THE ROLE OF TISSUE TRANSGLUTAMINASE (TG2) IN RENAL CELL CARCINOMA CELL ADHESION AND CELL MIGRATION

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ABSTRACT

THE ROLE OF TISSUE TRANSGLUTAMINASE (TG2) IN RENAL CELL CARCINOMA CELL ADHESION AND CELL MIGRATION

Transglutaminase (TG2) catalyzes Ca^{+2} dependent post-translational modification of proteins by forming covalent ε (γ-glutamyl) lysine linkages. TG2 also plays an important role in cell adhesion and migration by acting as a novel adhesion protein interacting with integrins and syndecan-4 (SDC4). Together with β1 integrin (ITGB1), TG2 expression has been shown increase in cancers with epithelial origin providing an enhanced drug resistance and metastatic potential. Renal Cell Carcinoma (RCC) is a common type of epithelial cancer comprising 90% of kidney cancer cases. In primary RCC tumors TG2 expression was found to be decreased while an increase in association with ITGB1 and SDC4 expression was found to be evident in the metastatic RCC tumors. This thesis aims to dissect the molecular mechanisms of TG2-induced metastatic potential in RCC using model cell lines. For this purpose, in primary A-498, Caki-2 and metastatic ACHN, Caki-1 RCC cell lines the role of TG2 in RCC cell migration and invasion on ITGB1 specific substrates was examined using Wound Scratch and Transwell Invasion Assay, respectively. The role of TG2 in the focal contact formation and cell adhesion was also analyzed through actin-stress fiber staining with FITC-labeled Phalloidin. Results indicated that while the fastest migration potential was found to be in metastatic Caki-1 and ACHN, the silencing of TG2 decreased their migration potential by %50. Results from Transwell experiments indicated that primary Caki-2 cells possessed the highest migration potential on fibronectin, while the metastatic RCC cell lines demonstrated the highest migration on collagen type I and laminin. Down-regulation of TG2 using siRNA technology led to a significant 50%-60% decrease in the invasive potential of all RCC cell lines suggesting that TG2 is not only important in cell migration but also in intravasation and extravasation stages of metastasis.

ÖZET

DOKU TRANSGLUTAMINAZ (dTG)'IN BÖBREK HÜCRE KARSİNOMLU HÜCRE YAPIŞMASI VE METASTAZINDAKİ ROLÜ

Transglutaminaz (dTG) Ca^{+2}'a bağımlı olarak ε(γ-glutamil) lizin bağları kurar ve böylece proteinlerin post-translasyonel değişikliklerini katalize eder. dTG'nin aynı zamanda hücre yapışma ve göçünde de yeni bir protein olarak önemli bir göre almakta olduğunu, integrinler ve sindekan-4 (SDC4) ile etkileşime girdiğini öne sürmektedir. β1 integrin (ITGB1) ile birlikte dTG'nin epitel kökenli kanser hücrelerine ilaç direnci ve metastatik özellik kazandırdığı ispatlanmıştır. Böbrek Hücre Karsinomu (BHK) böbrek kanseri vakalarının %90'ını kapsamakta olan yaygın bir epitel kanser türüdür. İlk defa primer BHK tümorlerinde dTG ekspresyonun azaldığı gösterilirken, metastatik BHK'larda ITGB1 ve SDC4 ekspresyonları ile birlikte artış gösteren dTG ekspresyonu ortaya konmuştur. Bu tezin amacı, seçilmiş BHK hücre hatlarında, dTG'nin moleküler mekanizmasını aydınlatmaktır. Bu amaç doğrultusunda, dTG'nin primer A-498, Caki-2 ve metastatik ACHN, Caki-1 BHK hücre hatlarının hücre göçündeki rolünü göstermek amacıyla Yara Yaratağı deneyi yapılmıştır. Ayrıca, BHK hücre hatlarının ITGB1 ligandları üzerine göç potansiyelleri Transwell Method ile analiz edilmiş olup, dTG'nin fokal kontak oluşumundaki rolü ve hücre adezyonu FITC etiketli Phalloidin ile Aktin-Stres-Fiber Boyama methodu kullanılarak florasans mikroskobu ile analiz edilmiştir. Çıkan sonuçlar en hızlı göç potansiyelinin metastatik Caki-1 ve ACHN hücrelerinde olduğunu göstermiş ve dTG ifadesinin susturulması sonucu göç potansiyellerinde %50 azalma bulunmuştur. Primer BHK hücreler fibronektin üzerine en çok göç potansiyeli gösterirken, metastatik BHK hatlarının kolajen tip1 ve laminin üzerindeki invazif etkisi yüksek bulunmuştur. dTG ifadesinin susturulması sonucu primer ve metastatik BHK hatlarının invazif potansiyellerinde %50 ile %60 düşüş yaşanması dTG'nin hücre göçü yanı sıra intravazasyon ve ekstravazasyonda da önemli olduğu öne sürmektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

µl	Microliter
Akt	Protein kinase B
APS	Ammonium Persulfate
ATP	Adenosine Triphosphate
ATTC	American Type Culture Collection
BSA	Bouvine Serum Albumin
BTC	Biotin Cadeverin, trifluoroacetate salt (N-(5-aminopentyl) biotinamide, trifluoroacetic acid salt)
Ca^{2+}	Free Calcium Ion
Col1	Collagen Type 1
c-Src	Proto-oncogene tyrosine-protein kinase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ECM	Extracellular Matrix
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
Erk	Extracellular signal-regulated kinases
FAK	Focal Adhesion Kinase
FAK	Focal adhesion kinase
FN	Fibronectin
HIF	Hypoxia-inducible factor
ITGB1	Integrin Beta 1
LM	Laminin
M	Molar
mDia1	Diaphanous-related formin
ml	Milliliter
MLCK	Myosin light chain kinase
mM	Millimolar
MMPs	Matrix metalloproteinases

mRNA	Messenger Ribonucleic acid
pH	Negative log of Hyrogen Ion Concentration
PI3-K	Phosphotidylinositol-3 kinase
PKCα	Protein Kinase C Alpha
PKL	Paxillin kinase linker
RCC	Renal Cell Carcinoma
Rho	Small GTP-binding protein
RPTEC	Renal Proximal Epithelial Cell
RTCC	Renal Transitional Cell Carcinoma
S.D.	Standard Deviation
SDC-4	Syndecan 4
siRNA1	Silencing Ribonucleicacid 1
siRNA6	Silencing Ribonucleicacid 6
TEMED	N,N,N’,N’- Tetramethylethylenediamine
TG2	Tissue Transglutaminase
TGF- β	Transforming Growth Factor Beta
TNM	Tumor, Node, and Metastasis
uPa	Urokinase plasminogen activator
VHL	Hippel-Lindau
WHO	World Health Organization

1. INTRODUCTION

1.1. KIDNEY PHYSIOLOGY

Kidneys are paired organs located at the both sides of the vertable column in the abdominal cavity. Kidneys perform mainly three roles in the body metabolism: (i) acid-base balance; (ii) synthesis of several hormones; and (iii) the most important role; excretion of nitrogenous waste which is metabolic end product. A normal adult's kidney size dimensions are generally 12x7x3 cm and weighs approximately 160 grams. Kidney has two major systems; parenchyma and collecting system. Cortex and medulla are the members of parenchyma system whereas the collecting system comprises ureters, calyces, and renal pelvis as shown in the Figure 1.1. Kidneys contain various specialized cell types in its structure therefore they are classified as anatomically complex organs [1].

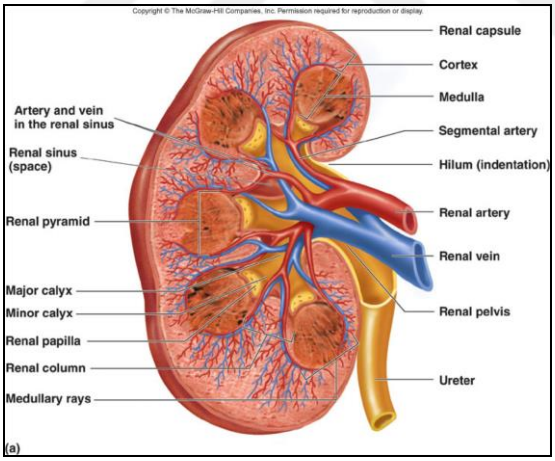

Figure 1.1 Kidney Anatomy and Physiology [2]

Functional unit of this system is called nephron which also contains specialized cells filtering the blood plasma. A nephron contains proximal tubule, renal corpuscle, loop of Henle, collecting duct and distal tubule. There are approximately 1 million nephrons in a kidney. Nephrons exist at the cortex (the outer layer of the organ) and medulla (the inner part of the kidney part; Figure 1.2) [1, 3]. Most part of the proximal tubule, renal corpuscle and a part of distal tubule is located in the cortex of the kidney whereas medulla part

consists of collecting ducts and the loop of Henle [3]. The head of the nephron is called renal corpuscle where urine is first formed. It contains glomerulus surrounded by the Bowman's capsule. As a first step of urine formation, blood is filtered by the glomerular capillaries and sent into Bowman's space and then delivered to renal tubules [1].

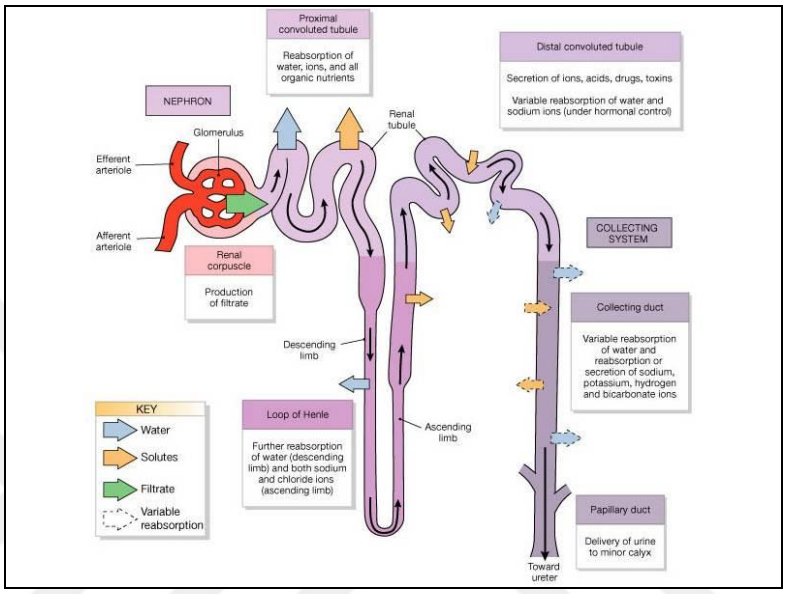

Figure 1.2. Nephron structure as a functional unit of kidney [4]

Given complex anatomy of kidney with specialized diverse cell types and its complex three-dimensional/structure, tissue engineering of artificial kidney has not been possible so far creating a world-wide donor problem in the treatment of kidney diseases such as chronic renal failure and kidney cancer [5, 6, 7].

1.2. KIDNEY CANCER

GLOBOCAN 2012 statistics demonstrate that approximately 14,1 million cancer cases and 8,2 million cancer deaths occurred in 2012. Cancer survival rate decreases most of the time not only due to late stage diagnosis but also limited access of treatment. Kidney cancer derived from kidneys is divided in two classes; renal transitional cell carcinoma (RTCC) and renal cell carcinoma (RCC). RTCC arises from the renal pelvis whereas RCC type is originated from renal parenchyma part of the kidney. Studies demonstrated that 90% of kidney cancer cases exist in RCC nature [8, 9].

In the light of cancer studies and statistics, it is demonstrated that kidney cancer generates nearly 2% of all cancer types worldwide. Unfortunately, about 250,000 new incidence and 100,000 deaths/year is being reported every year. According to the global cancer statistics in 2012, kidney cancer seen in women and men are 10% and 20%, respectively. Moreover in kidney cancer patients, death rates are found to be 6% in men and 3% in women. These rates are shown to be similar in Turkey population. The highest kidney cancer incidence and death rates are found to be among American Indians/Alaska Natives suggesting that obesity and over smoking in this population may induce this imparity [10, 11].

1.3. RENAL CELL CARCINOMA

Renal cell carcinoma (RCC) is the most widespread type of kidney cancer seen at a rate of 90% worldwide. Diagnosis of RCC can be characterized by its poor prognosis and high potential for metastasis. Cell invasion mechanism of RCC tumors is not known well, yet. The formation rate of RCC is approximately 210,000 new cases/year, bringing a mortality of ~100,000 deaths/year among world. The disadvantage of poor prognosis, results in RCC to be diagnosed at metastatic stage [12, 13]. Diagnosis at metastatic stage causes resistance against radiation therapies as well as chemotherapy. In most cases the gene called von Hippel-Lindau (VHL), located at chromosome 3p25-p26, mutation causes RCC disease. Von Hippel–Lindau is a tumor suppressor gene. Mutations in that gene causes VHL syndrome, which is inherited only one mutation in the protein causing lost or altered function of the protein. Any sporadic mutation in the second copy of the protein results in carcinomas, especially leading kidneys and forming renal clear cell adenocarcinoma. VHL protein is a member of a protein complex that contains elongin B/C, cullin-2, and having ubiquitin ligase E3 activity. A transcription factor, hypoxia-inducible factor (HIF), playing a main role in the gene expression via oxygen, is controlled by this protein complex via ubiquitination and degradation [14]. VHL protein marks target proteins in order to get ready for degration by its E3 ubiquitin ligase activity. HIF1a is the most studied target, inducing angiogenesis and expression of angiogenesis related factors [15]. Activation of HIF enhances angiogenesis, leading increased glucose intake which is necessary for tumor growth. Renal cell carcinomas demonstrate constructive activation of HIF in oxygenated areas, although HIF is dominantly active in hypoxic environments. Active VHL protein synthesized normal cells regulate HIFα via hydroxylation in oxygenated environment [15].

In the presence of oxygen, 2-oxoglutarate, iron, and, HIF becomes inactive via HIF hydroxylases. This reaction results in a formation of a binding site in the protein transcript of the VHL gene. HIF1α is polyubiquitylated for degradation by proteases via pVHL. On the other hand, in hypoxic environment, accumulated HIFα subunits bind to HIFβ (transcription factor activating other genes encoding proteins such as vascular endothelial growth factor (VEGF). HIFβ activated genes are involved in angiogenesis. Moreover, cells having mutated VHL gene cannot handle these reactions and they are unable to destroy the formation of dimmers so that even in oxygenated areas, they have hypoxic behavior [16].

In general, it is mostly seen that single tumor is generated within one of the kidneys. Moreover, in some cases, more than one tumor formation can be found in one kidney [17].

World Health Organization (WHO) presents that RCC has four major histological types; clear cell RCC (80-90%), papillary RCC (10-15%), chromophobe RCC (4-5%), and collecting-duct (1%) [18,19]. The difference in these types depends on the histological and molecular genetic changes [17].

Several treatments have been applied in the treatment of RCC. Treatment type is selected depending on the tumor size and tumor location, its local and distant spread and finally the effect of tumor to renal function. Various treatments of RCC can be classified as; (i) adjuvant therapy, (ii) surgery/laparoscopic surgery, (iii) therapeutic approaches (as an alternative to surgery), (iv) surgical treatment of metastatic RCC (tumor nephrectomy), (v) surveillance following radiotherapy for metastases in RCC, (vi) radiotherapy, and (vii) surveillance after nephrectomy [9, 20].

One of the most useful and efficient prognostic factors for RCC is grading. TNM (Tumor, Node, and Metastasis) is commonly used not only for RCC but also for other carcinoma types. TNM specifies the anatomical extension of tumor and tumor diffusion into the lymph nodes. Distant metastasis is another prognostic factor commonly used in RCC prognosis. Another grading system being used in RCC is called Fuhrman Nuclear Grading System. This system locks on the morphology of nucleus. It measures tumor nuclear size, shape and also it can detect nucleoli at 20 microns diameter, too and performs grading in

RCC. In recent years, studies reported that TNM System is much more useful and reliable than Fuhrman Nuclear Grading System in the prognosis of RCC [5].

1.4. INTEGRINS

Integrins exist in a wide range of organisms, from sponge to mammals. They are obligate heterodimers having α and β subunits. So far, 19 α and 8 β subunits have been reported in mammals. Integrins are responsible for cell adhesion, and cell signaling required for the cell survival through the interaction with extracellular matrix (ECM) ligands. Integrins are cell surface receptors interceding cell-cell and cell-ECM adhesions [21].

In tumor cell de-differention phase, integrin family receptors have a key role as cell adhesion receptors. Moreover, they play a role in tumor cell attachment to the tumor stromal ECM and to the basement membrane of capillaries and vessels during cancer metastasis and colonization. Accumulating data implicated a strong correlation between aberrant TG2 and β1/β5 integrin expression for tumor cell migration, invasion and drug resistance leading to the development of metastatic phenotype [22, 23]. In order to mediate cell-cell and cell-matrix interactions, integrins work together with other cell adhesion molecules such as cadherins and syndecans. Integrins on the cell surface, bind to the ECM ligands such as vitronectin, fibrinogen, fibronectin, collagen, and laminin [24].

