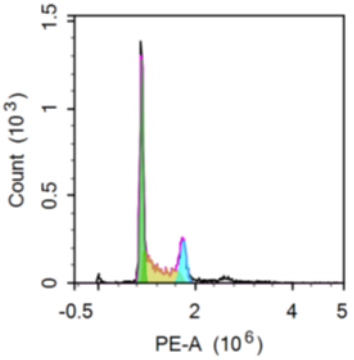
Cell cycle analysis of MDA-MB-231 cells revealed no significant change with TAGLN overexpression (**Figure 3.30**).

MDA-MB-231 E1



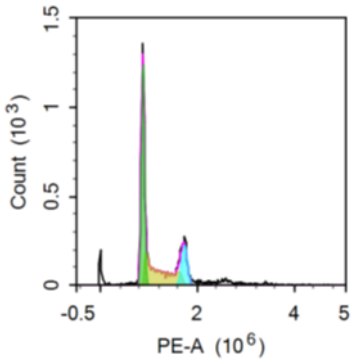
RMS	16.65 %
Freq G0/G1	38.01 %
Freq S	31.64 %
Freq G2/M	13.97 %

MDA-MB-231 E2



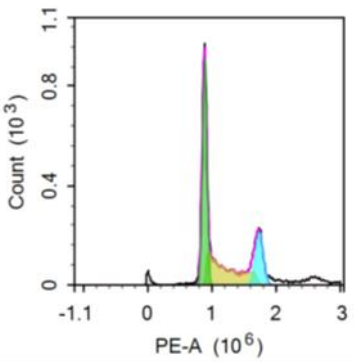
RMS	17.05 %
Freq G0/G1	41.69 %
Freq S	30.64 %
Freq G2/M	15.45 %

MDA-MB-231 T1



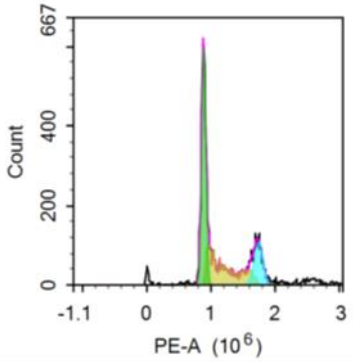
RMS	18.05 %
Freq G0/G1	40.96 %
Freq S	28.15 %
Freq G2/M	13.86 %

MDA-MB-231 T2



RMS	13.00 %
Freq G0/G1	40.16 %
Freq S	29.16 %
Freq G2/M	17.33 %

MDA-MB-231 T3



RMS	9.80 %
Freq G0/G1	42.01 %
Freq S	30.70 %
Freq G2/M	16.17 %

Figure 3.31. Propidium iodide staining of MDA-MB-231 cells revealed no change in cell cycle phases with TAGLN overexpression.

Colonies were seeded one day before and then they were collected and fixed. There is no significant change in cell cycle phase percentages with TAGLN overexpression. The experiment was performed once.

3.3.3. Overexpression of TAGLN in MDA-MB-361 Cells

3.3.3.1. Overexpressing TAGLN Gene in MDA-MB-361 Cells

MDA-MB-361 cells which were transfected with pCMV6 TAGLN vector had the larger second band above the intrinsic TAGLN band. With the transfection of MDA-MB-361 cells with pCMV6 vector, TAGLN expression has increased by 25.13% when compared to pCMV6 Entry vector transfected cells (**Figure 3.32** and **Figure 3.33**).

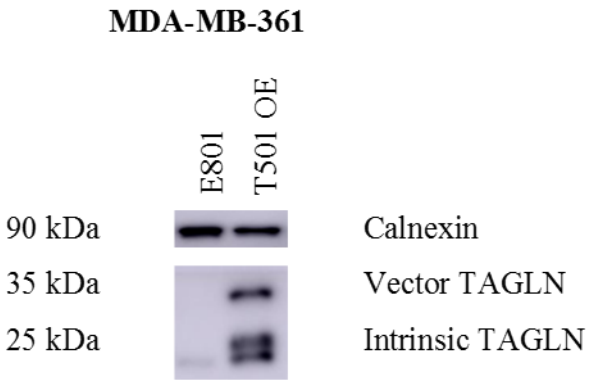


Figure 3.32. TAGLN gene was overexpressed in MDA-MB-361 cells with pCMV6 TAGLN vector.

Calnexin was used as loading control. Data was normalized to Calnexin protein levels. The experiment was performed twice.

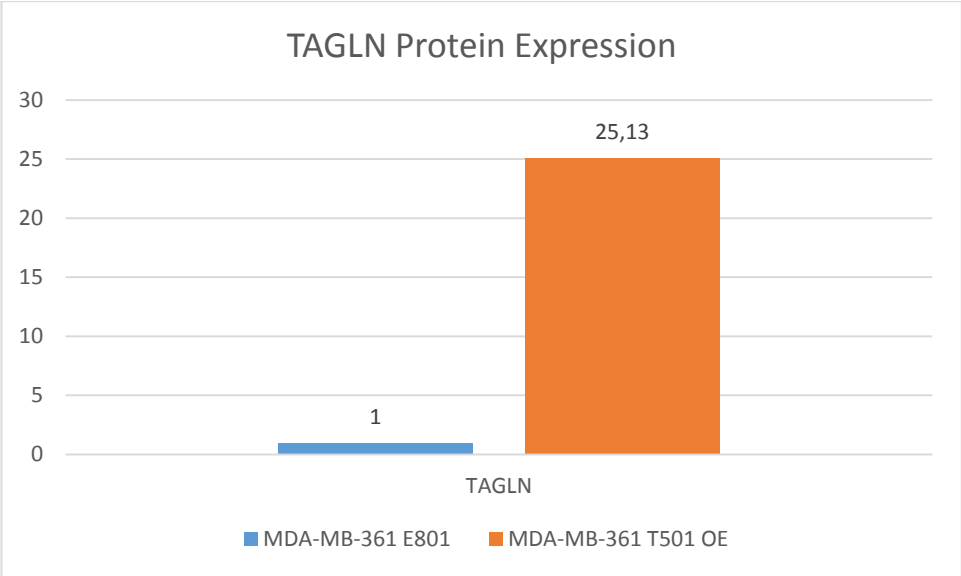


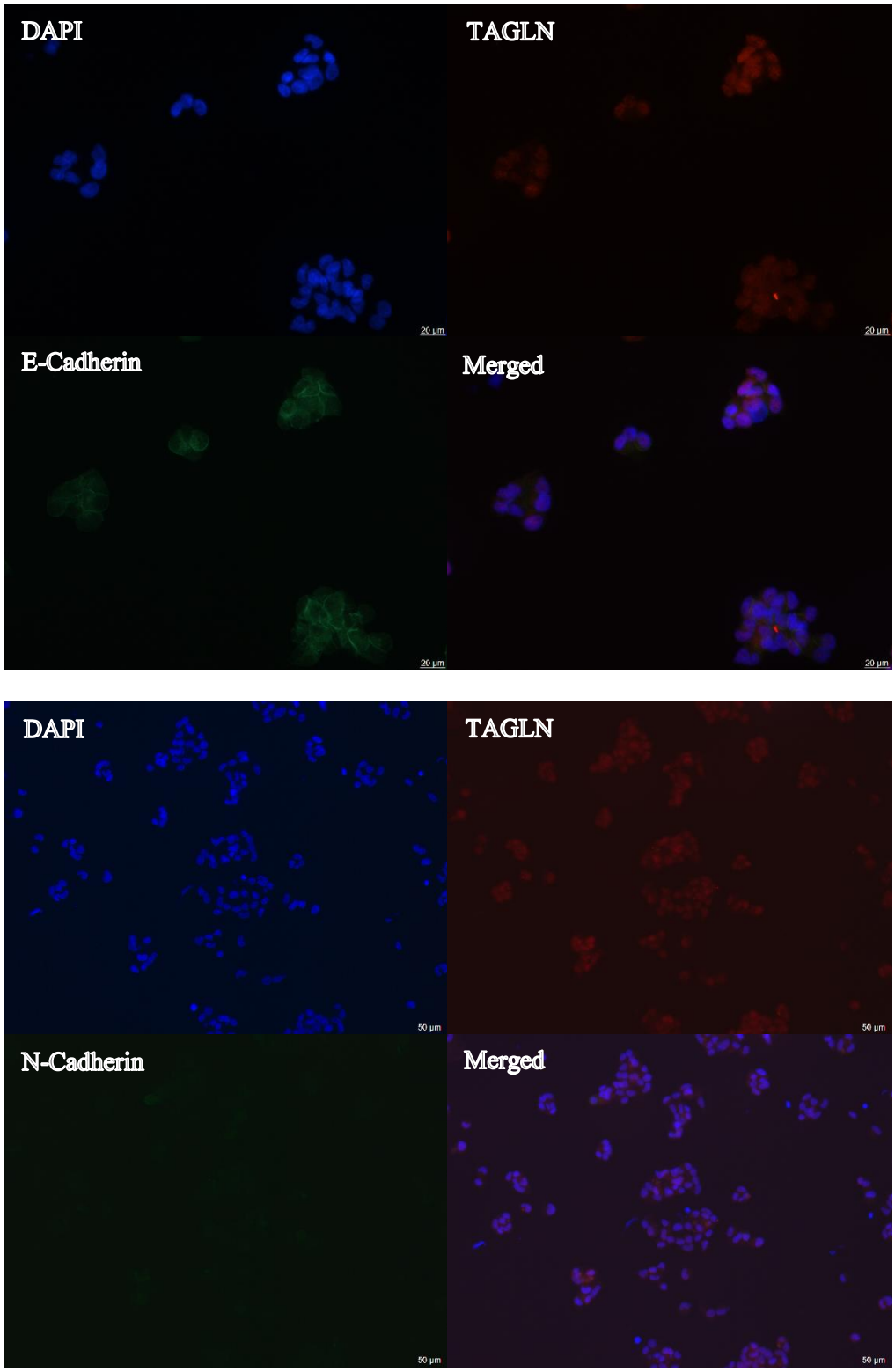
Figure 3.33. TAGLN gene overexpression was quantified in MDA-MB-361 cells.

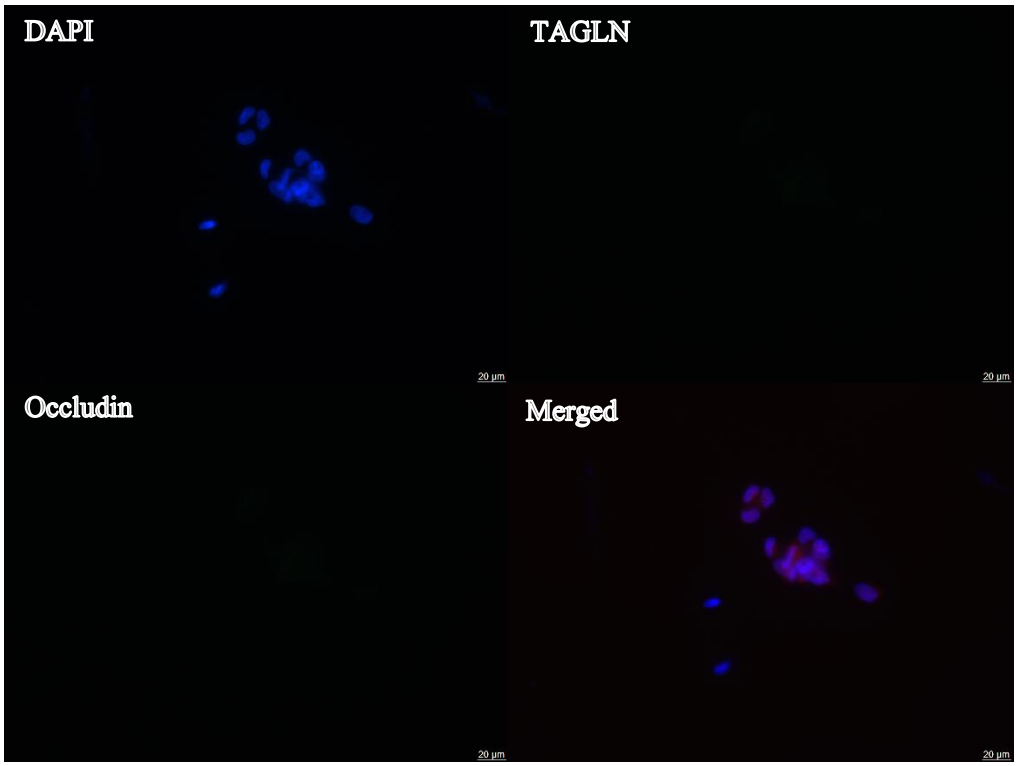
Quantification was performed for MDA-MB-361 overexpression cells by comparing them to pCMV6 Entry vector transfected MDA-MB-361 T501 cells. Protein expressions were normalized to Calnexin protein levels. Image J was used for quantification.

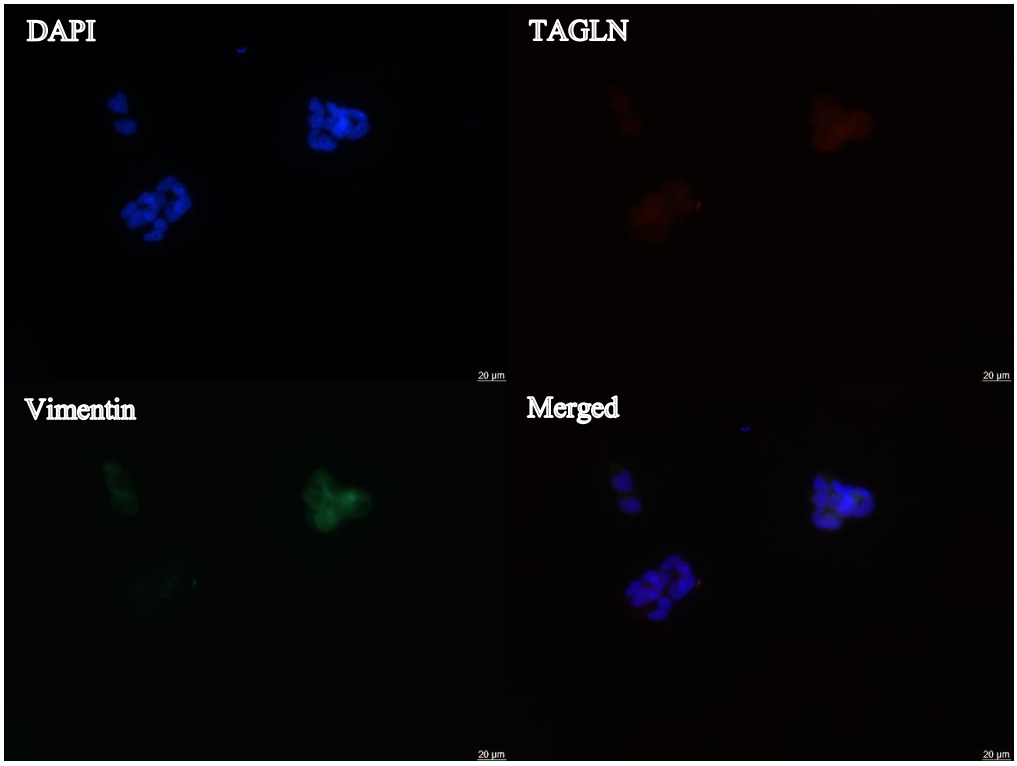
3.3.3.2. Immunofluorescence Analysis of EMT Markers in MDA-MB-361 Cells Transfected with pCMV6 TAGLN Vector and Control

With immunofluorescence staining, with the overexpression of TAGLN gene, while there was decrease in N-Cadherin and Vimentin expression, there was an increase in E-Cadherin expression (**Figure 3.34**).