Integrins are divided into three functional groups as (i) vascular integrins, (ii) cell-cell adhesion integrins and (iii) cell-ECM adhesion integrins. Integrins with β1 subunit form the majority of receptors that recognizes the ECM ligands [25, 26]. As the anchorage of the cell to ECM is critical for cell survival, the binding of the integrin to its specific ECM ligand generates not only a linkage with the cell cytoskeleton, but also leads the production of wide range of transduction signals affecting the gene expression, cell shape and behavior [27].

Integrins have the ability to change the cellular behavior by both recruiting and activating the signaling proteins. Non-receptor tyrosine kinase family including focal adhesion kinase (FAK) [28] and proto-oncogene tyrosine-protein kinase c-Src (which form a dual kinase complex with FAK) [29] are the examples of signaling proteins in interaction with

integrins. When integrin β1 binds to its ECM ligand, it immediately recruits and activates FAK. Activated FAK not only activates phosphotidylinositol-3 kinase (PI3-K) via Src and/or an IRS and lead the activation of Akt (protein kinase B), but also may directly recruit Grb2-Sos (or indirectly via Src) and stimulate the Raf/MEK/Erk (extracellular signal-regulated kinases) pathway via activating the small GTPase Ras. The Raf/MEK/Erk pathway then likewise functions in the cell survival pathway [27]. As the FAK–Src complex is formed, it can either bind to various adaptor proteins (such as p130Cas and paxillin) or phosphorylate them. To activate FAK and/or Src, various integrin regulated linkages exist in normal cells [30]. As FAK–Src becomes activated, the complex promotes cell motility, cell survival, and cell cycle progression. FAK and Src also play a role in promoting tumor angiogenesis and metastasis. Studies demonstrated that the FAK–Src complex is found to be activated in many tumor cells. It is known that signals sent from the FAK-Src complex lead to tumor growth and metastasis [31]. One of the most important targets in integrin-mediated signaling is actin stress fiber formation. FAK-Src complex activity activates p130Cas–Crk complex leading to lamellipodium formation by promoting Rac GTPase activity in migratory cells. Phosphorylation of paxillin kinase linker (PKL), paxillin and p130Cas by FAK–Src complex directly affect the focal adhesion formation as well as affecting the focal adhesion turnover rates. FAK and Src catalytic activities promote gene expression changes in VEGF and proteases such as urokinase plasminogen activator (uPa) and matrix metalloproteinases (MMPs). MMP-2, MMP-9 and uPA secretion rates are promoted to be increased when extracellular signal regulated kinase 2 (ERK-2) and c-Jun N-terminal kinase (JNK) are elevated. Therefore, activation and the localization of proteases at the leading edge of the migratory tumor cells promote matrix degradation and mediated by integrins. In other words, FAK–Src signaling events regulate cell invasion/metastasis dependent on a linkage to integrins [31].

Actin stress fibers in the context of integrins requires integrin clustering and integrin occupancy. After the the FAK/Src complex is formed, PAK is activated by Cdc42/Rac, it binds to PIX. Bounded PAK to PIX then associates with PKL- paxillin [32, 33]. Therefore, it can be concluded that activated PAK leads Rac induced cell adhesions. PAK phosphorylates proteins such as MLC and myosin light chain kinase (MLCK) that control adhesion complex assembly/ disassembly [34]. In other words, ligand binding to integrins promote integrin clustering in the cell membrane. This integrin occupancy then activates

Src and FAK and forms the FAK/ Src complex. Activated paxillin by FAK/Src complex induces the CAS and CRK activation and lead DOCK180 to get activated. Induced Rac by the activation of DOCK180 triggers both PAK and ARP2/3 to mediate the cell migration. ARP2/3 is the key mechanism for stress fiber formation. Mechanism suggest that FAK regulates the cell motiliy in both recruiting Src and activating the RAS pathway including actin polymerization via FAK/Src pathway. Integrin-signaling pathways also include Rho (small GTP-binding protein). Giry et al. showed that the activation of small Rho family of small GTPase (RhoA, RhoB and RhoC) by FAK stimulates actin stress fiber formation [35]. Association of actin and myosin units promotes actin stress fiber assembly. Stress fibers are placed along the base of the cell attached through its integrin-rich focal adhesions [36]. Although RhoA, RhoB and RhoC show nearly 80% similarity in their sequence, extensive majority of studies on stress fiber signalling pathways focused on RhoA as being the major regulator of stress fiber formation [37]. Activated RhoA then induces ROCK/ROK protein kinases [38] and mDia1 (diaphanous-related formin) [39]. RhoA-mediated ROCK-1 and ROCK-2 (serine/threonine kinases) functions in prominent stress fibre formation [40]. ROCK phosphorylates MLC and inhibits myosin phosphatase. As the myosin phosphatase is inhibited, MLC phosphorylation increases and leads to also an increase in MLC phosphorylation leading to actomyosin contractility. On the other hand, to support the stress fiber formation [37]. Moreover, it has been shown that the association of integrins with TG2 on the cell surface mediates the FAK/Src, p190RhoGAP activities leading the up-regulation of the activation levels of RhoA GTPase and its target ROCK, respectively. The activation pathway promotes the increased focal adhesion formation, actin stress fiber formation and additionally increased actomyosin contractility in the TG2 expressing cells [41].

1.5. SYNDECANS

One of transmembrane heparan sulfate proteoglycan family is the syndecans consisting of a transmembrane core protein covalently attached to unbranched heparan sulfate polysaccharide chains. Syndecans (SDCs) bind extracellular ligands both through their covalently attached heparan sulphate chains and core proteins [42]. They play a role in various cellular functions such as cell proliferation, cell-cell and cell-matrix adhesion, growth-factor signaling, and anticoagulation [42]. Studies implicated that during wound

repair of mice, Syndecan1 (SDC1) and Syndecan4 (SDC4), expression was induced, explaining the regulating property of SDCs in cell proliferation and migration [43]. Syndecans can function as co-receptors or activators for molecules such as growth factors and components of the matrix [44].

Only ubiquitously expressed member of the syndecan family is SDC4 which can be expressed in cells from different origins. ECM proteins recognize the heparan sulfate chains of SDC4 and recognized SDC4 acts a co-receptor for integrins leading to cell adhesion via promoting mitogen-activated protein kinase (MAPK) signaling pathways [45, 42].

Moreover, SDC4 can bind to fibronectin (FN) and contribute the integrin-based adhesion by acting as an accessory signaling molecule via activating protein kinase C α (PKCα) and Rho. Thus, it plays a role in the formation of focal adhesions and stress fibers with ITGB1 by interacting with different domains of FN [46, 47]. SDC4 studies also showed that SDC4 associates with focal adhesions containing ITGB1 or ITGB3 subunits, and those that formed on substrates of LM, FN, Col1 and vitronectin [48]. In recent year work from our laboratory demonstrated TG2 as another binding partner for SDC4. Upon binding to FN, TG2 is recognized by SDC4 as an adhesion protein and intercede an RGD-independent cell adhesion and survival signaling cooperatively with the ITGB1 [49, 50, 51]. Recently, Bass et al. indicated that SDC4 stimulates the integrin trafficking in fibroblast cells by acting as an integrin regulator [52]. Cell migration requires the stabilization of integrin-containing adhesions, cycles of cytoskeletal contraction (as late-phase events) and membrane protrusion, respectively [53]. Therefore, while indicating the activation of RhoG, Rac1 and reorganization of β1 integrin in the early phase of migratory pathway, Bass et al. also classified SDC4 as the first sensor for fibronectin initiating the cell migration of fibroblasts that SDC4 recruits the integrin trafficking [52].

1.6. TISSUE TRANSGLUTAMINASE

1.6.1. Transglutaminases

Transglutaminases are broadly disseminated protein family found in vertebrates, invertebrates, micro-organisms, and plants. Transglutaminases are mostly found in many tissues and body fluids of vertebrates [54]. Transglutaminase enzymes catalyze the transfer of acyl groups to primary amino groups of several compounds and can serve as acyl acceptor substrates. Acyl donors are the glutamine side chain of proteins as well as hydroxyl groups of T-hydroxiceramides [54, 55]. Transglutaminases are also called cross-linking enzymes as they generate ester bonds between glycine side-chains of proteins and hydroxylipids or isopeptide crosslinks between glycine and lysine side-chains of proteins. Transglutaminase enzymes crosslink and stabilize protein or protein-lipid assemblies by implication [56].

In ε-(γ)-glutamyl crosslinking reactions transglutaminase enzymes perform post-translational modifications of other proteins by forming an isopeptide bond between ε-amino group of lysine residue and γ-carboxamide group of glutamine residue of the proteins. Formation of isopeptide bond resists to proteolytic and mechanical degradation [5].

In amine incorporation reactions, polyamines and primary amines both serve as substrates for transglutaminases. At the end of the reaction, (γ- glutamyl)-polyamine bond formation occurs by the incorporation of glutamine into poly/primary amine resulting in a polypeptide-bound glutamine and a primary amine [55].

Studies also demonstrated that TGs remove the amide group from the glutamine residue of proteins and lead to the hydrolysis of amides. Furthermore, it is found that tissue transglutaminase (TG2) and Factor XIIIa show isopeptidase activity and play role in the hydrolysation of ε-(γ-glutamyl) lysine isopeptide bond, demonstrating the role of transglutaminases in both catalyzing and breaking of ε-(γ-glutamyl) lysine bonds [5].

All tissue transglutaminase types are Ca^{+2} dependent, in a 9-membered family (seven of them were characterized at protein level) that is composed of examples for transglutaminases are; TG in keratinocytes (TG1), ubiquitously expressed tissue TG (TG2), epidermal TG in epidermis (TG3), prostate TG in prostate (TG4), and TGX (TG5) epithelial tissue, blood coagulation factor XIIIa in plasma, circulating zymogen factor (FXIII) and catalytically inactive erythrocyte band 4.2. These proteins are encoded by different genes and being heterogeneous in size in the range between 80-110 kDa [57].

1.6.2. Transglutaminase 2 Activity

TG2 is a mammalian enzyme that is expressed ubiquitously such as nucleus, mitochondria, cytoplasm, cell surface and extracellular matrix (ECM). In mammals, multiple types of tissue transglutaminases have been identified. TG2 is a unique member of the transglutaminase family. TG2 is a multi-functional protein with distinct catalytic activities.

TG2 is transcripted by the TGM2 gene which is 32.5 kb located on chromosome 20 with 13 exons and 12 introns [58].

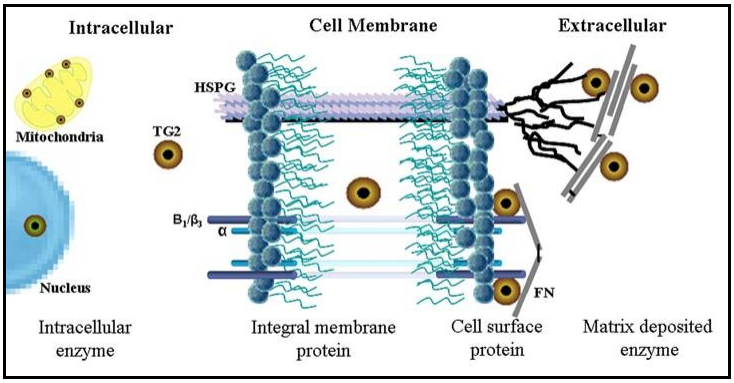

Figure 1.3. Presence of TG2 in cell and out of the cell [59]

It is known that TG2 is involved in many biological disorders such as cancer to neurodegenerative diseases. Studies have been reported that transamidating activity of TG2 is an essential molecule for the cancer cell survival [60]. Unlike most of the other transglutaminase family members, TG2 can exist in both intracellular and extracellular spaces of tissue. As shown in Figure 1.3. intracellular TG2 is mostly seen in the cytosol of

the cell. On the other hand, smaller amounts of TG2 also exists in nucleus part and located within the organelle, mitochondrion. In addition, intracellular TG2 plays a role in apoptosis by interacting with retinoblastoma protein in the nucleus to protect cell against apoptosis. The enzyme can also deposited out from the cytosol by passing through the cell membrane. The mechanism has not been explained and characterized, yet [61, 62]. In the extracellular space, TG2 plays a role in cell adhesion, ECM stabilization, cellular proliferation, wound healing, and cellular motility via crosslinking proteins.

1.6.3. Tissue Transglutaminase in Cancer

During tumor formation, there are several main important processes such as; cell adhesion, cell migration, angiogenesis, extracellular matrix (ECM) homeostasis. These processes are playing important role in both tumor formation and progression. Cancer is a multi-step process starting as a small mass of cells forming a primary tumor through tumor progression and tends to form metastatic tumors after accumulating the necessary mutations. Cells can exist in ECM, attaching to ECM components or exist as attached one cell to another. In order to have the main biological process such as; adhesion, signaling, cell survival and proliferation; normal cells are in need to attach ECM. Inability of attaching into ECM leads an impairment cell proliferation and induces tumor formation and also increases invasive and metastatic potential of the cells [63].

1.6.4. Tissue Transglutaminase and Cell Adhesion/Migration

Cell-cell and cell-extra cellular matrix (ECM) adhesion is essential for a multi-cellular existence (cell survival). Cell adhesion not only provides cell support via mediating cell positioning, but also provides micro environmental sensing. Cell adhesion to ECM involves signaling mechanisms which control the attachment, spreading and the formation of focal adhesions and stress fibers [64].

Although integrins and syndecans are the cell adhesion receptors interceding cell adhesion, their functional contributions to cell–ECM interactions are not well known [65].

TG2 is a multi-functional protein that involves several biological processes. Studies demonstrated that TG2 can exist in both intracellular and extracellular spaces of tissue. TG2 in extracellular space of tissue can act a signaling molecule. TG2 stabilizes extracellular matrix (ECM) via crosslinking. Hereby, TG2 promotes cell-matrix interactions [9]. Externalized TG2 plays a key role in cell-ECM interactions, cell survival, cell adhesion, cell spreading, and cell migration via its transamidating activity or novel cell adhesion protein function by activating β1 integrins and syndecan-4 (SDC4) [66]. As TG2 come out from the cell membrane it can interact with β1 or β3 integrins (ITGB1, ITGB3) in association with fibronectin (FN). Therefore, TG2 on the cell surface can serve as co-receptor for FN independently from its transamidating activity [60]. FN is a glycoprotein and one of the main components in ECM that plays a role in embryogenesis, angiogenesis, and wound healing [67]. FN binds to αIIBβ3 and α5β1 integrins via its RGD (consists of arginine,glycine and asparagine) region. TG2 and FN generate a complex that is recognized by cell surface heparin sulfate proteoglycans. This association induces cell adhesion and survival by implication. [60, 66, 68].

Jones et al studied on ECV304 cell line to investigate the function of TG2 enzyme on cell adhesion using anti-oligonucleotides. Study indicated that the cell adhesion potential of the cells and FN polymerization were reduced when TG2 was down-regulated in these cells [56]. A study reported that TG2 manipulates cell adhesion and cell spreading independently from its catalytic activity [69]. In another study, the role of TG2 as a novel cell-surface adhesion protein in cell motility as well as its matrix-crosslinking ability was demonstrated [70]. It is found that via regulation of phospholipase C activity, TG2 controls the dynamic adhesion formation in cell spreading and cell migration. Due to the crosslinking activity of TG2, it plays an important role in regulating ECM remodeling [70]. Membrane associated TG2 on the cell surface, can also be implied as an active exo-enzyme, managing the catalysis of crosslinkages in ECM [71]. It is also found that TG2 activity on cell surface does not only crosslink fibronectin (FN) and collagen but also play a non-enzymatic role in ECM as an ITGB1 co-receptor [52]. Thus, TG2 supports RGD independent cell adhesion to FN [49, 60, 72, 73].

Cell motility is functionalized both by lamellipodia protrusion and contraction. A lamellipodium protrudes from the cell and attaches to the substrate when the cell becomes

polarized. Cell adhesion provides necessary traction to generate the pulling force and retraction of the tail [74]. The cells start to exert contractile force on ECM by integrins. It was suggested that actin stress fibers may provide contractile forces necessary for the cell migration in the leading edge of the cell [75]. On the other hand, actin stress fibers are found to be more evenly distributed in the cortex of stationary (primary) cells [53, 76], suggesting that non-polarized stress fibers might inhibit cell migration [77].

1.7. AIM OF THE STUDY

The aim of the study is to investigate and demonstrate the importance of TG2 in RCC. Until now, studies showed that decreased TG2 expression and also decreased TG2 activity was found in primary tumors resulting in the generation of a more destabilized ECM necessary for the primary tumor growth. On the hand, increased TG2 expression and activity in addition to the increased integrin β1 has been reported to cause drug resistance and increased metastatic potential in tumor cells [49, 60, 61, 78, 79].

In the light of these accumulating evidence and previous data from our laboratory, this study is designed to investigate the effect of TG2 in cell adhesion and cell migration in renal cell carcinoma (RCC). In order to demonstrate the effect of TG2 on cell adhesion and cell migration in RCC, *in vitro* model primary A-498 and Caki-2; and metastatic Caki-1 and ACHN RCC cell lines were used.

2. MATERIALS

2.1. INSTRUMENTS

- Inverted Phase Contrast Microscope (Nikon Eclipse TS-100)
- Flourescence Microscope (Nikon 80i Eclipse Fluorescence Microscope)
- Laminar Flow Cabinet (ESCO Labculture Class II Biohazard Safety Cabinet Type 2A)
- CO_2 Mammalian Incubator (Nuaire NU5510/E/G)
- Centrifuge (Hettich Mikro 22R, Germany and SIGMA 2-5 centrifuge)
- pH meter (Hanna Instruments PH211)
- Vortex (Stuart SA8)
- Mini- PROTEAN Tetra Cell Elecrophoresis System (Bio-Rad 165-8001)
- Chemiluminescence (ChemiDoc XRS+) Imaging System (Bio- Rad)
- -80^{o} C freezer (Thermo Forma -86C ULT Frezer)
- Sonicator (Bandelin Sonopuls)

2.2. EQUIPMENTS

- T-25, T-75, T-150 Cell Culture Flasks (TPP)
- 6- well, 24- well plates (Grenier-Bio)
- Cryovials (TPP)
- Micro pipettes 1000- 200- 10µl (Expell)
- Centrifuge Tubes 50-15 ml (Isolab)
- Centrifuge Eppendorfs 2- 1 ml (Isolab)
- Serological Pipettes 25-10-5-2 ml (Axygen)

2.3. CHEMICALS

Cell Culture Media:

- Dulbecco's Modified Eagle's Medium, high glucose (Sigma D6429 and Gibco 41966)
- McCoy's 5A Medium (Thermo Scientific SH 302000.01, USA and PAN Biotech P04-00501)

Growth Supplements:

- Fetal Bovine Serum (FBS) (Sigma F9665)
- Non-Essential Amino Acid (NEAA) (Sigma M7145)
- 2- Propanol (AppliChem A3928)
- Absolute Ethanol (AppliChem A3928)
- Acrylamide/ Bis-acrylamide (29:1) (Sigma A3574)
- Ammonium Persulfate (Sigma A3678)
- Bovine Serum Albumin (Sigma A7030)
- Dulbecco's Phosphate Buffer Saline (DPBS) (PAN BiotechP04-53500)
- Ethylenediaminetetraacetic acid (EDTA) (Merck 108418)
- Fibronectin from Human Plasma (Sigma F0895)
- Collagen Type-I from Human Placenta (Sigma C7774)
- Laminin from Human Placenta (Sigma L6274)
- Glycine (Merck 104169)
- Mounting Reagent (Santa Cruz Biotechnology)
- N,N,N',N'- Tetramethylethylenediamine (TEMED) (Sigma T7024)
- Paraformaldeyde (Merck)
- Penicillin- Streptomycin (Pen/Strep) (Thermo Scientific SV 30010)
- Phalloidin FITCH Labelled (Sigma P5282)
- Tris-base (Merck 108387)
- Tris-HCl (Merck 108219)
- Triton-100X (Biomatik Corporation)

- Trizol (Invitrogen 15596-018)
- Trypsin-EDTA (Biochrom L2153)
- Tween-20 (Merck 822184)

2.4. KITS and SOLUTIONS

- Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare RPN2135)
- Amersham Hybond- ECL Nitrocellulose Membrane (GE Healthcare RPN303D)
- Amersham Rainbow Protein Marker (GE Healthcare RPN800E)
- Bovine Serum Albumin, Protein Standard (Sigma P0834)
- RIPA Lysis Buffer (Santa Cruz sc-24948)
- Hs_TGM2_1 FlexiTube siRNA (20nmol/tube) (Qiagen SI00743715)
- Hs_TGM2_6 FlexiTube siRNA (20nmol/tube) (Qiagen SI03055465)
- AllStars Negative Control siRNA (20nmol/tube) (Qiagen 1027281)
- RNAifect Transfection Reagent (Qiagen 301605)

2.5. ANTIBODIES

2.5.1 Primary Antibodies

- Mouse- anti-TG2 Antibody (Thermo Scientific, Labvision Cub 7402, MS-224-P)
- Mouse- anti- Integrin β1 Antibody (Santa Cruz, sc-8978)
- Rabbit- anti- Syndecan-4 Antibody (Invitrogen)

2.5.2. Secondary Antibodies

- Anti- rabbit IgG Peroxidase Conjugate (Sigma A0545)
- Anti- mouse IgG Peroxidase Conjugate (Sigma A4416)

2.6. CELL LINES

- A-498, Primary Human Kidney Epithelial Carcinoma, Adherent (ATCC Number : HTB-44)
- Caki-2, Primary Clear Cell Carcinoma of Human Kidney, Adherent (ATCC Number : HTB-47)
- ACHN, Metastatic Renal Cell Adenocarcinoma (Lung), Adherent (ATCC Number : CRL-1611)
- Caki-1, Metastatic Kidney Clear Cell Carcinoma (Skin), Adherent (ATCC Number: HTB-46)

3. METHODS

3.1. Cell Culturing Methods

3.1.1. Cells and Cell Culture Conditions

A-498, Caki-2, ACHN and Caki-1 cell lines were cultured periodically at 37ºC, 5% (v/v) CO2 and 95% (v/v) air. A-498 and ACHN cells were cultured according to the manufacturer's recommendations, in Dulbecco's Modified Eagle's Growth Medium (DMEM) containing 10% (v/v) FBS and 100 units/ml Penicillin and 100µg/ml Streptomycin, 0.1 mM MEM non-essential amino acid solution, whereas, Caki-1 and Caki-2 cells were cultured in McCoy's 5A Medium) containing 10% (v/v) FBS and 100 units/ml Penicillin and 100µg/ml Streptomycin, 0.1 mM non-essential amino acid solution. Cells passages were performed every 3 days as the confluency reaches to 70%.

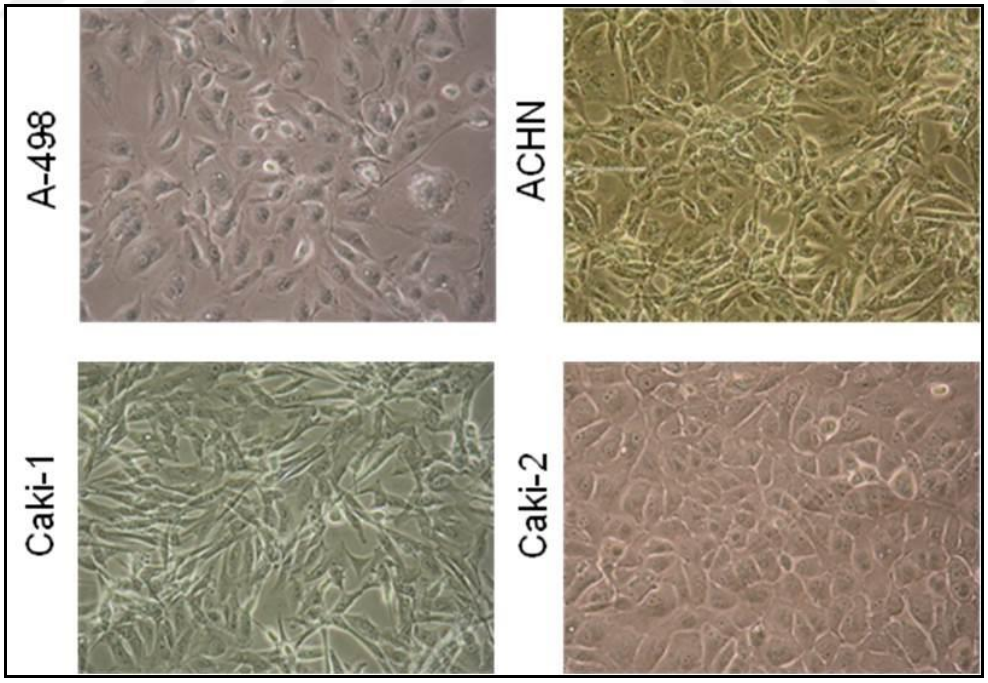

Figure 3.1. Images of RCC cell lines taken using the 20x objective after 48 hrs of incubation

Caki-1 cells demonstrated star-like long rectangular shapes, while Caki-2 cells showed a more spherical morphology. Furthermore, A-498 cells displayed stretched cubic forms and ACHN cells showed elongated structure similar to Caki-1 cell line.

3.1.2. Cell Passaging

Cell monolayer was washed with Phosphate Buffer Saline (PBS), pH= 7.4 and then treated with trypsin (0.5 g/L) in 2 mM PBS.EDTA solution, pH= 7.4 for 5 min in 37ºC mammalian incubator. In order to block the trypsin activity, cells were collected in 10% (v/v) Fetal Bovine Serum (FBS) at two times the volume of trypsin added and centrifugated at 300xg for 5 min. Supernatant was discarded and pellet was resuspended with complete media in order to get the intended dilution.

3.1.3. Determination of Cell Number

Following the centrifugation cell pellets were resuspended in growth media (Section 3.1.2). 10 µl aliquots of the suspension were placed in hemocytometer. Cells in the middle area were counted by four times using inverted microscope and then average number of cells was calculated. Cell number per milliliter is calculated according to the formula "counted number of cells x dilution factor/mm^2x chamber depth".

3.1.4. Cell Freezing

Freezing mix is prepared by mixing 10% (v/v) Dimethyl sulfoxide (DMSO) and 90% (v/v) FBS. Obtained cell pellets were resuspended in 1 ml of freezing mix per vial to be frozen. Vials were then aliquoted and placed on ice and taken immediately to -80ºC. After an overnight-incubation vials were transferred into liquid N_2 tank for longer storage.

3.1.5. Cell Thawing

Cryovial containing the frozen cells is taken out from the liquid nitrogen storage or – 80ºC. Cells were immediately thawed by gently swirling the vial at 37°C. Cell suspension was then placed into 15 ml Falcon and 5-10 ml of pre-warmed complete growth medium was

transferred onto the suspension drop wise. Cell suspension was centrifugated at 300xg for 5 min. Supernatant was decanted without disturbing the cell pellet and cells were resuspended with complete growth medium and transferred into the appropriate culture vessel (preferably T25). In the following day, media was changed for the removal of remaining DMSO. Cells were passaged when they reached to 70% confluency.

3.2. siRNA SILENCING

The target sequences of siRNA1 and siRNA6 on the mRNA of TGM2 gene are 5'-CCGCGTCGTGACCAACTACAA-3' and 5'- CACAAGGGCGAACCACCTGAA-3' siRNA Silencing master mix was prepared according to the data sheet of Qiagen. 19.23µl of 20nmol siRNA stock (Non-silencing, siRNA1 or siRNA6), 80.77µl of Buffer ECR, and finally 30µl of RNAifect was added into microfuge tube. The master mix was vortexed gently and then incubated at RT for 15 min in order to form a complex. The complex was given into 2% (v/v) FBS containing growth media on the cell monolayer in a drop wise manner. Cells were then incubated at 37^{o}C for 36 hours (hrs).

3.3. MEASUREMENT OF TG2 AND INTEGRIN B1 PROTEIN LEVELS IN RCC CELL LINES

3.3.1. Preparation of Total Cell Lysates

Cells were seeded into 6-well plates (A-498 and Caki-2 cells 200,000; ACHN and Caki-1 cells 300,000) and incubated overnight in media containing 10% (v/v) FBS. Following day, cells were brought to quiescent state by serum starvation and incubated at least for 16 hrs at 37^{o}C. Cell monolayer was washed once with PBS, pH 7.4 and cells were lysed in 30µl of complete RIPA (1X TBS, 1% Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.004 % sodium azide) lysis buffer containing 1% protease inhibitor cocktail, 1mM PMSF, and 1 mM sodiumorthovanadate. Cell lysates were collected by the help of a cell scraper into an eppendorf and sonicated on ice with a probe sonicator. Samples were stored at -80^{o} C.

3.3.2. Lowry Assay

BSA standards were prepared by serial dilution of 2 mg/ml BSA Solution at working range of 0.05-0.50 mg/ml. Determination of the protein content was performed by addition of 1µl unknown samples diluted in 4µl dH_2O into the wells of a microtiter plate. In order to generate a standard curve by 5µl from each BSA standard was also used. Wells were then incubated with 25 µl Reagent A and 200 µl Reagent B at dark in RT for 15 min. Absorbance values were then measured at 750 nm. Standard curve graph was plotted using the absorbance values of BSA standards vs their concentrations in order to calculate the protein content in the unknown samples. 30µl of protein samples were loaded into wells.

3.3.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Stacking gel and separating gels containing 8% or 10% (w/v) polyacrylamide were poured into cassettes in order to separate proteins. Preparation of the gels was performed according to the Table 3.1.

Table 3.1. SDS-PAGE polyacrylamide gel ingredients and concentrations

Stock Solutions	**Final % Acrylamide Concentration (w/v)**	
For Separating Gel	8%	10%
30% (w/v) acrylamide/ 0,8% bisacrylamide	4 ml	5 ml
1,5 M tris-HCl containing 0,4% (w/v) SDS, pH=8,8	3.75 ml	3.75 ml
dH_2O	7.25 ml	6.25 ml
10% (w/v) APS	0.05 ml	0.05 ml
TEMED	0.01 ml	0.01 ml
For Stacking Gel		

Table 3.1. SDS-PAGE polyacrylamide gel ingredients and concentrations (continue)

30% (w/v) acrylamide/ 0,8% bisacrylamide	0.65 ml	0.65 ml
0,5 M Tris-HCl containing 0,4% (w/v) SDS, pH=6,8	1.25 ml	1.25 ml
dH_2O	3.05 ml	3.05 ml
10% (w/v) APS	0.025 ml	0.025 ml
TEMED	0.005 ml	0.005 ml

3.3.4. Western Blotting

Proteins were separated on the gel by SDS-PAGE Method according to their molecular weight. Proteins on the gel were then transferred to nitrocellulose membrane (0.22µm and 0.45µm) using Western Blotting Technique. Nitrocellulose membrane was equilibrated in cold transfer buffer, pH=8.3 before the procedure. Sponges and filter papers were also placed into a box containing the ice cold transfer buffer, pH=8.3. Gel was removed from the glass plate and washed with dH_2O in order to rinse the SDS from the cells. Gel sandwich was then prepared (Figure 3.1.).

CASSETTE
Black Side
Sponge
2 Filter Papers
Gel
Nitrocellulose Membrane
2 Filter Papers
Sponge
Transparent Side

Figure 3.2. The arrangement of the transfer cassettes

The sandwich cassette was placed into the cassette holder provided that the gel side was facing to the cathode and the membrane side was facing to the anode. The cassette holder was then placed into the transfer tank filled with the ice cold transfer buffer and an ice box was also placed into the transfer tank to eliminate the heat generated during the transfer process. Western Blot Transfer was performed at 150mA for 90 min. Membrane was washed 3 times with TBS-Tween (TBS-T), pH=7.4 (100 mM Tris-HCL, 9% NaCl, 0,5% (v/v) Tween) for 15 min and blocked in the blocking solution composed of 5% (w/v) non-fat dry milk in TBS-T, pH=7.4 for 1 hr on shaker at RT. Blocking solution was renewed and primary antibodies against TG2 and ITGB1 in blocking solution was separately added onto the membranes. Membrane was then incubated with the primary antibody overnight at 4^0C on a shaker. 1:10.000 dilution was used for mouse monoclonal anti-TG2 antibody Cub7402, and 1:10.000 dilution for anti -ITGB1 antibodies. Membrane was then washed 2 times for 15 min with TBS-T and blocked with non-fat milk solution for 20 min at shaker Blocking solution was renewed and secondary antibodies were added. 1:10.000 dilution was preferred for anti-mouse peroxidase and 1:2000 for anti-rabbit peroxidase antibodies. Antibody added membranes were incubated for 2 hrs at shaker. At the end of the incubation time, nitrocellulose membranes were washed 3 times with TBS-T for 5 min and then membranes were equilibrated with PBS for 10 min at RT. TG2 band images were taken by Bio-Rad Molecular Imager ChemiDoc XRS+ using ECL-Amersham Imaging Solution.

3.4. ACTIN PHALLOIDIN STAINING

Phalloidin-FITC was dissolved in DMSO at 1 mg/ml and stored at -20^o C. Transfected cells (see Transfection section) were detached and counted. Cells were seeded in 8-well glass permanox-chamber slides (200,000cells/ml) and allowed to attach and spread. Medium was discarded and cell monolayer was carefully rinsed with PBS, pH 7.4 without detaching the cells from the surface. Cells were fixed using freshly made 3.7% (w/v) paraformaldehyde in PBS for 15 min at RT. Chambers were washed with PBS, pH 7.4 for 3 times for 5 minutes. Fixed cells were then permeabilized in 0.1% (v/v) Triton X-100 in PBS, pH 7.4 at RT for 15 min. After the washing steps, PBS buffer supplemented 3% (w/v) Bovine Serum Albumin (BSA) solution was added onto the sample wells and chamber was incubated at RT for 30 min. Actin stress fibers were stained using fluorescein

isothiocyanate (FITC)-labeled phalloidin. Phalloidin Stock solution was diluted in a ratio of 1:100 in 3% BSA and cell monolayer was incubated with 125µl/well of 10 µg/ml FITC-labeled phalloidin overnight in a dark humidified chamber at 4ºC. Wells were then washed with PBS, pH 7.4 3 times for 5 minutes and excess PBS on the slide was discarded. After removal of the plastic wells, a few drops of Mounting Reagent was applied on the slide and a rectangular cover-slip was placed on the slide at 45 degree angle and the edge of the cover slip was lowered slowly to avoid the formation of trapped bubbles. Excess mounting reagent was removed with a filter paper and edges were covered with a transparent nail-polisher. Slide was dried up for 5 min at RT and images were taken using Nikon 80i Eclipse fluorescence microscope. Slides were then stored at -20ºC.