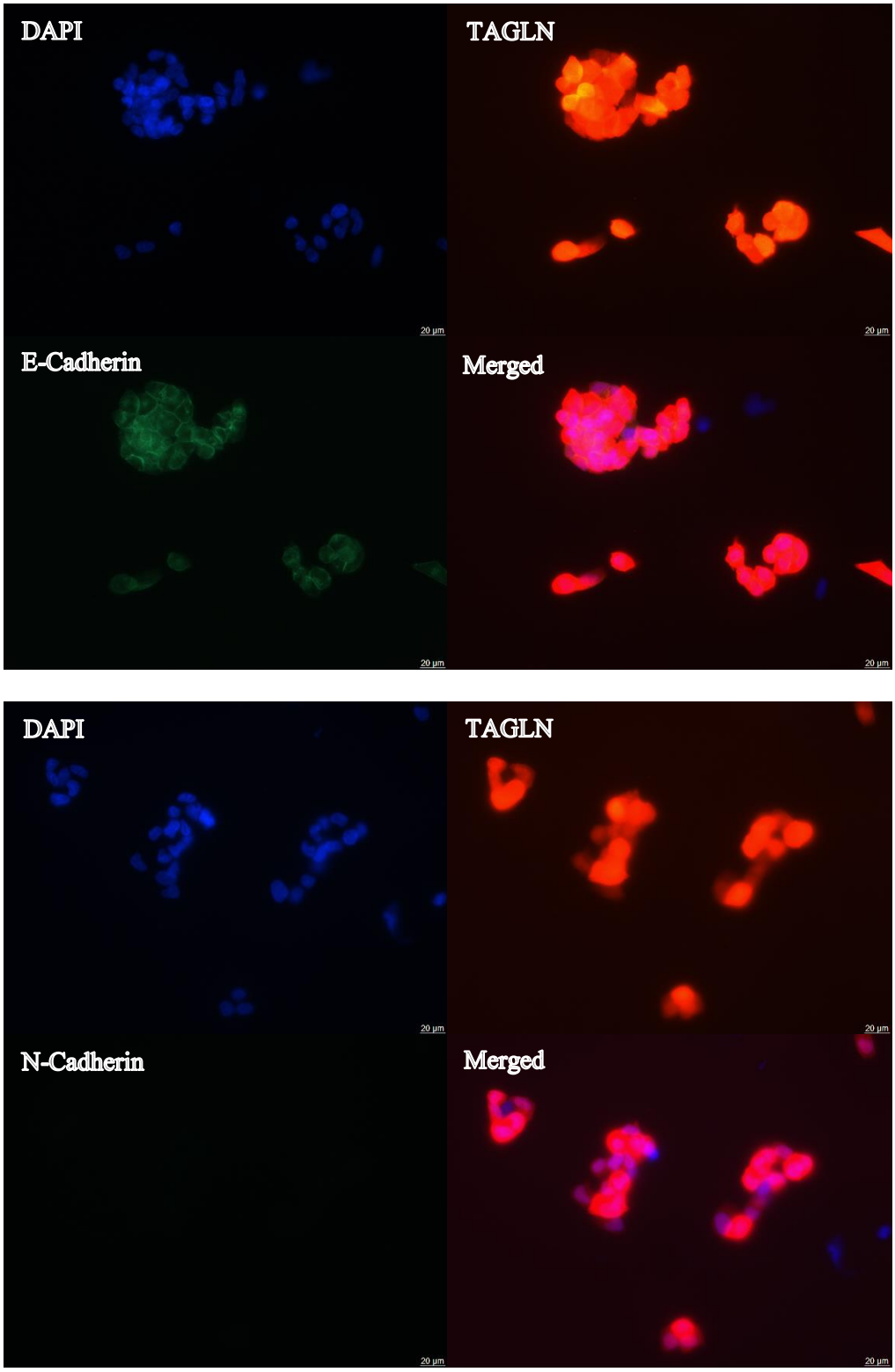
A) MDA-MB-361 E801




DAPI
TAGLN
20 µm
Occludin
Merged
20 µm
20 µm


DAPI
TAGLN
20 µm
Vimentin
Merged
20 µm
20 µm

B) MDA-MB-361 T501 OE



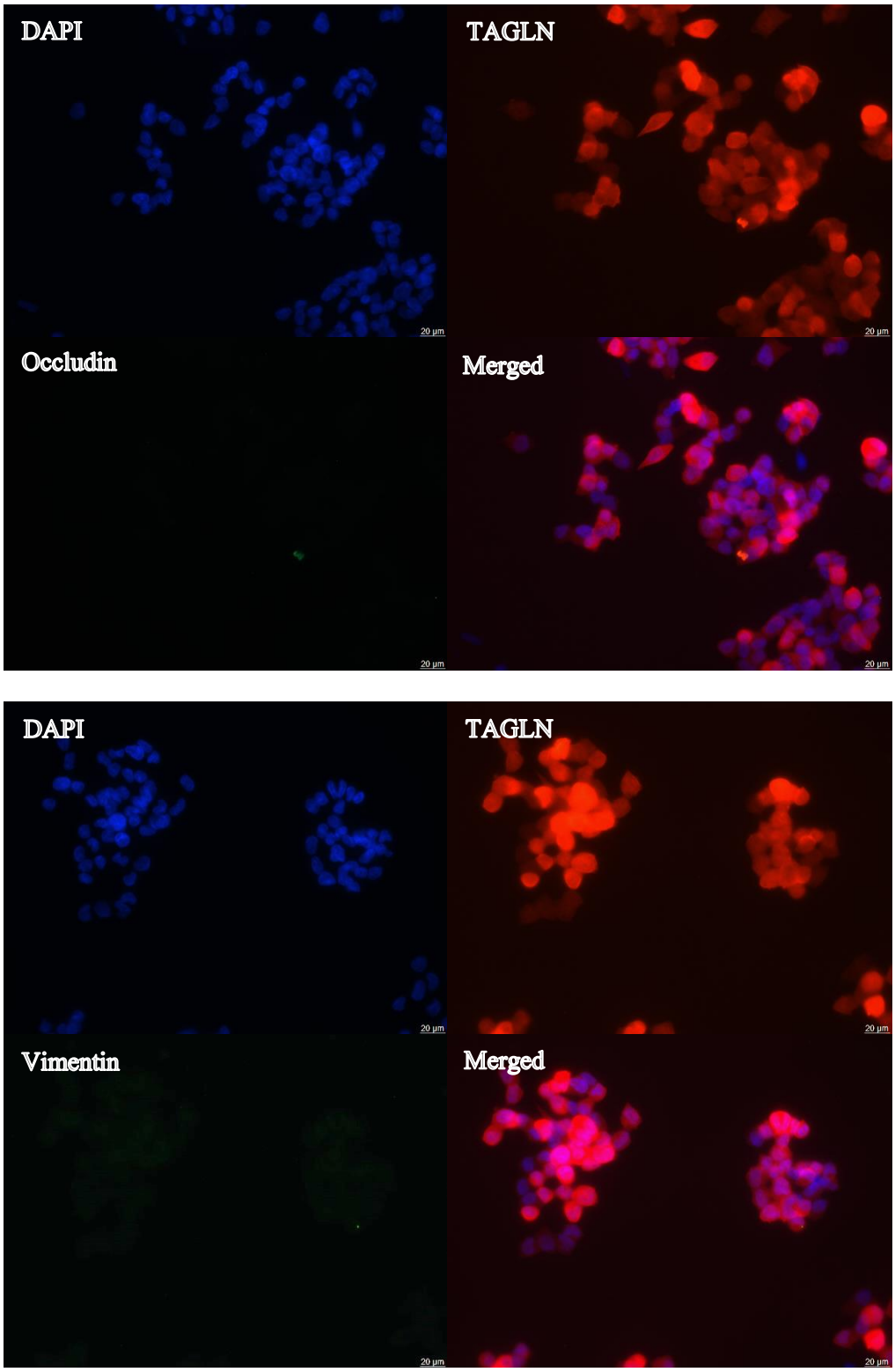


Figure 3.34. Overexpressing TAGLN gene in MDA-MB-361 cells effected the expression of epithelial marker E-Cadherin and mesenchymal markers N-Cadherin and Vimentin.

*With the overexpression of TAGLN gene, while there was decrease in N-Cadherin and Vimentin expression, there was an increase in E-Cadherin expression (**A** and **B**). Images were taken at 40x (except for MDA-MB-361 E801 TAGLN-N-Cadherin staining which is at 20X. The scale bar is 20 µm. The staining was performed twice.*

3.3.3.3. Colony Formation Assay of MDA-MB-361 Cells Transfected with pCMV6 TAGLN Vector and Control

With TAGLN overexpression, there was decrease in colony numbers of MDA-MB-361 cells when compared to pCMV6 Entry vector transfected cells. Statistical significance was determined by using unpaired two-tailed t-test ($p<0.05$) (**Figure 3.35**, **Figure 3.36** and **Table 3.11**).