3.5. PREPARATION OF β1-INTEGRIN SUBSTRATE MATRICES

3.5.1. Coating of Transwells and Plates with Fibronectin

Transwells were coated with fibronectin (FN) diluted in 50 mM Tris-HCl, pH 7.4. at 5µg/ml concentration. Coated transwells were incubated at 4ºC overnight or at 37º C for 90 min. At the end of the incubation, excess amount of FN was removed and washed for once with 100µl of 50 mM Tris-HCl.

3.5.2. Coating of Transwells and Plates with Collagen Type-1

Transwells were coated with collagen type-1(Col1) at 40µg/ml concentration dissolved in SFM (Serum Free Media) DMEM. Coated transwells were incubated at 37º C for 2 hrs. At the end of the incubation, excess amount of Col1 was discarded and washed once with PBS, pH 7.4.

3.5.3. Coating of Transwells and Plates with Laminin

Transwells were coated with 10µg/ml laminin (LM) diluted in PBS, pH 7.4. Coated transwells were incubated at 37º C for 2 hrs. At the end of the incubation excess amount of LM was discarded and washed once with PBS, pH 7.4.

3.6. Determination of Invasive Potentials of RCC Cell Lines on Integrin B1 Ligands by Transwell Method

Due to size difference among the cell lines, A-498 and Caki-2 cells were seeded 200,000cells/well while Caki-1 and ACHN cells were seeded 300,000cells/well in 6-well plates in complete growth medium and incubated overnight at 37^{o}C. Following day, cells were incubated with SFM for at least 16 hrs to achieve quiescent state in cells. At the end of the incubation, cells in 2% (v/v) FBS containing media were transfected by siRNAs directed against TG2. siRNA transfection was performed as described in Section 3.2. Depending on the real-time analysis performed before, siRNA1 and siRNA6 were determined to be the most effective in silencing the TG2 expression in all the RCC cell lines. The complex was added in a dropwise manner into the media covering the cell monolayer. Cells were then incubated at 37^{o}C for 36 hrs. After incubation time, cells were trypsinized and counted. A cell suspension containing 325,000cells/ml was prepared in AIM-V media and 200µl from this cell solution was seeded into transwells coated with FN, Col1 and LM. Transwells were then incubated at time points of 3 and 6 hrs for FN, 6 and 10 hrs for Col1 and finally 10 and 14 hrs for LM in order to investigate the invasive potentials of RCC cell lines.

3.7. Cell Migration Potentials via Wound Scratch Assay

A-498 and Caki-2 cells were seeded 200,000 cells/well; Caki-1 and ACHN cells were seeded 300,000 cells/well in 12-well plates in complete growth medium and incubated overnight at 37^{o}C. Following day, complete medium was replaced with SFM for 16 hrs in order to achieve quiescent state. At the end of the incubation, cells were transfected with siRNA1 and siRNA 6 (Section 3.2). Overnight incubated transfected cells were then scratched using a tip along one direction. This time point assumed as T=0 hr. RCC cell lines were incubated for 48 hrs and every 24 hrs images were taken. 10 non-overlapping digital images covering the central area of each well were captured at 4X magnification at T=0, T=24 and T=48 hrs using ECLIPSE TE200 fluorescence microscope.

3.8. STATISTICAL ANALYSIS

All data were presented as the mean ± standard error (S.E.). Statistical analysis was performed using One-way ANOVA with Tukey post test was performed using GraphPad Prism version 5.03 for Windows, Graphpad Software, San Diego California USA, and Image J Program. P- values < 0.05 were considered statistically significant.

4. RESULTS

4.1. TG2 and INTEGRIN β1 PROTEIN LEVELS IN RCC CELL LINES

In order to investigate the TG2 and ITGB1 protein expression levels in RCC cell lines, cell were seeded at densities as mentioned before (Material Methods Section 2.4.) RCC cell lysates were collected and used in the detection of TG2 and ITGB1 via Western Blotting. β-actin was used to ensure the equal loading of each sample. Rabbit polyclonal anti-β1 integrin antibody (1:1000 dilution) was used to detect β1 integrin bands, mouse monoclonal anti-TG2 antibody (1:1000 dilution) was used to detect TG2 bands, and finally mouse monoclonal anti β-actin (1:1000 dilution) was chosen for the β-actin detection via Western Blotting.

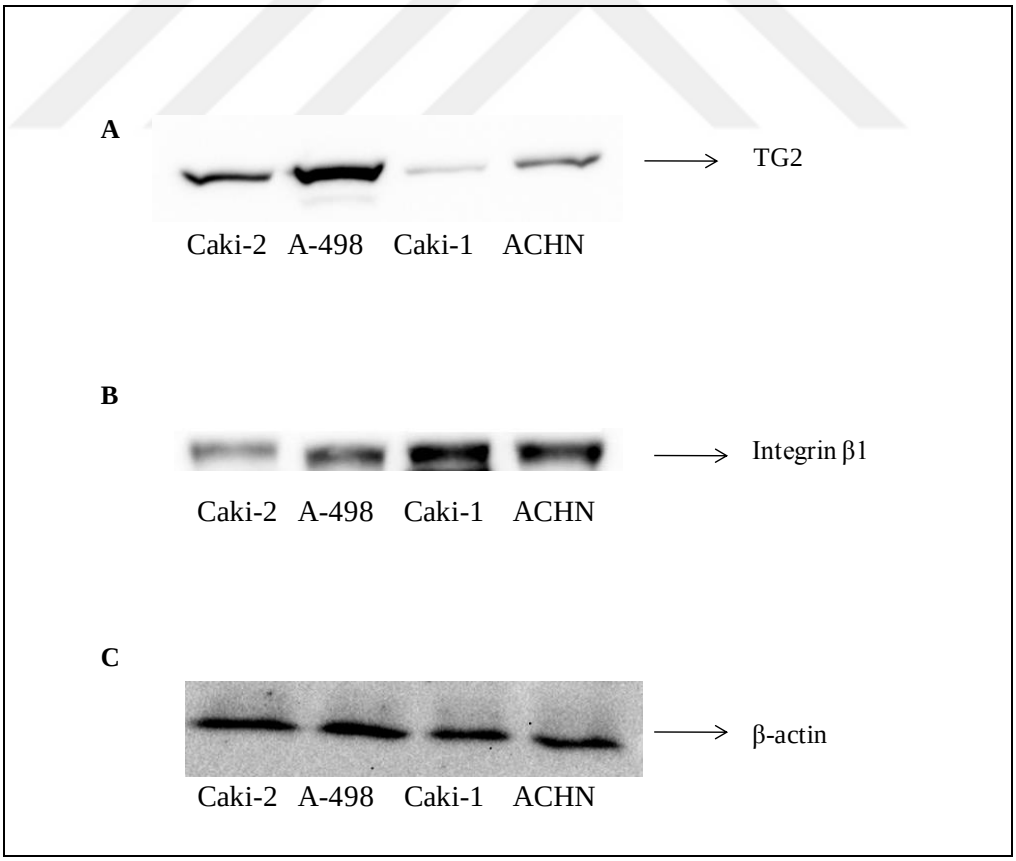

Figure 4.1. Western blot analysis in RCC cell lines
A. TG2, **B.** Integrin β1 and **C.** β-actin blots

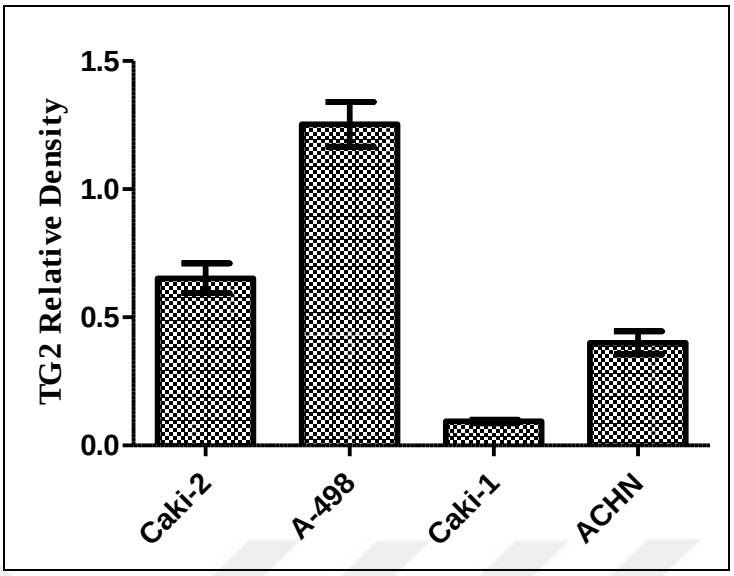

Figure 4.2. Relative Density for TG2 in RCC cell lines. Normalization was done by dividing the TG2 signal by that of actin within each experiment. The results are representative of three independent experiments

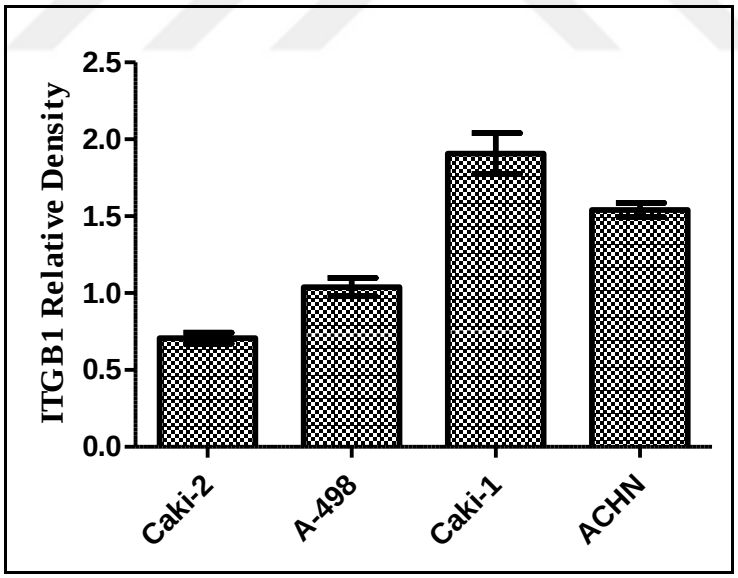

Figure 4.3. Relative Density for ITGB1 in RCC cell lines. Normalization was done by dividing the ITGB1 signal by that of actin within each experiment. The results are representative of three independent experiments

4.2. DOWN REGULATION OF TG2 EXPRESSION IN RCC CELL LINES

Cells were treated with siRNA1 and siRNA6 separately in order to silence TG2 expression for 36 hrs. The first and second lanes show TG2 protein level in non-treated control and in non-silenced samples, respectively. Third lane indicates the siRNA1 silenced TG2 protein levels. Moreover, the fourth lanes which were the last ones, demonstrate the siRNA6 silenced TG2 protein level in that specific RCC cell line. The histogram was produced as introduced above (Figure 4.4.-4.5.-4.6.-4.7.).

Western Blot Analysis demonstrated a distinct reduction in TG2 protein level in siRNA1 and siRNA6 transfected RCC cell lines. The treatment of cells with NS siRNA did not lead any significant change in the levels of TG2 when compared to that of the control. siRNA silencing reduced TG2 protein level in A-498 cell line by 57% using siRNA1 and by 42% using siRNA6. siRNA treatment was most effective on Caki-1 cells in that 92% and 83% reduction was obtained in TG2 protein levels after siRNA1 and siRNA6 transfection, respectively. TG2 protein expression was decreased by 72% using siRNA1 and by 70% using siRNA6 in Caki-2 cells. Finally, a significant 84% decrease in TG2 protein expression was found in ACHN cells following siRNA1 transfection while a 64% reduction was recorded for siRNA6 treatment. Results confirmed the efficacy of siRNA silencing in TG2 expression of the selected RCC cell lines (Figure 4.4.-4.5.-4.6.-4.7.).

TG2 protein expression levels were shown to be elevated in primary RCC cell lines (Caki-2 and A-498) compared to metastatic RCC cell lines (Caki-1 and ACHN). A-498 cells demonstrated the highest TG2 protein level among four RCC cell lines. A-498 cells expressed approximately 2-fold higher levels of TG2 than Caki-2 cells ($P<0.05$) while metastatic ACHN cells expressed TG2 protein 4.2-fold higher than Caki-1. Primary Caki-2 cells expressed 7-fold higher TG2 protein than metastatic Caki-1 ($p<0.05$) (Figure 4.1.-4.2.)

On the other hand, ITGB1 protein expression levels were found to be higher in the metastatic cell lines than the primary cell lines. The highest ITGB1 protein level was found in Caki-1 which was 1.2-fold higher than that of ACHN ($P<0.05$). A-498 cells

demonstrated a 1.4-fold higher ITGB1 protein level compared to Caki-2 ($P<0.05$) (Figure 4.1.-4.3.).

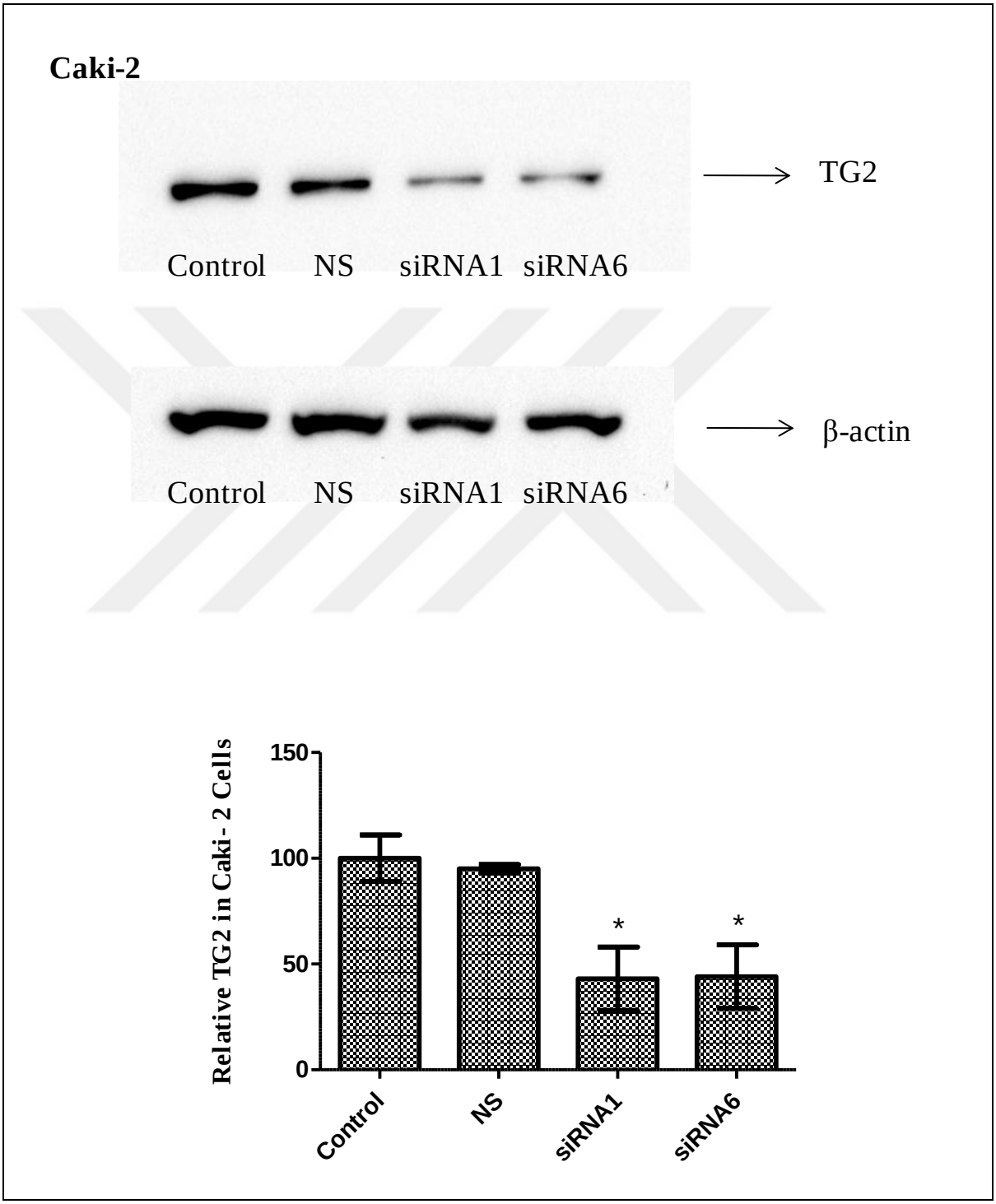

Figure 4.4. TG2 protein expression levels in Control, NS, siRNA1 and siRNA6 treated Caki-2 cells via Western Blot Analysis. *P < 0.05 shows the significant difference versus control

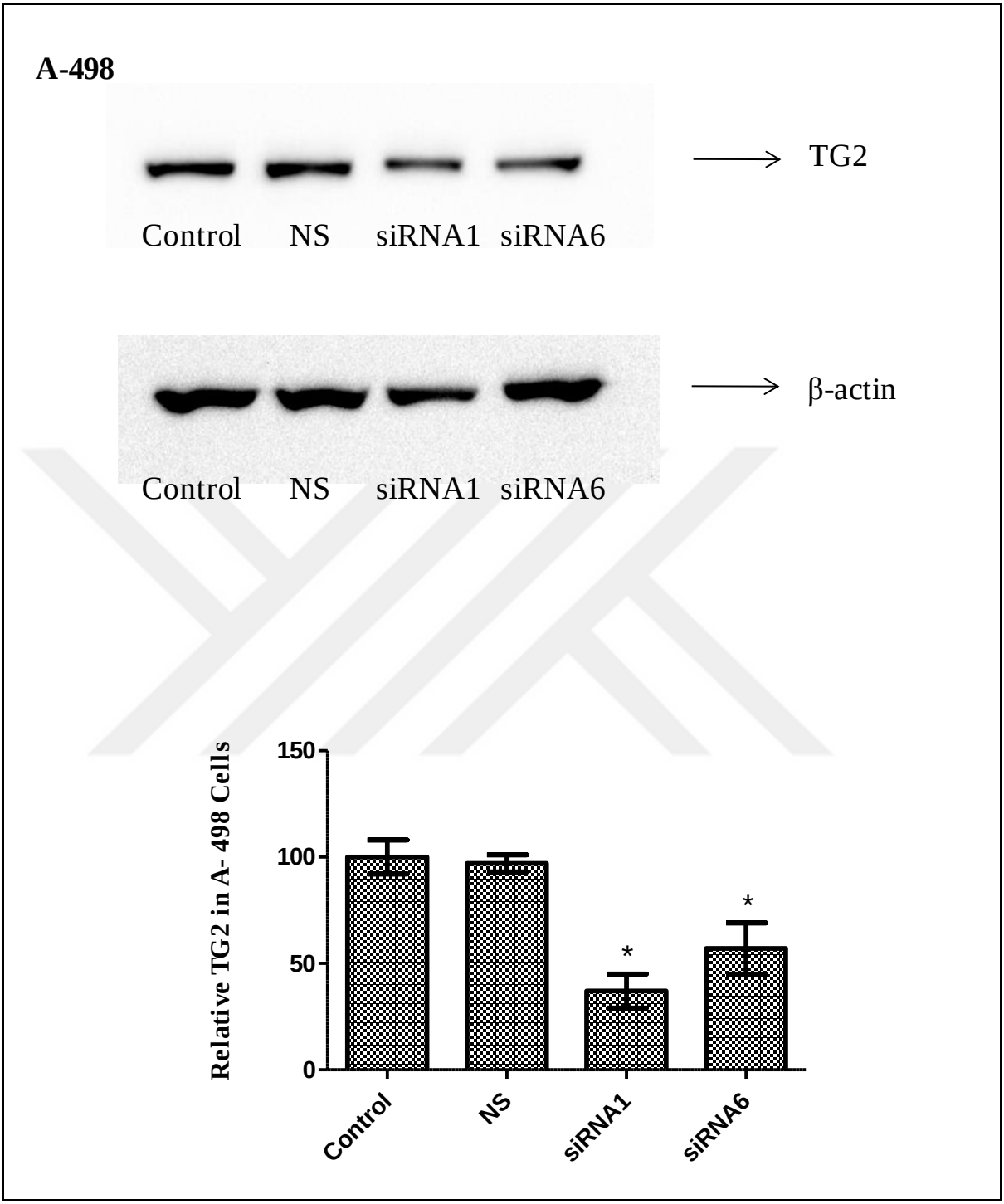

Figure 4.5. TG2 Band Percentage in Control, NS, siRNA1 and siRNA6 treated A-498 cells via Western Blot Analysis. *P < 0.05 shows the significant difference versus control

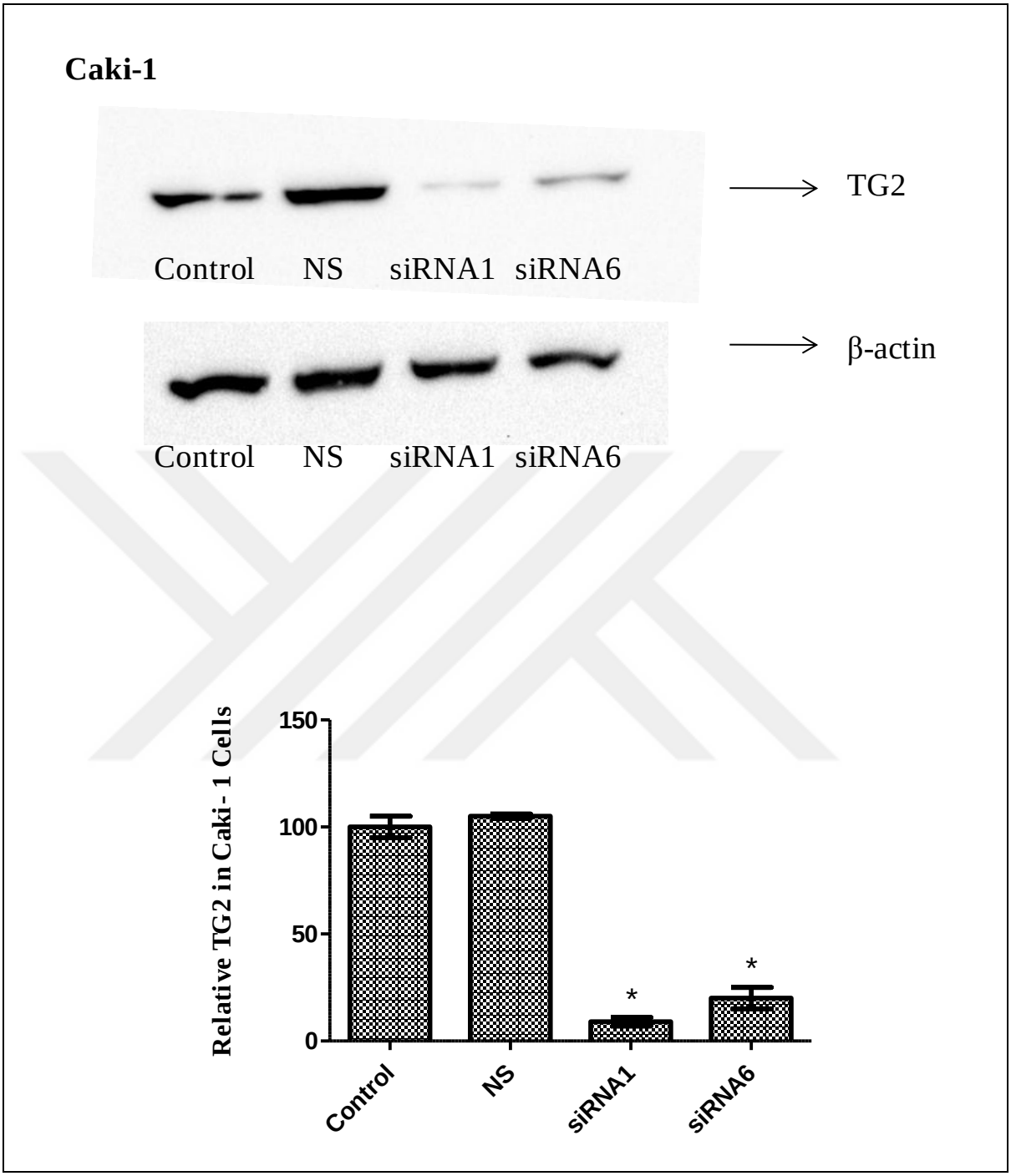

Figure 4.6. TG2 Band Percentage in Control, NS, siRNA1 and siRNA6 treated Caki-1 cells via Western Blot Analysis. *P < 0,05 shows the significant difference versus control

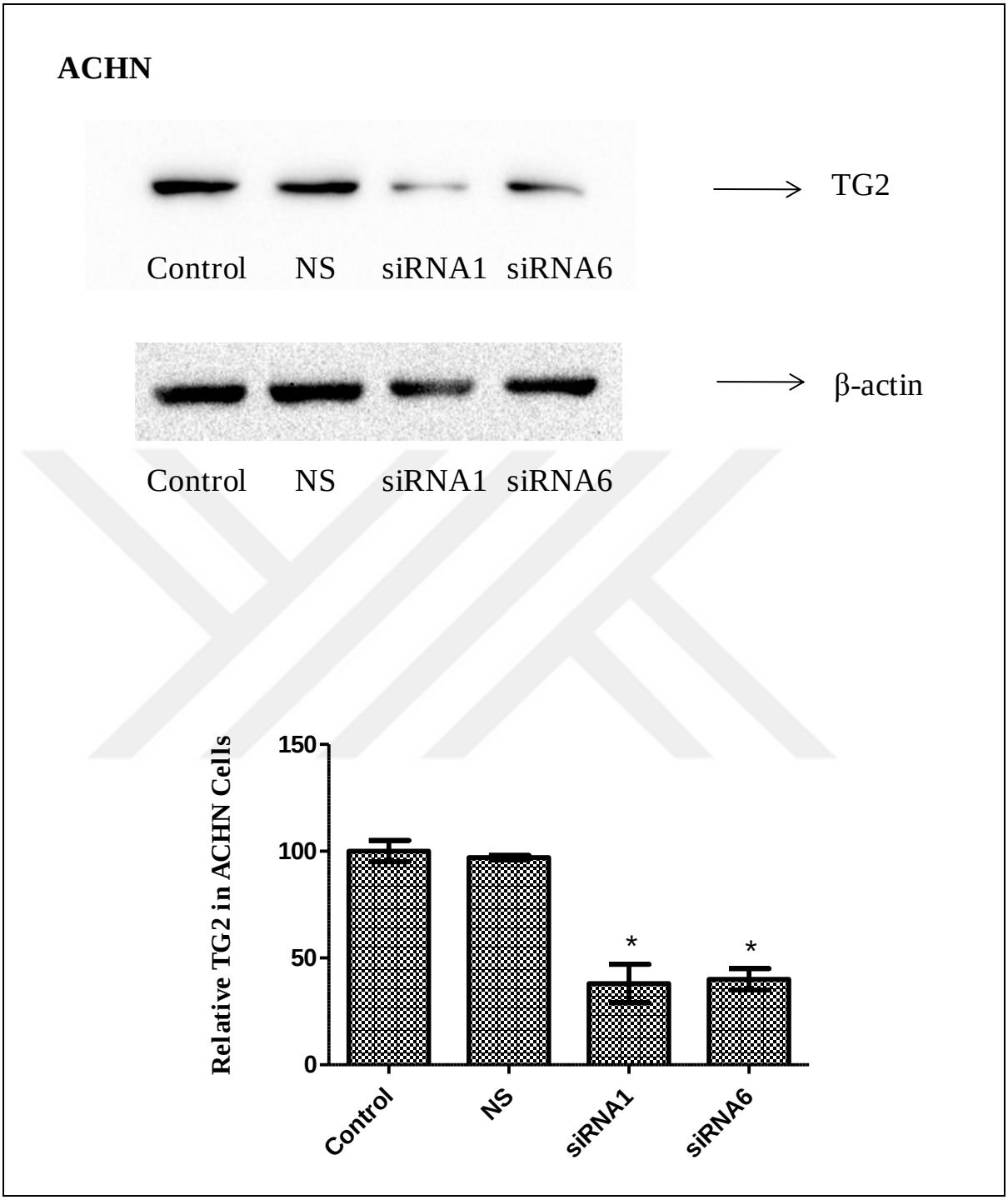

Figure 4.7. TG2 Band Percentage in Control, NS, siRNA1 and siRNA6 treated ACHN cells via Western Blot Analysis. *P < 0,05 shows the significant difference versus control

4.3. EFFECT OF TG2 DOWNREGULATION ON ACTIN STRESS FIBER FORMATION

The effect of TG2 on the cell adhesion and actin stress fiber formation was analyzed by fluorescence microscopy utilizing FITC-phalloidin. NS (Non-silencing RNA) was used as a negative control meanwhile siRNA1 and siRNA6 were used as silencing RNA against TG2 gene expression. Stained actin stress fibers were visualized at 100X magnification as described in section Images were a representative of three separate experiments.

In general, all control groups of four RCC cell lines displayed perfect cell adhesion resulting in dense appearance of actin stress fibers with a flat morphology and sufficient actin stress fiber formation. Actin stress fibers formed both in control and NS groups of cells were seemed to be elaborated and well formed. Although siRNA1 and siRNA6 treated RCC cell lines were attached, the cells failed to form actin stress fibers or did form less dense stress fibers.

Primary RCC cell line Caki-2 appeared to be more sensitive to TG2 down regulation compared to the other RCC cell lines. siRNA6 silenced Caki-2 cells displayed 7% of actin stress fiber formation while siRNA1 treated Caki-2 cells demonstrated 10% of actin stress fiber formation of the control group. For Caki-2 cells, the actin stress fiber formation was more restricted to the cell periphery while A-498, Caki-1, and ACHN cells formed longitudinal stress fibers in bundles. Data showed that siRNA6 transfection reduced actin stress fiber formation 79% in A-498 cells and only 30% of actin stress fiber formation was achieved in A-498 cells silenced via siRNA1, compared to the control group (Figure 4.8.).

Metastatic RCC cell lines ACHN and Caki-1 also displayed drastic difference in the formation of actin stress fibers. Caki-1 cells could only formed 16% of actin stress fibers when treated with siRNA1 and similarly siRNA6 treated Caki-1 cells only showed 17% of actin stress fiber formation of the control. ACHN cells showed 73% and 69% decrease in the fiber formation when transfected with siRNA1 and siRNA6, respectively, when compared to the NS siRNA treated control (Figure 4.9.). These results suggest that TG2 has a significant role in actin stress fiber formation in RCC cell lines hence the silencing of TG2 could be a promising therapeutic approach in the treatment of metastatic RCC.

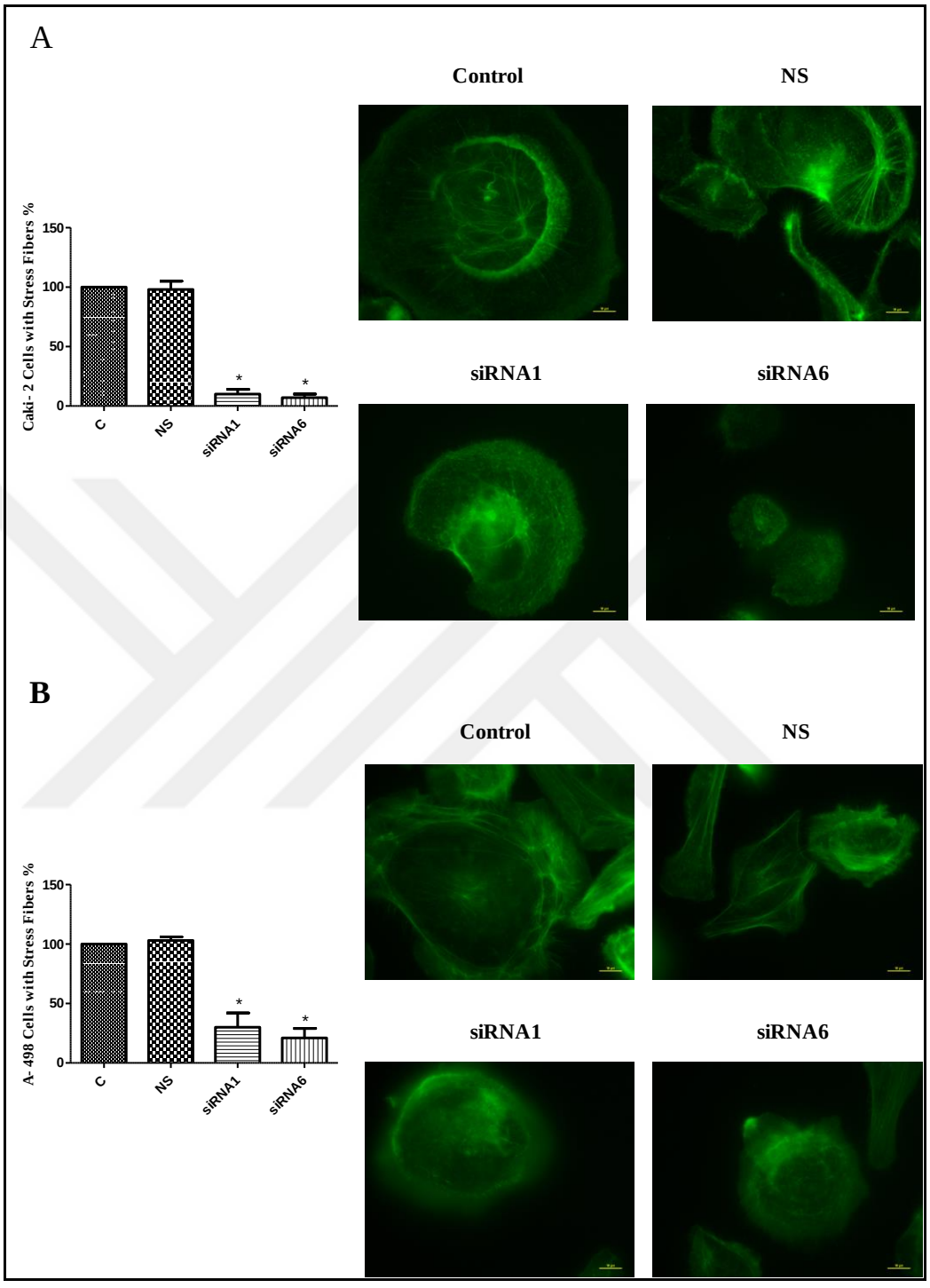

Figure 4.8. Effect of Transglutaminase II (TG2) on actin stress fiber formation in primary renal cell carcinoma Caki-2 (A) and A-498 (B) cell lines. The results are representative of three independent experiments. *P < 0.05 shows the significant difference versus the corresponding control group. Images were taken at 100X magnification. Bar, 10µm