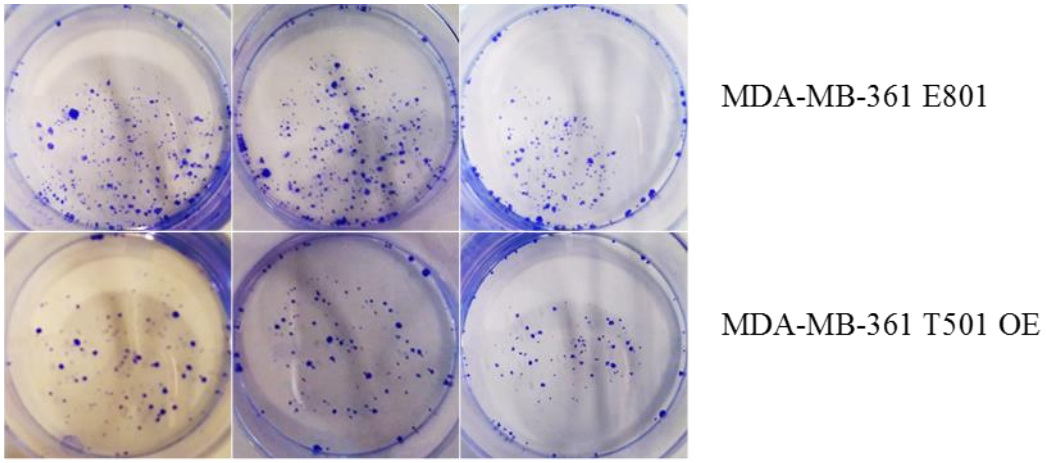


Figure 3.35. Overexpressing TAGLN gene has decreased the colony formation capacity of MDA-MB-361 cells.

Cells were seeded in equal numbers in each well. They were cultured in normal growth medium with Geneticin Selective Antibiotic until colonies became visible to eye. After 1-2 weeks, cells were fixed with ice-cold methanol and then stained with crystal violet solution and colonies were counted. Experiment had three replicates per sample and was performed once.

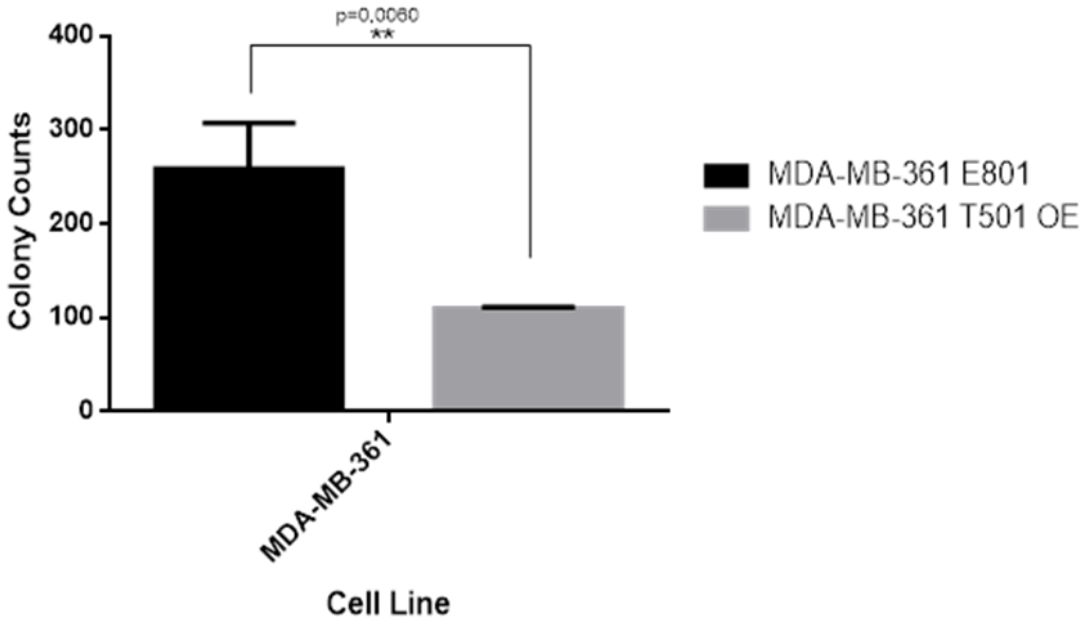


Figure 3.36. Overexpressing TAGLN Gene has caused the formation of less colonies in MDA-MB-361 cells.

Cells were seeded in equal numbers in each well after colonies are formed, cells are fixed and colonies are counted. Unpaired two-tailed t-test was used for analysis (P < 0.05).

Table 3.11. Statistics of colony formation of MDA-MB-361 cells overexpressing TAGLN and control.

Unpaired two-tailed t-test was used for analysis (P < 0.05).

Unpaired t test	
P value	0.0060
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.321 df=4

3.3.3.4. Cell Viability Assay of MDA-MB-361 Cells Transfected with pCMV6 TAGLN Vector and Control

Overexpressing TAGLN gene has decreased the viability of MDA-MB-361 cells at Day 4 at the statistical level. Statistical significance was determined by using unpaired two-tailed t-test ($p<0.05$) (**Figure 3.37** and **Table 3.12**).

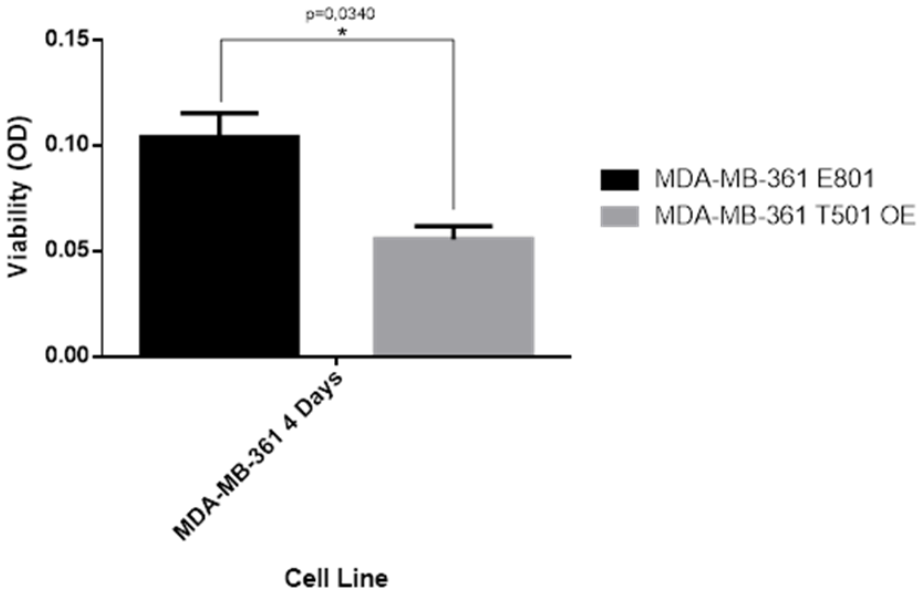


Figure 3.37. Overexpressing TAGLN gene has decreased the viability of MDA-MB-361 Cells.

Cells were seeded in equal numbers in each well and they were fixed and stained with SRB dye at the specified day. Unpaired two-tailed t-test was used for analysis ($p<0.05$). The experiment had two replicates per sample and was performed once.

Table 3.12. Statistics of viability of MDA-MB-361 cells overexpressing TAGLN and control.

Unpaired two-tailed t-test was used for analysis ($p<0.05$).

Unpaired t test	
P value	0.0340
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.284 df=2

3.3.3.5. qRT-PCR Analysis of EMT Marker Panel in MDA-MB-361 Cells Transfected with pCMV6 TAGLN Vector and Control.

While TAGLN overexpression was confirmed at the protein level, at the mRNA level TAGLN expression is reduced. mRNA level of epithelial CDH1 was increased, mesenchymal marker CDH2 was decreased and mesenchymal markers FN, MMP9 and SNAI2 were increased compared to empty vector transfected cells (**Figure 3.38** and **Table 3.13**).

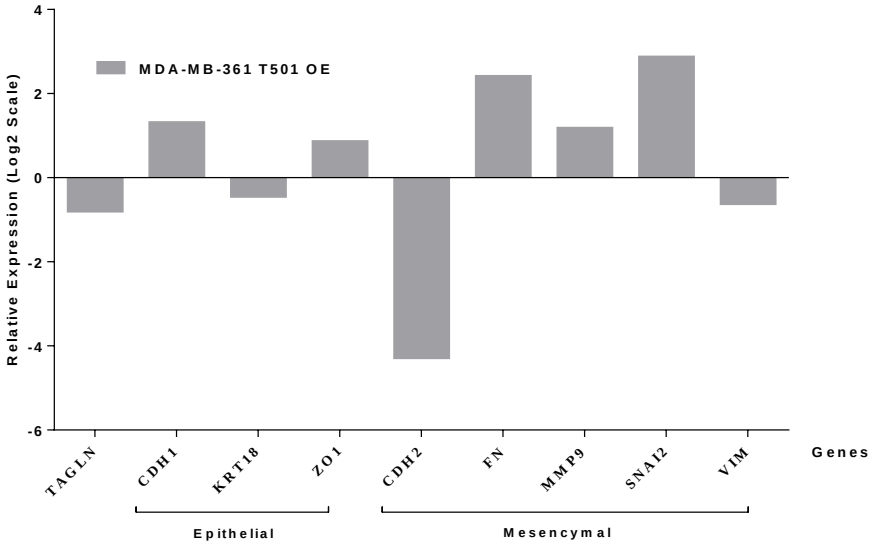


Figure 3.38. qRT-PCR analysis of TAGLN and EMT markers in MDA-MB-361 cells overexpressing TAGLN and control.
All data was normalized to GAPDH expression and then to empty vector transfected treated samples while drawing the graph. All data are in Log2 scale. The experiment had two replicates per sample and was performed once (except for TAGLN and GAPDH primers which were tested twice).

Table 3.13. Expression values that were used in Figure 3.28.

	MDA-MB-361 T501 OE
TAGLN	-0.80
CDH1	1.32
KRT18	-0.45
ZO1	0.86
CDH2	-4.28
FN	2.41
MMP9	1.18
SNAIL	2.87
VIM	-0.62

Table 3.14. Summary table of complete results.

Fluorescence intensity were used to make comparisons between samples. Statistically significant results in colony formation and viability assays were included. qRT-PCR TAGLN included all data regardless of magnitude whereas qRT-PCR epithelial or mesenchymal markers included data which are greater than +1 and smaller than -1. Numerical changes in cell cycle phases were given.

	siRNA Transfection		**Overexpression Transfection**		
	MDA-MB-157	**MDA-MB-231**	**MDA-MB-157**	**MDA-MB-231**	**MDA-MB-361**
Western Blotting (Vimentin)	Decrease	Decrease	Increase	Increase	N/A
Immunofluorescence (E-Cadherin)	No change	No change	No change	No change	Increase
Immunofluorescence (Vimentin)	Decrease	Decrease; Localize to nucleus	Increase	Increase	Decrease
Colony Formation	Increase	Decrease	No change	Increase (Dose Dependent -T2 Only)	Decrease
Viability	Increase	Decrease	No change	Increase (Dose Dependent- T2 Only)	Decrease
qRT-PCR TAGLN	Decrease	Decrease	Decrease	Increase in T1 and T3; Decrease in T2	Decrease
qRT-PCR Epithelial Markers	No change	Increase in CDH1	Increase in CDH1 and KRT18	Decrease in CDH1; KRT18 and ZO1 (T1 Only)	Increase in CDH1
qRT-PCR Mesenchymal Markers	Increase in FN and MMP9	Decrease in CDH2; Increase in FN and MMP9	No change	Decrease in FN (T1 Only)	Decrease in CDH2; Increase in FN, MMP9 and SNAIL
Cell Cycle	Decrease in G0/G1 Phase; Increase in S Phase; Decrease in G2/M Phase	Increase in G0/G1 Phase; Decrease in S Phase; Increase in G2/M Phase	No change	No change	N/A

Chapter 4. Discussion

Transgelin (TAGLN) is an actin-binding protein. It is highly expressed in fibroblasts and smooth muscle cells. In smooth muscle cells, it takes part in processes including motility and differentiation and also has a role in the formation of stress fibers.