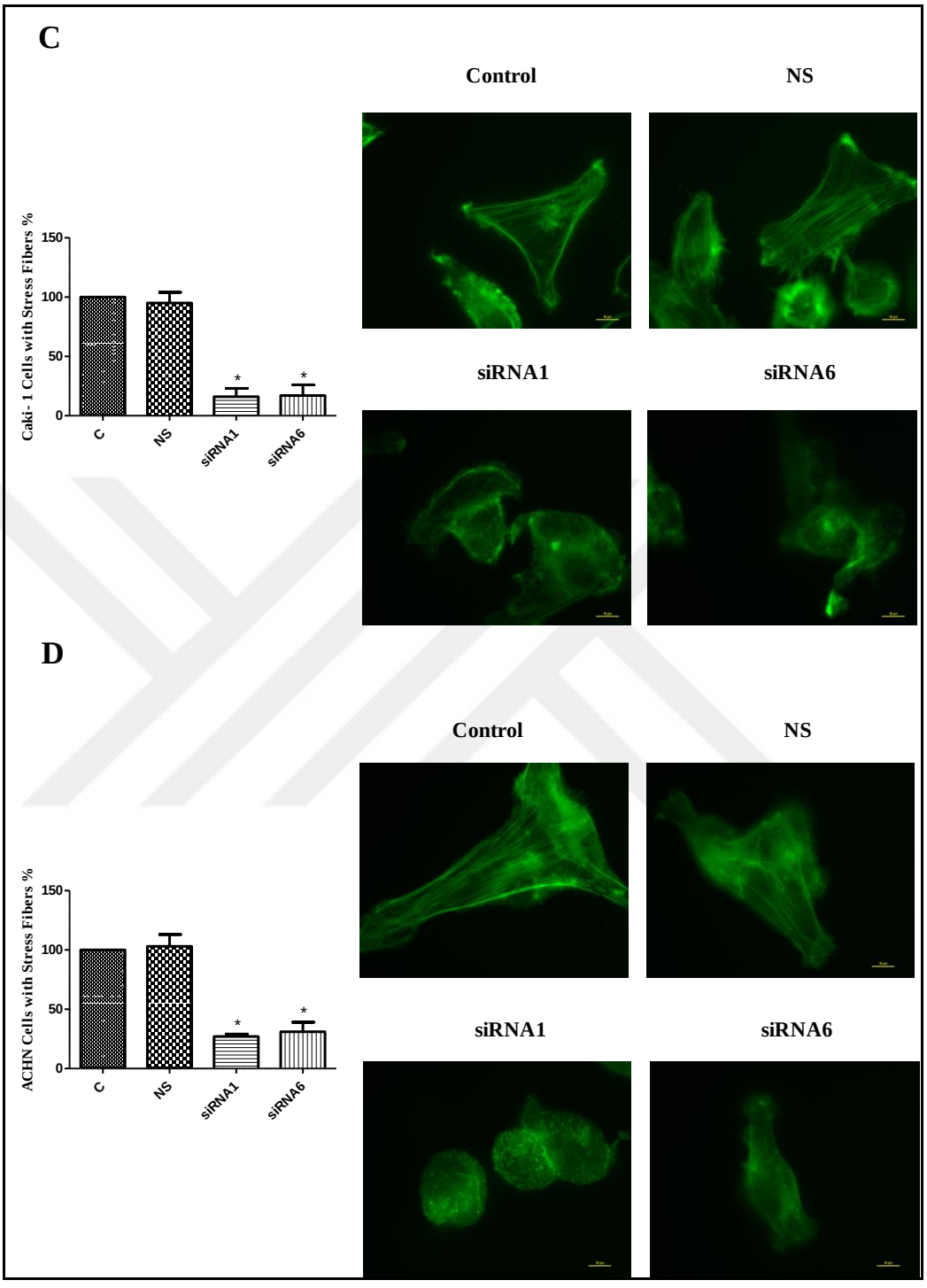

Figure 4.9. Effect of Transglutaminase II (TG2) on actin stress fiber formation in metastatic Renal Cell Carcinoma Caki-1 (C) and ACHN cell lines (D). The results are representative of three independent experiments. *P < 0,05 shows the significant difference versus the corresponding control group. Images were taken at 100X magnification. Bar, 10µm

4.4. EFFECT OF TG2 DOWNREGULATION ON MIGRATION OF RCC CELL LINES VIA WOUND SCRATCH ASSAY

In order to see the effect of TG2 on the migration of RCC cell lines, A-498 and Caki-2 cells were seeded 200,000 cells/well; Caki-1 and ACHN cells were seeded 300,000cells/well in 12-well plates and Wound Scratch Assay was performed. In order to determine the cell number to be seeded, optimization experiments were performed. Experiment was not performed for 72 hrs as wound areas were totally closed after 48 hrs.

4.4.1. Wound Scratch Cell Number Optimizations

In order to determine the cell number to be seeded into 12-well plates, Caki-1 and ACHN cells were seeded in different concentrations as 900.000 cells/well, 1 million cells/well, 1.2 million cells/well, and 1.3 million cells/well. Cells were incubated overnight in order to observe the confluency in the 12-well plate (Figure 4.10). Moreover, A-498 and ACHN cells were seeded in different concentrations as 100.000 cells/well, 200.000 cells/well, 300.000 cells/well, 400.000 cells/well, 500.000 cells/well, 600.000 cells/well. Following day, cell monolayer was scratched and incubated for 24 to 48 hrs (Figure 4.11.).

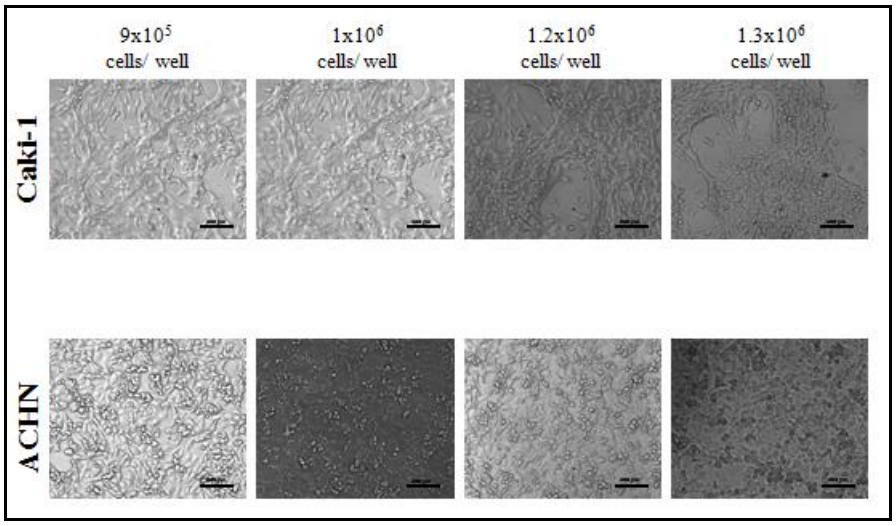

Figure 4.10. Caki-1 and ACHN cells were seeded in different concentrations into 12-wells for cell number optimization. Images were taken after cells monolayers were incubated overnight. Bar, 500µm.

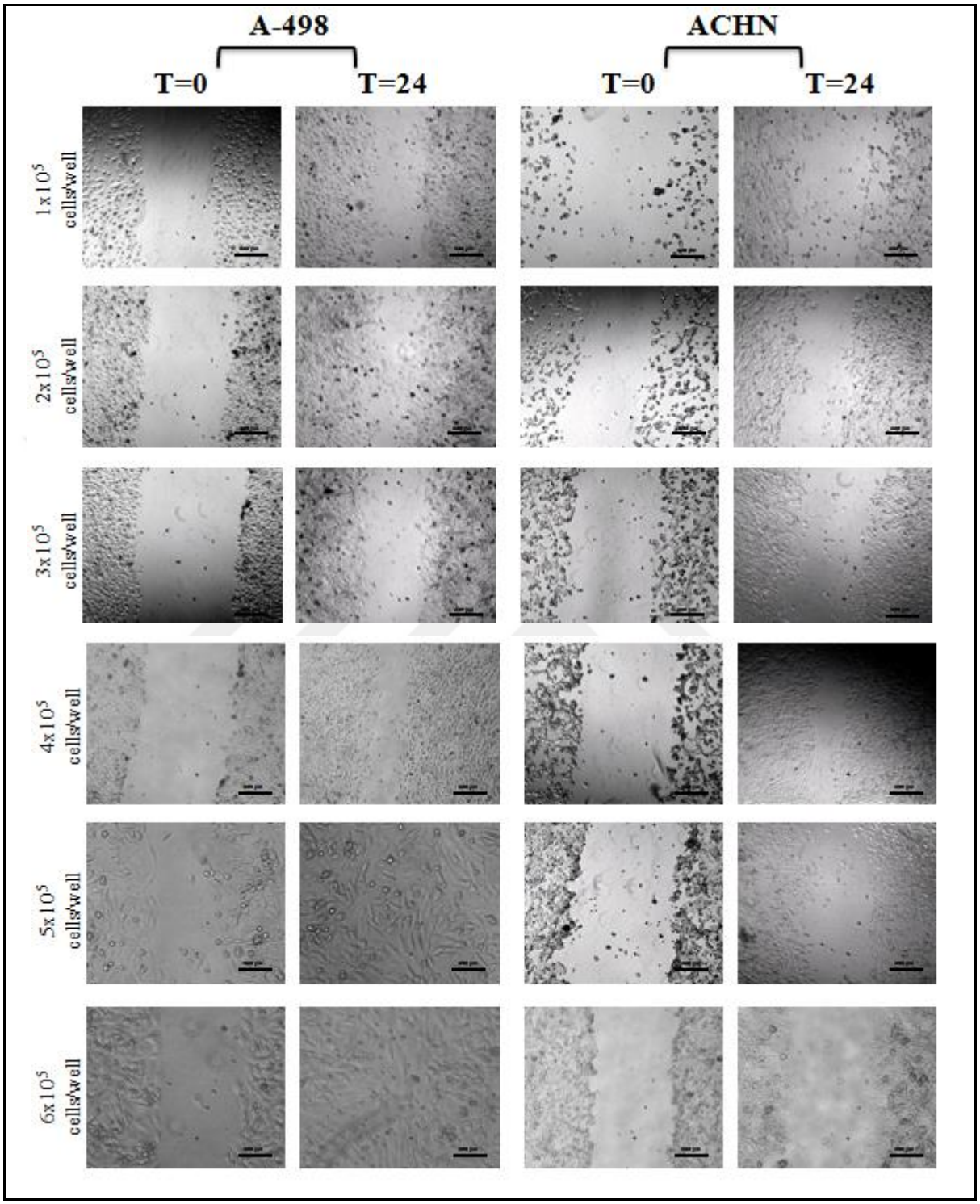

Figure 4.11. A-498 and ACHN cell lines were seeded at different concentrations. After incubation overnight, cell monolayer was scratched and the migration images were taken after 24 hrs. Bar, 500µm.

4.2.2. Effect of TG2 on Cell Migration

In this study, in order to demonstrate the effect of TG2 on the cell migration, RCCcells were treated with siRNA1 and siRNA6 against TG2 gene expression. Images of scratched cell monolayers were taken in different time points, such as T=0, T=24 and T=48 hours. NS treated RCC cells were used as a control. The scratched area was found to be totally closed at the end of 48 hours for each of four RCC cell lines.

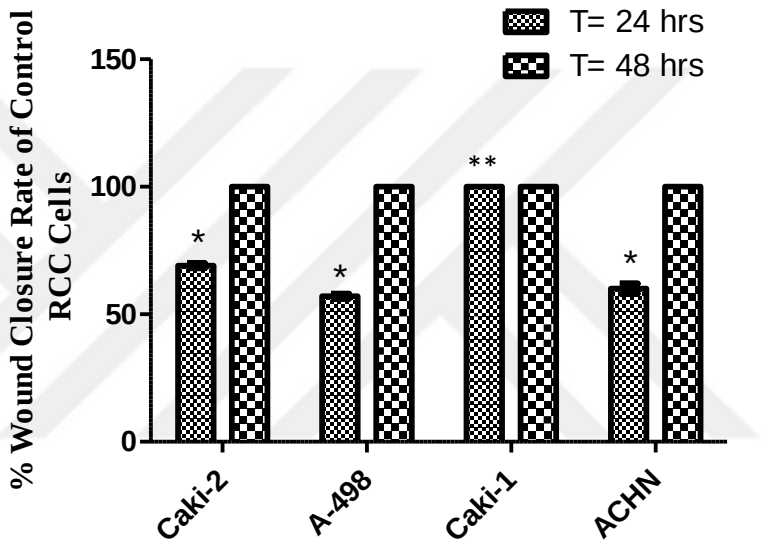

Figure 4.12. Determination of the migration potentials of RCC cell lines at T= 24 hours and T= 48 hours. The results are representative of three independent experiments. *P < 0.05 shows the statistically significance between two different time points recorded for each cell line. ** represents the statistical difference of Caki-1 (P < 0.05) among the four RCC cell lines

Migration potentials of primary and metastatic RCC cell lines demonstrated that metastatic Caki-1 cells had the highest migration potential with displaying a complete scratch closure at the end of 24 hours. Following Caki-1 cells, Caki-2 cells migrated 69% of the scratch area and metastatic ACHN cells migrated 60% of the wound area whereas primary A-498 had the slowest migratory potential with 57% at the end of 24 hours. It was found that all of the RCC cells totally closed the scratch area after 48 hours.

Primary Caki-2 cells appeared to be more sensitive to TG2 down regulation compared to the other primary RCC cell line A-498. While primary RCC cell line Caki-2 cells closed 70% of the wound area at 24 hours, siRNA1 silenced Caki-2 cells could only closed the 20% of the wound area and siRNA6 treated Caki-2 cells could only show 13% of migration potential through the scratch area at the end of 24 hours. While approximately 88% of the wound area was closed at 48 hours by Caki-2 cells, siRNA1 treated and siRNA6 treated Caki-2 cells could only closed the scratch by 30% and 15%, respectively (Figure 4.13.). Results demonstrated that primary A-498 cells closed approximately 72% of wound area at 24 hours. siRNA1 and siRNA6 silenced A-498 cells displayed a similar migratory potential and closed the 40% of the wound area when compared to the control groups at 24 hours. Down-regulation of TG2 by siRNA did not affect the migration of A-498 in long term as both siRNA treated and control groups of A-498 cells totally closed the scratch area at the end of 48 hours (Figure 4.14.).

Metastatic Caki-1 cells had the highest migration potential displaying a total scratch closure at the end of 24 hours whereas siRNA1 treated Caki-2 cells demonstrated 57% decrease in the scratch closure. Data showed that siRNA6 transfection reduced 69% wound area closure in Caki-2 cells at 24 hours. Results showed that the scratch area closure potential of siRNA1 treated Caki-2 cells could only reached to 54% from 43% at the end of 48 hours. siRNA6 silenced Caki-2 cells displayed only 12% increase in the rate of scratch closure when compared to the control groups at 48 hours shown in Figure 4.15. Metastatic ACHN closed 28% of the wound area at 24 hours whereas ACHN cells were found to be closed the scratch area completely at 48 hours. siRNA1 and siRNA6 transfection affected ACHN cell migration by 50% at both time points compared to control groups. siRNA1 treated ACHN cells could only display a migratory potential of 12% at 24 hours and 53% at 48 hours whereas siRNA6 transfected ACHN cells showed 15 % and 53% of migratory potential at 24 and 48 hours, respectively shown in Figure 4.16. Data showed that the most affected RCC cells by the down-regulation of TG2 was Caki-2, with an average of 75% inhibition in the migratory rate at the end of 24 hours. While the average inhibition of cell migration in siRNA transfected metastatic Caki-1 was found to be 63%, that of siRNA treated ACHN was 41% by the end of 24 hours. The least affected RCC cell line by the siRNA transfection down-regulating TG2 was found to be primary A-498 cells by 32% inhibition in migration into the scratch area. For the 48-hour time point, wound scratch

results were found to be in parallel with the 24-hour data. Caki-2 cells were the most affected ones by the down-regulation of TG2 demonstrating an average of 22% inhibition in the migratory rate at the end of 48 hours. While the cell migration inhibition in siRNA transfected Caki-1 cells was found to be 57%, that of siRNA treated metastatic ACHN was 46% by the end of 48 hours. The least affected RCC cell line by the siRNA transfection against TG2 was found to be primary A-498 cells which closed the scratch area completely at 48 hours demonstrating no inhibitory effect by siRNA transfection. The down-regulation of wound scratch results suggest that TG2 has a significant role in RCC cell migration hence the silencing of TG2 could be a promising therapeutic approach in the treatment of metastatic RCC.