In a previous study performed in breast cancer, TAGLN was found to be downregulated by promoter DNA hypermethylation in 19 out of 21 breast tumor tissues when compared to paired normal tissues. It was also revealed that survival among breast cancer patients is more likely if patients have higher TAGLN expression or lower promoter methylation of TAGLN (**20**). This finding proposes a role for the TAGLN gene as a tumor suppressor in breast cancer. In another study performed in colorectal cancer tissues, TAGLN expression was found to have been reduced in colorectal cancer tissues compared to normal healthy tissues. Colorectal cancer tissues were also identified to have higher promoter methylation compared to normal healthy tissues and patients with unmethylated TAGLN promoter had increased survival time (**42**). Yet another study in colorectal cancer also claimed the previously stated findings (**28**). These findings supported a possible role for TAGLN as a tumor suppressor gene. However, while some studies claimed TAGLN to be a tumor suppressor gene, there were several others proposing an oncogenic role for TAGLN in other types of cancers. For example, in a study on nerve sheath tumors, TAGLN expression was upregulated via hypomethylation and TAGLN therefore acted as an oncogene (**19**). These findings showed TAGLN to take on different roles in different cancers via promoter methylation.

In addition, there were some other studies claiming TAGLN gene function either as a tumor suppressor or oncogene in different cancer tissues. Briefly, TAGLN downregulation has been documented in colon, breast and prostate tissues (**15**, **28**, **31**, **32** and **33**) and its upregulation was documented in gastric and nerve sheath tumors (**19** and **34**).

To the best of our knowledge, the functional effect of TAGLN gene expression has not been studied in detail in breast carcinoma cell lines. The aim of this study was therefore to identify the functional role of TAGLN in breast cancer development.

In our study, wild type breast cancer cell lines and a non-tumorigenic breast cell line were co-stained with TAGLN and EMT markers. BT-474 cells which have been reported as epithelial displayed high E-Cadherin and no Vimentin expression supporting our expectations and TAGLN was observed in the nucleus and throughout the cell body (**Figure 3.1**). MCF-12A cells which were reported as epithelial displayed low E-Cadherin and high Vimentin expression in contrast to our expectations (**Figure 3.2**). One study might explain the conflicting behavior of MCF-12A cells such that these cells were claimed to have a heterogeneous makeup, composed of cells with epithelial and mesenchymal phenotypes (**37**). Also, in another study, the FACS method was used to separate MCF-12A cells to their sub-populations and there were isolated cells with mesenchymal phenotype in a heterogeneous mixture of cells (**43**). MDA-MB-157 cells which were reported as mesenchymal displayed high Vimentin expression supporting our expectations and TAGLN was observed in the nucleus and throughout the cell body (**Figure 3.3**). MDA-MB-231 cells which were reported as mesenchymal displayed high Vimentin expression supporting our expectations and TAGLN was observed in the nucleus and throughout the cell body (**Figure 3.4**). MDA-MB-361 cells which were reported as epithelial displayed medium E-Cadherin expression supporting our expectations and TAGLN was observed at low levels throughout the cell body (**Figure 3.5**).

TAGLN gene expression was silenced or overexpressed and functional analysis was performed in selected breast cancer cell lines in this study. Selection of the respective breast cancer cell lines included two criteria; the relative TAGLN expression of a cell line with respect to the other cells and the morphological status of the cells. Epithelial MDA-MB-361 cells which express low amounts of TAGLN were therefore selected to overexpress the TAGLN gene. Mesenchymal MDA-MB-157 and MDA-MB-231 cells which express more TAGLN compared to the MDA-MB-361 cells were selected to silence and overexpress the TAGLN gene. The phenotypes of cells were also taken into account in order to observe any possible change with respect to initial cell morphology.

TAGLN expression was silenced by transfecting with TAGLN siRNA. Western blotting analysis was performed to see changes at the protein levels of TAGLN and Vimentin. The presence of TAGLN and mesenchymal protein Vimentin expression was observed in MDA-MB-157 and MDA-MB-231 cells by immunofluorescence staining (**Figure 3.3** and **Figure 3.4**). The protein intensities of TAGLN and Vimentin were decreased in both MDA-MB-157 and MDA-MB-231 cells in Western blot analysis (**Figure 3.8** and **Figure 3.9**). Any possible changes in the morphologies of cells and re-localization of proteins upon TAGLN silencing were assessed with immunofluorescence staining. According to the Human Protein Atlas online database, TAGLN protein is mainly localized to the mitochondria, microtubules and cytosol in cells (**45**). The E-Cadherin protein, which is mostly expressed in epithelial cells, is mainly localized to the plasma membrane, cell junctions and the Golgi apparatus (**46**). The Vimentin protein, which is mostly expressed in mesenchymal cells, localizes to the intermediate filaments (**47**). In our experiment, MDA-MB-157 cells still had elongated cell morphology following TAGLN silencing, whereas MDA-MB-231 cells seemed to lose their elongated morphology. Although the TAGLN protein silencing was showed with western blotting analysis, immunofluorescence staining of TAGLN protein did not showed a significant decrease in MDA-MB-157 cells.

In MDA-MB-231 cells, the TAGLN protein seems to lose its localization in the cytoplasm and it re-localizes more to the nucleus. E-Cadherin staining was not expected in mesenchymal origin of cell lines MDA-MB-157 and MDA-MB-231. The E-Cadherin staining was mostly at the nucleus which might be a background staining.

In MDA-MB-157 cells, Vimentin staining seemed to remain at the cytoplasm but less signal was observed compared to non-targeting siRNA transfected cells.

Upon TAGLN silencing in MDA-MB-231 cells, Vimentin protein together with TAGLN protein appeared to re-localize to the nucleus more than the cytoplasm (**Figure 3.10** A and B). While MDA-MB-157 cells did not experience strong changes in the cell morphology except for the decrease in the cytoplasmic Vimentin protein upon TAGLN silencing, MDA-MB-231 cells started to become rounded and re-localizations of TAGLN and Vimentin to the nucleus was observed. This re-localization might be the reason for the changes in the cell morphology from elongated to rounded morphology. TAGLN is an actin binding proteins and Vimentin is known

to co-localize with microtubules. The cytoskeleton of cells is made of microtubules, actin filaments and intermediate filaments. These structures together have roles in the cellular organization, cell division and cell morphology (**48**). So, it might be therefore be assumed that there is a relationship between newly gained cell morphology and the localizations of TAGLN and Vimentin. MDA-MB-231 cells which display single-cell migration or multicellular streaming as the mode of migration in cancer normally have mesenchymal and elongated cell morphology which aids in migration (**49**). The loss of elongated phenotype might cause a decrease in the tendency of cell migration. Although migration analysis should be performed by using stably TAGLN silenced cells.

We analyzed the TAGLN overexpression effect on cell morphology, cell viability, cell cycle and expression levels of EMT markers in MDA-MB-157, MDA-MB-231 and MDA-MB-361 cells.

The Western blot analysis showed that TAGLN overexpression in both MDA-MB-157 and MDA-MB-231 cells led to more Vimentin expression compared to the control vector transfected cells (**Figure 3.16**, **Figure 3.17**, **Figure 3.24** and **Figure 3.25**) and also immunofluorescence staining results showed that the same cells kept their elongated morphology upon TAGLN overexpression.