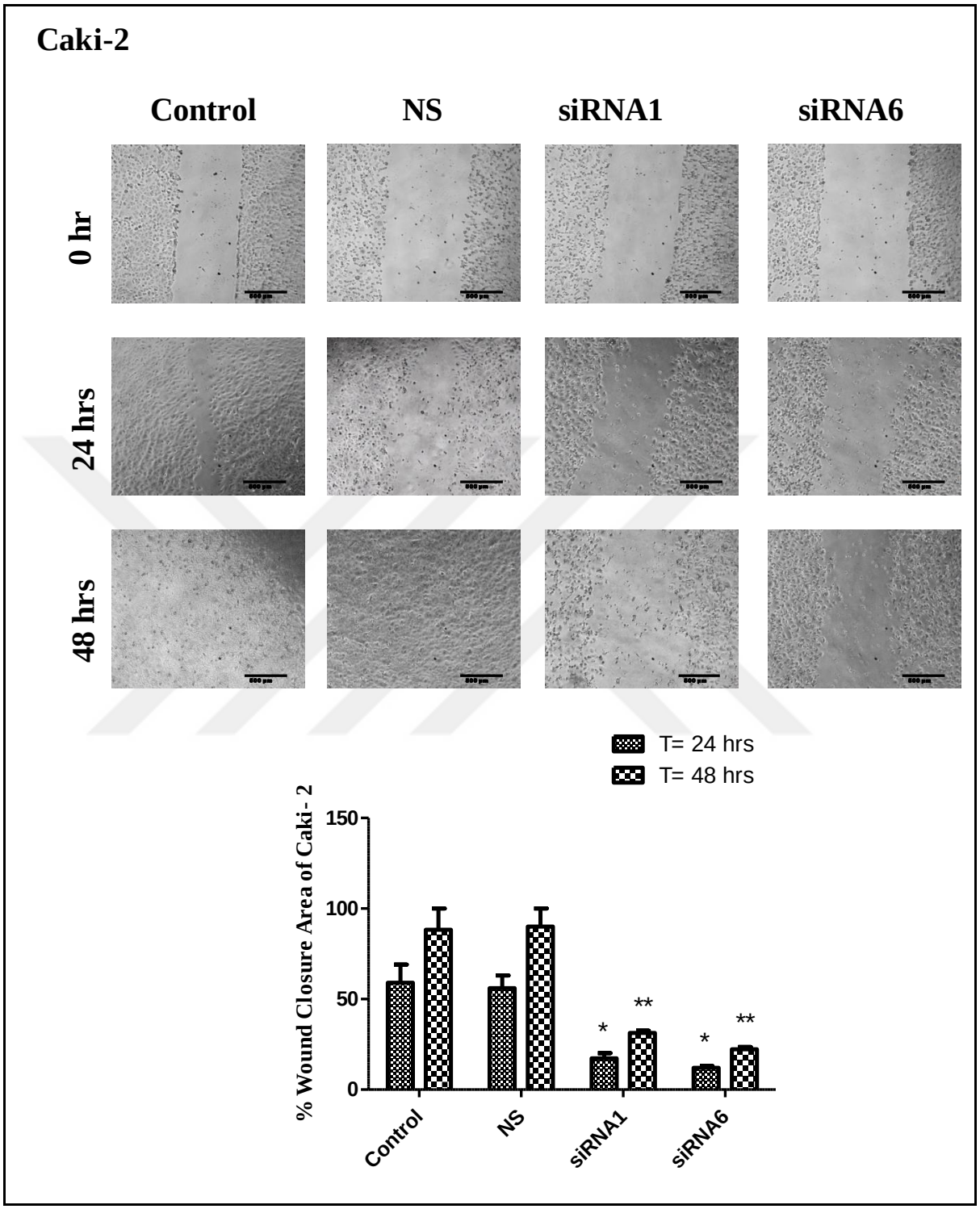

Figure 4.13. Effect of TG2 on the cell migration potential of Caki-2 cells at 24 and 48 hrs time points. *P < 0.05 shows statistically significant difference versus corresponding control T= 24 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 48 hrs. Bar, 500µm.

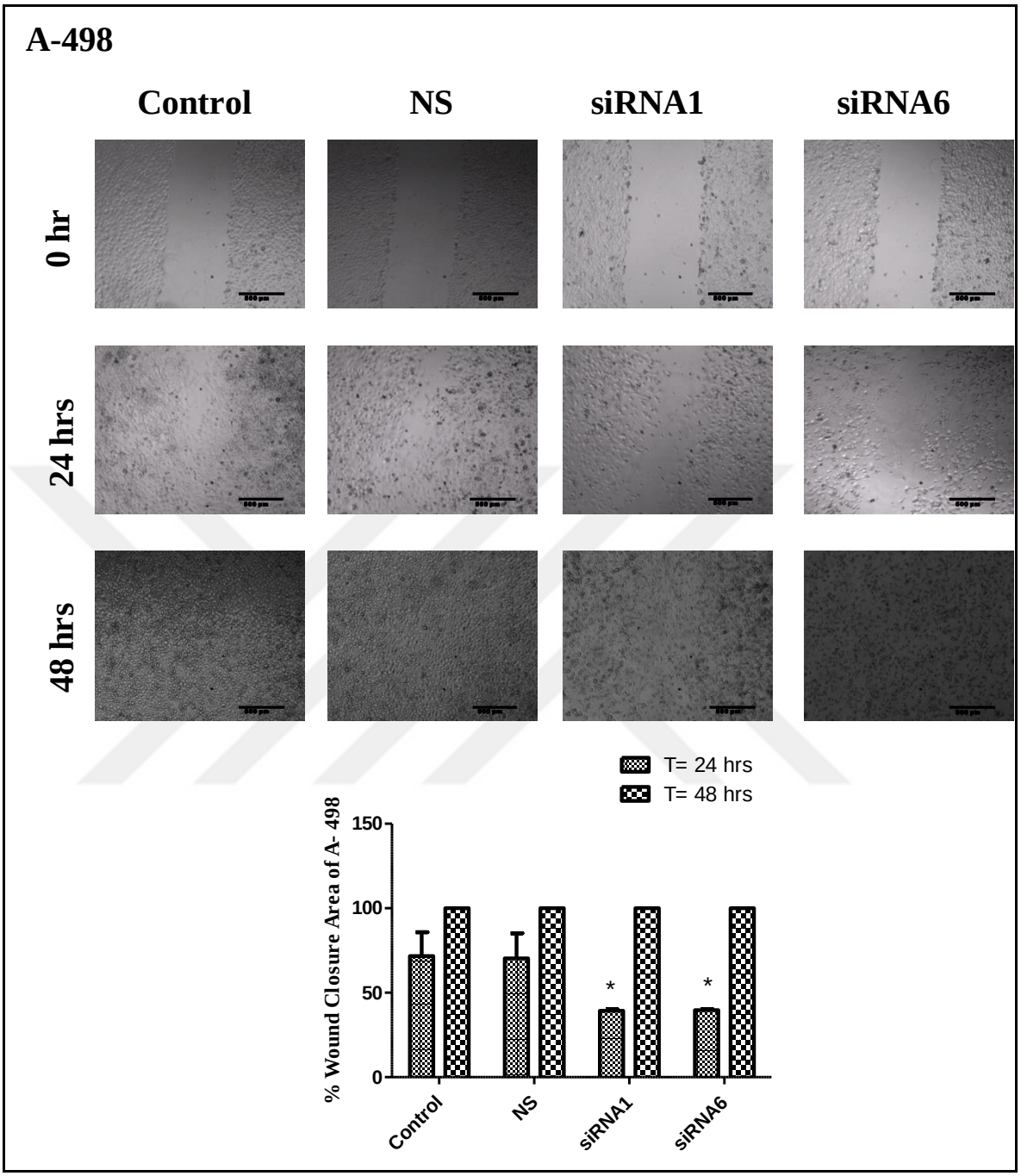

Figure 4.14. Effect of TG2 on the cell migration potential of A-498 primary cell line at 24 and 48 hours time points of Wound Scratch Assay. . *P < 0.05 shows statistically significant difference versus corresponding control T= 24 hrs Bar, 500µm

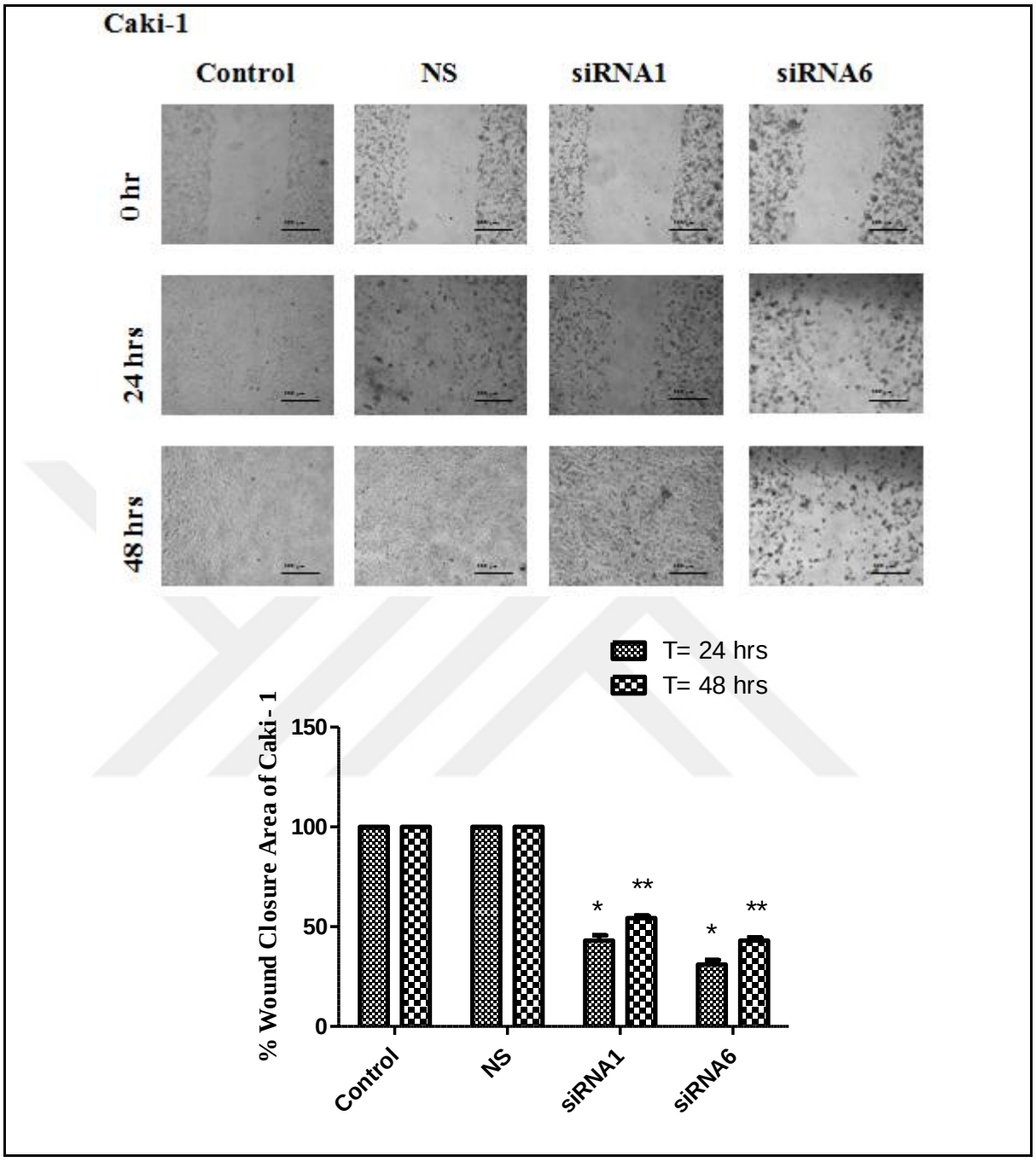

Figure 4.15. Effect of TG2 on the cell migration potential of metastatic Caki-1 cell line at 24 and 48 hours time points of Wound Scratch Assay. . *P < 0.05 shows statistically significant difference versus corresponding control T= 24 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 48 hrs. Bar, 500µm

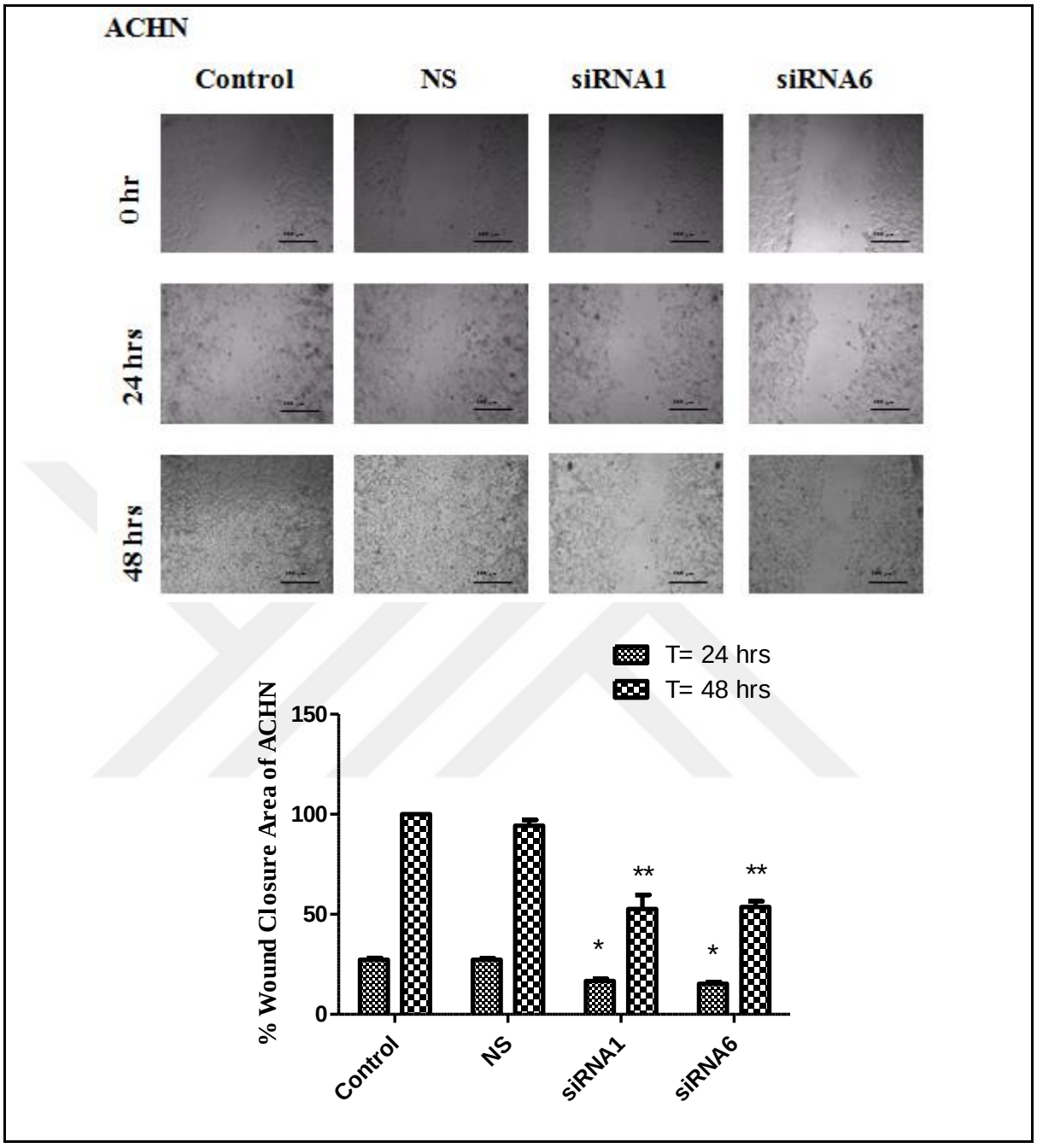

Figure 4.16. Effect of TG2 on the cell migration potential of metastatic ACHN cell line at 24 and 48 hours time points of Wound Scratch Assay. *P < 0.05 shows statistically significant difference versus corresponding control T= 24 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 48 hrs. Bar, 500µm

4.5. EFFECT OF TG2 DOWNREGULATION ON INVASION OF RCC CELL LINES ON INTEGRIN β1 SUBSTRATES

Analysis of the cell migration via transwell experiment was performed on RCC cell lines (Caki-2, A-498, Caki-1, and ACHN) on ITGB1 substrates FN, Col1, and LM. In order to investigate the effect of TG2 on cell migration, TG2 expression was silenced using siRNA technology. The number of cells migrated through the bottom of the transwells were calculated by summing up five images from non-overlapping fields. In data analysis, the number of NS siRNA treated cells migrated though the bottom of ITGβ1 substrate-coated transwells was considered as 100% and the number of migrated siRNA1 and siRNA6 transfected cells were normalized against that of NS control.