Although Vimentin expression intensity was high in MDA-MB-157 cells, TAGLN expression was not very strong in immunofluorescence staining (**Figure 3.18**).

In MDA-MB-231 cells, the intensity of fluorescence signal depicting TAGLN expression was slightly higher in MDA-MB-231 T2 OE cells when compared with empty vector transfected cells. The Vimentin overexpression was apparent in all three TAGLN overexpressing cells. E-Cadherin staining was again at the nucleus and did not change upon TAGLN overexpression. Hence, E-Cadherin staining might be a background staining for the nucleus (**Figure 3.26**). As stated above, TAGLN and Vimentin are known to interact with the cytoskeleton elements of actin filaments and microtubules respectively. TAGLN overexpression might be affecting actin filaments which in turn might alter microtubules and associated proteins such as Vimentin. TAGLN silencing and overexpression in MDA-MB-157 and MDA-MB-231 cells inhibited and supported the initial mesenchymal morphology of cells respectively.

However, our statement is based on only Vimentin expression level. Therefore more EMT markers should be tested to confirm if mesenchymal marker expression increases upon TAGLN overexpression.

In MDA-MB-361 cells, the cells protected their grape-like morphology upon TAGLN overexpression which was highly apparent with enhanced red fluorescence signal in TAGLN overexpressed cells: E-Cadherin expression was increased whereas N-Cadherin and Vimentin expression were decreased with immunofluorescence staining (**Figure 3.34**). This created a completely reverse reaction to TAGLN overexpression in mesenchymal MDA-MB-157 and MDA-MB-231 cells. TAGLN overexpression in MDA-MB-361 cells therefore supported the initial characteristics of cells. This result that contradicts those obtained with TAGLN overexpression. This might be due to TAGLN's dual role in epithelial to mesenchymal transition. TAGLN might be enhancing mesenchymal properties in mesenchymal cells and enhancing epithelial properties in epithelial cells. The case where TAGLN acted differently in epithelial cells was previously shown by silencing the TAGLN gene in epithelial MCF10A cells which resulted with an increase in Vimentin protein levels and more elongated cell morphology (**3**).

It was interesting to observe that upon TAGLN ectopic expression in MDA-MB-361 cells, the endogenous TAGLN expression level was also affected with ectopic TAGLN vector expression and showed increase in the protein intensity (**Figure 3.32**). The pCMV6 vector which was used to overexpress TAGLN gene in MDA-MB-157, MDA-MB-231 and MDA-MB-361 cells had the C-terminal Myc-DDK tags in its structure (**50**). So, in order to assess the actual level of ectopic expression of TAGLN gene anti Myc or Anti-DDK antibodies can be used in western analysis.

In the immunofluorescence staining experiments, 4% formaldehyde was used for fixation. While staining with E-Cadherin antibody in epithelial cells appeared well, there were background staining in mesenchymal cells which were not expected to show E-Cadherin expression. Formaldehyde is generally recommended to be used before staining of nuclear proteins. However, due to background staining issue with our samples, ice-cold methanol or TCA fixations could have been used in the staining of cytoplasmic proteins. Methanol fixation was also documented to be useful in

immunofluorescence staining since it provides high immunofluorescence staining with low background (**51**).

The effect of TAGLN expression on the cells were assessed by using two cell viability assays, colony formation and SRB assays. The colony formation assay is an *in vitro* cell survival assay which is based on the ability of a single cell to grow into a colony. In this assay, single seeded cells were tested for their ability to undergo unlimited number of cell division. After the altered gene expression in the cells, only a fraction of cells retain their capacity to form colonies which is an indication of cell proliferation (**52** and **53**). SRB assay is *in vitro* cytotoxicity assay which can be used to test the effect of altered gene expression on cell viability. SRB dye binds to the amino acid residues of proteins and this binding gives an estimation of protein mass. Protein mass is directly proportional to the cell count. After seeding equal numbers of cells for two or more different groups, cells can be grown for a certain time period and then the ones with higher proliferative capacity will have a greater protein mass and in turn more SRB staining. While two tests aim for the same goal to understand the proliferation capacity of cells, the colony formation assay is more time-consuming and laborious than SRB assay. In addition, colony formation assay requires cells to be in strict single cell suspension while seeding them and also this assay might not be suitable for cells with low colony-forming ability. SRB assay, on the other hand, might give incorrect results if it becomes overpopulated with cell count or staining might be deficient or in excess (**53**). In our experiment, how the altered TAGLN expression can affect the cells' capacity to form colonies and their proliferation capacity was investigated. For colony formation assay, mesenchymal MDA-MB-157 and MDA-MB-231 cells do not form colonies but they can spread out whereas epithelial MDA-MB-361 cells can form tight colonies. Upon silencing of TAGLN expression in MDA-MB-157 cells, cell spreading and cell viability seems to be enhanced significantly (**Figure 3.11**, **Figure 3.12**, **Table 3.2**, **Figure 3.13** and **Table 3.3**). However, upon TAGLN overexpression, cell spreading and cell viability did not change significantly, proposing a dose dependent effect of TAGLN expression (**Figure 3.19**, **Figure 3.20**, **Table 3.5**, **Figure 3.21** and **Table 3.6**). MDA-MB-157 cells are mesenchymal primary cells and are not metastatic. In these cells, TAGLN might be functioning as a tumor suppressor gene and acting at a dose dependent manner since TAGLN silencing increased proliferative capacity of cells

but its upregulation in MDA-MB-157 cells did not change the cell proliferation capacity (**Figure 3.16** and **Figure 3.17**).

MDA-MB-231 cells do not form colonies but mostly show cell spreading. It was observed that the cell viability and spreading were decreased in TAGLN silenced MDA-MB-231 cells (**Figure 3.11**, **Figure 3.12**, **Table 3.2**, **Figure 3.13** and **Table 3.3**). Upon TAGLN overexpression, one TAGLN overexpressing colony (MDA-MB-231 T2 OE) showed significant increase in cell spreading and cell viability compared to empty vector transfected cells (**Figure 3.27**, **Figure 3.28**, **Table 3.8**, **Figure 3.29** and **Table 3.9**). MDA-MB-231 cells are mesenchymal metastatic cells and in these cells, TAGLN might be functioning as an oncogene and acting at a dose dependent manner since TAGLN silencing decreased proliferative capacity of these cells while its upregulation in cell clone with highest TAGLN expression, MDA-MB-231 T2 OE, was sufficient to increase cell proliferation.

Upon TAGLN overexpression in MDA-MB-361 epithelial cells, colony formation capacity and cell viability decreased significantly (**Figure 3.35**, **Figure 3.36**, **Table 3.11**, **Figure 3.37** and **Table 3.12**). TAGLN might be functioning as a tumor suppressor since its overexpression decreased proliferative capacity. This tumor suppressor or oncogenic capacity of TAGLN gene should be confirmed with further experiments, such as cell invasion assays and in vivo mouse experiments.

Colony formation assays could have been performed on agar to observe cell growth in an anchorage-independent manner. In normal colony formation assay, which we have performed in our analysis, cells are spread onto a tissue culture plate in growth medium which provides the basal membrane and extracellular matrix to the cells. There, cells were grown until visible colonies were formed, then colonies were fixed and counted. The colony formation assay only provides information about colony formation capacities of the tested cells. In soft agar colony formation assay, normal cells cannot grow in an anchorage-independent manner, however, transformed cells can grow and divide without binding to a substrate (**54**). The soft agar colony formation assay with TAGLN expression altered cells can be performed to gain information whether the

TAGLN expression level can affect transformation and metastatic potentials capacities of these cells.

In order to understand how TAGLN expression effects EMT markers at the mRNA level, qRT-PCR was set up with siRNA transfected cells and overexpressing clones and their controls. Log2 value for TAGLN was included for all data regardless of magnitude whereas Log2 value for epithelial or mesenchymal markers was included for data which are greater than +1 and smaller than -1.

Upon TAGLN silencing in MDA-MB-231 and MDA-MB-157 cells, the decreased in TAGLN mRNA level was detected by qRT-PCR and this decrease was also confirmed with Western blot analysis (**Figure 3.14** and **Table 3.4**).

In TAGLN overexpressing cell clones from MDA-MB-231, MDA-MB-157 and MDA-MB-361 cells unexpectedly showed the decreased in TAGLN mRNA level compare to the empty vector transfected cells with qRT-PCR. But Western blot analysis with same cells showed increase in TAGLN protein level (**Figure 3.22**, **Table 3.7**, **Figure 3.30**, **Table 3.10**, **Figure 3.38** and **Table 3.13**). This unexpected outcome with overexpression clones may be due to the fact that there is either post-transcriptional modifications of TAGLN mRNA or TAGLN protein might be stabilized via binding actin residues after it is translated and stable TAGLN protein reduces TAGLN mRNA through a negative feedback-loop mechanism. Silencing the TAGLN gene in MDA-MB-157 cells resulted in increase in the expression of Mesenchymal markers FN and MMP9 (**Figure 3.14** and **Table 3.4**). Western blotting and immunofluorescence analysis previously revealed TAGLN expression to promote mesenchymal characteristics of MDA-MB-157 cells, so it was unexpected to see the increase in FN and MMP9. In a study in HT1080 cells of fibrosarcoma origin, TAGLN was found to be a novel regulator of MMP-9 expression and overexpression of TAGLN decreased MMP9 mRNA and protein levels and also siRNA mediated silencing of TAGLN in WI-38 lung fibroblasts elevated MMP9 synthesis in the same study (**22**). This reverse relationship of TAGLN expression with MMP9 was also shown in breast cancer tissues (**32** and **22**). It has been shown that the expression of FN and MMP9 are concurrent and FN even induces MMP9 expression. This study was performed by culturing MDA-MB-231 cells with FN and it was revealed that pro-

MMP9 expression was induced (**40** and **41**). This finding might explain how FN induces MMP9 expression but it is not clear how TAGLN takes part in this process. Silencing the TAGLN gene in MDA-MB-231 cells resulted in increase in the expression of epithelial marker CDH1, decrease in the expression of mesenchymal marker CDH2 and increase in the expression of mesenchymal markers FN and MMP9 at the mRNA level (**Figure 3.14** and **Table 3.4**). In this study, TAGLN expression seems to be promoting mesenchymal characteristics of MDA-MB-231 cells, it was unexpected to see in the increase in FN and MMP9. But these increases are in the mRNA level and needs to be confirmed at protein level.

In TAGLN overexpressing MDA-MB-157 cells, epithelial markers CDH1 and KRT18 was upregulated at the mRNA level, however, upregulation of CDH1 could not be observed at the immunofluorescence image. In TAGLN overexpressing MDA-MB-231 cells, decrease in epithelial markers CDH1, KRT18 and ZO1 and decrease in mesenchymal marker FN were observed at the mRNA level in one cell clone only, MDA-MB-231 T1 OE. TAGLN overexpression was not significantly correlated with mesenchymal markers' expression levels in other two cell colonies of MDA-MB-231. In TAGLN overexpressing MDA-MB-361 cells there were an increase in epithelial marker CDH1, decrease in mesenchymal marker CDH2 at the mRNA level which is in parallel to the immunofluorescence staining results for CDH1 and CDH2. Additionally, mesenchymal markers FN, MMP9 and SNAI2 were upregulated at the mRNA level. It must again be stressed that western blotting analysis should be performed with more markers to draw a more solid explanation for the role of TAGLN in EMT process in the breast cancer.

There is a phenomenon called the partial EMT which can define the migration properties or invasiveness of cells. Cellular plasticity occurs during cancer progression such that changes from epithelial to mesenchymal phenotype and from mesenchymal to epithelial phenotype can occur. While it was generally accepted that cells experience EMT and gain metastatic features, a newly established concept, partial EMT can occur such that at the same time both epithelial and mesenchymal markers are expressed and cells become more invasive in cancer (**55**).

Cell cycle analysis was performed with Propidium Iodide Staining on cells with altered TAGLN expression. Overexpressing TAGLN in MDA-MB-157 and MDA-MB-231 cells had no significant effect on cell cycle (**Figure 3.23** and **Figure 3.31**).

Silencing of the TAGLN expression in MDA-MB-157 cells resulted in slight increase in S phase (**Figure 3.15**). Since the colony formation/cell spreading and viability assays revealed increase proliferation capacity of the cells upon TAGLN silencing, this increase in the S phase might account for the observed behavior of these cells.

In conclusion, TAGLN expression might have an effect on Epithelial to Mesenchymal Transition (EMT) by altering the expression of the EMT markers E-Cadherin and Vimentin. This effect also takes the original morphology of the respective cell lines into the account such that TAGLN expression promotes the initial morphology. In this study, the effect of TAGLN expression on cell proliferation was also studied and TAGLN was found to act as a tumor suppressor in MDA-MB-157 and MDA-MB-361 cells and as an oncogene in MDA-MB-231 cells. Since the metastatic capacities of the native cells are different, the TAGLN expression level may have different affect in different breast carcinoma cell lines.

Chapter 5. Future Perspectives

Our analysis revealed that TAGLN gene might have a role in EMT transition by altering the expressions of the EMT markers E-Cadherin and Vimentin. To understand the role of in EMT, more EMT markers are required to be tested in TAGLN expression altered cells both with immunofluorescence and Western blot analysis. At the mRNA level, TAGLN silencing increased the expression of metastasis mediator MMP9. This finding should be confirmed at the protein level and the role of the TAGLN gene in invasiveness or metastatic potential or tumor formation capacity should be studied in both in vitro and in vivo systems. In *in vitro* systems, matrigel chamber assays and solid agar colony formation assays can be performed to analyze the invasive capacity of the cells. In *in vivo* systems, cells with altered TAGLN gene expression can be injected into mammary fat pad of mice to observe their tumor formation capabilities. The luciferase gene containing cells with the altered TAGLN expression can also be injected through tail of the mice to observe metastatic capability of TAGLN. The Zebra fish can also be used for in vivo experiments. TAGLN silenced or overexpressed breast tumor cells can be injected into the zebrafish abdomen to investigate the metastatic and invasion behavior of these cells. The in vivo mice experiments are currently under progress in our lab. The long term effect of TAGLN gene silencing can be assessed by creating TAGLN gene knockdown with CRISPR/Cas9 system in luciferase expressing cells. These cells can be used for *in vivo* experiments. TAGLN interact with actin cytoskeleton and therefore it may have a role in the formation of focal adhesions. The cells with altered expression of TAGLN can be analyzed for focal adhesion molecules and phospho specific FAK presence can be assessed by detecting with anti-phopho FAK antibody with Western blot and immunofluorescence staining.

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