4.5.1. Invasion of RCC Cell Lines on Fibronectin

In order to investigate the effect of TG2 on cell migration, RCC cell lines were seeded at densities as mentioned before (Material Methods Section 2.4..) on the upper chamber of transwells coated with FN and allowed to transmigrate for 3 and 6 hours. It was recorded that number of cells invaded through FN coated transwells was 341 for Caki-2, 221 for A-498, 236 for Caki-1, and 252 for ACHN at the end of 3 hours. At the end of 6 hours, 414 Caki-2, 330 A-498, 366 Caki-1, and 383 ACHN cells migrated through the FN coated transwell. Result of three independent experiments demonstrated that the primary RCC cell line Caki-1 showed the highest invasive potential on FN (Figure 4.17.).

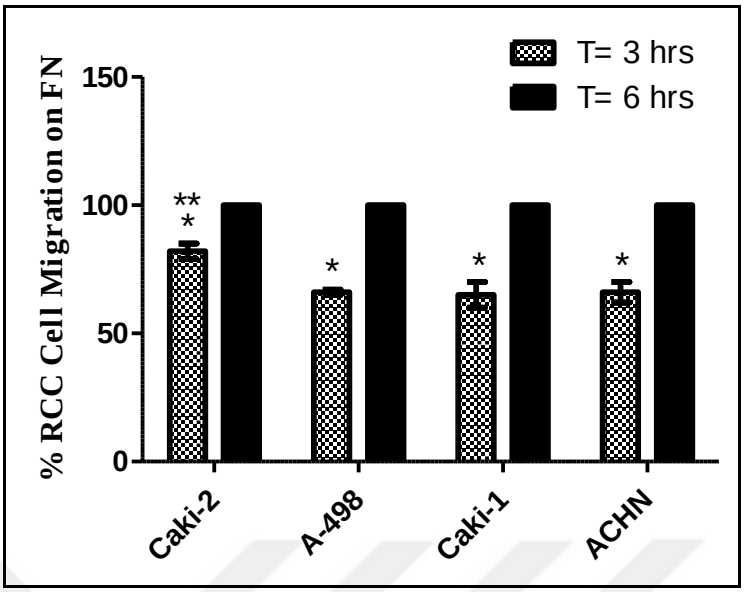

Figure 4.17. Determination of the invasive potentials of RCC cell lines on fibronectin at T= 3 hours and T= 6 hours. The results are representative of three independent experiments. *P < 0.05 shows the statistically significance between two different time points recorded for each cell line. ** represents the statistical difference (P < 0.05) of Caki-2 at T=3 hrs among the four RCC cell lines

Number of Caki-2 cells migrated through FN at early time point (T= 3 hours) was 341, whereas number of migrated cells at late time point (T= 6 hours) was 414. In siRNA1 transfected Caki-2 cells, the number migrated cells through FN was found to be 156 at 3 hours and this number could only reached to 178 cells at the end of 6 hours. The results were similar for siRNA6 transfected Caki-2 cells in both time points in that 157 cells and 203 cells were found to transmigrate through FN at the end of 3 and 6 hours, respectively. Overall, Caki-2 cells had a significant decrease in their invasive potential on FN when treated with siRNA1 and siRNA6 (Figure 4.18.).

Number of migrated A-498, was 221 at early time point and 330 at late time point. (T= 6 hours for FN) Results for primary RCC cell line A-498 cells were also similar to that of Caki-2 cells. 87% of primary A-498 cells invaded through FN at early time point whereas siRNA1 and siRNA6 transfected A-498 cells could only migrated at 52% and 40%, respectively at the end of 3 hours. By the end of 6 hours, the migration percentage of

siRNA1 and siRNA6 treated A-498 cells were similar to the early time point (T= 3 hours) at 22% and 17% respectively (Figure 4.18.).

In metastatic RCC cell line Caki-1, at the early time point (T= 3 hours) 92% of Caki-1 cells proteolytically degraded FN and migrated through the bottom of the transwell while only 33 % of siRNA1 and siRNA6 transfected Caki-1 cells could invade through FN. After 6 hours, siRNA1 and siRNA6 treated Caki-1 cells migration through FN was recorded as 38% and 40%, respectively (Figure 4.19.).

Suppression of TG2 expression using siRNA1 transfection in metastatic ACHN cells decreased the cell migration by 31% at the end of 3 hours and 22% after 6 hours. Similarly, siRNA6 treated ACHN cells demonstrated 49% decrease at T= 3 hours and 42% decrease at T= 6 hours in cell migration through FN (Figure 4.19.).

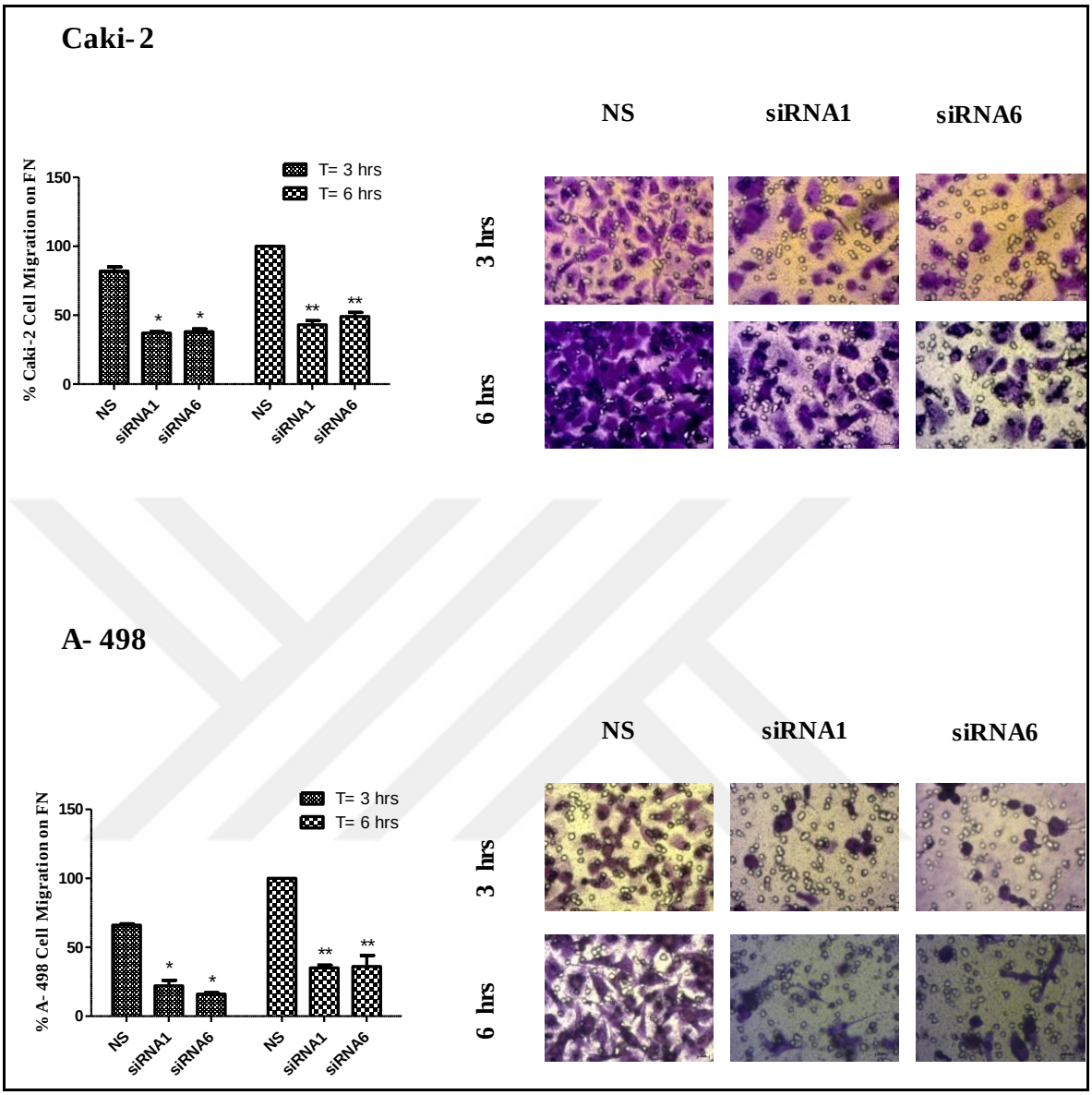

Figure 4.18. Determination the effect of TG2 on the invasive potentials of primary RCC cell lines Caki-2 and A-498 on ITGB1 ligand FN at T= 3 hours and T= 6 hours. The results are representative of three independent experiments. *P < 0.05 shows statistically significant difference versus corresponding control T= 3 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 6 hrs. Bar, 20µm

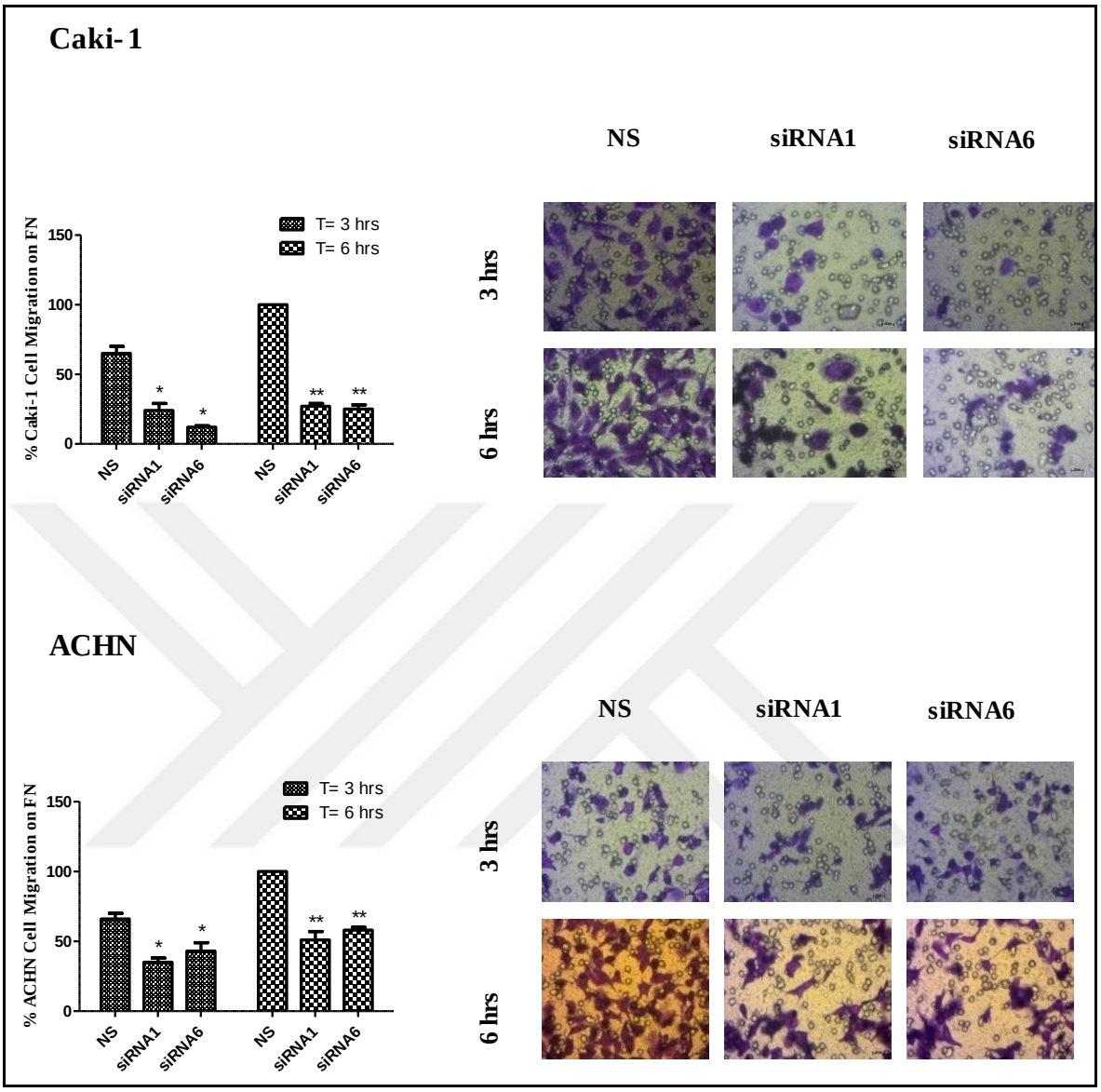

Figure 4.19. Determination the effect of TG2 on the invasive potentials of metastatic RCC cell lines Caki-1 and ACHN on ITGB1 ligand FN at T= 3 hours and T= 6 hours. The results are representative of three independent experiments. *P < 0.05 shows statistically significant difference versus corresponding control T= 3 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 6 hrs. Bar, 20µm

4.5.2. Invasion of RCC Cell Lines on Collagen Type-1

To investigate the effect of TG2 on RCC cell migration through Collagen type-1, cells were seeded at densities as mentioned above on the upper chamber of transwells coated with collagen type-1 (Col1) and allowed to transmigrate for 6 and 10 hours.

Number of migrated metastatic Caki-1 and ACHN through the bottom surface of Col1 coated transwells at the end of 6 hours, were 356 and 608, respectively. For primary cell lines A498 and Caki-2, number of migrated cells were 394 and 435, respectively at the end of 6 hours. At the end of 10 hours time interval, it was quantified that the migrated cell number was 510 for Caki-2, 419 for A-498, 586 Caki-1, and 681 for ACHN. Metastatic ACHN cells migrated the most at both early and late time points (T= 6 hours and T= 10 hours) (Figure 4.20.).

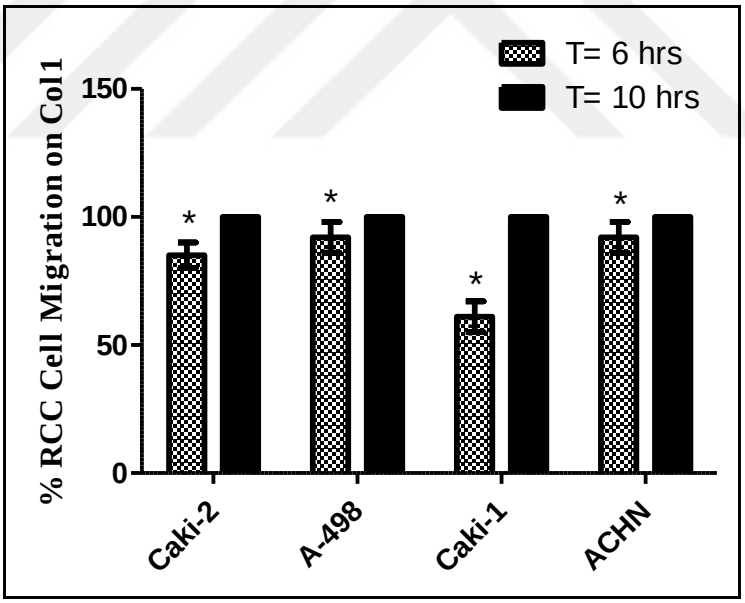

Figure 4.20. Determination of the invasive potentials of RCC cell lines on Collagen Type-1 at T= 6 hours and T= 10 hours. The results are representative of three independent experiments. *P < 0.05 shows the statistically significance between two different time points recorded for each cell line

Suppression of TG2 expression levels using siRNA1 led to an approximate 59% reduction in the invasive potential of Caki-2 on Col1 at the early time point T=6 hours, 68% reduction was found for the late time point T=10 hours. siRNA6 transfected Caki-2 cells showed a 58% decrease in cell migration through Col1 after 6 hours and a 66% decrease at the end of 10 hours. Overall, when treated with siRNA1 and siRNA6 Caki-2 cells demonstrated similar invasive potential at both early and late time points (Figure 4.21.)

Both siRNA1 and siRNA6 transfected primary A-498 cells demonstrated a similar result for Col1 at early time point T=6 hours, with 59% and 61% of migration potential of the control NS. After 10 hours of cell migration, it was found that siRNA1 treated A-498 cells showed 74% decrease in cell migration on Col1 and a 72% reduction in the invasive potential was recorded for A498 on Col1 when treated with siRNA6 (Figure 4.21.)

Suppression of TG2 via siRNA technology showed drastic reduction in the invasive potential of metastatic Caki-1 cells. Caki-1 cells showed low level of invasion and migration on Col1 (17%) at both time points (T=6 hours and T= 10 hours) when transfected with siRNA6. Similarly, siRNA1 transfected Caki-1 cells could only migrate 20% through Col1 after 6 hours. Interestingly, siRNA1 transfected Caki-1 cells demonstrated an increase in their migration potential through Col1 with 63% of NS control after 10 hours (Figure 4.22.).

Metastatic ACHN cells displayed 20% of invasive potential on Col1 after 6 hours when transfected with siRNA1. After 10 hours, the invasive potential of siRNA1 treated ACHN increased to 53% compared to control group. While 50% of siRNA6 transfected ACHN cells migrated through Col1 at early time point (T=6 hours) and cell migration potential could only rise to 58% after 10 hours of time interval (Figure 4.22.).

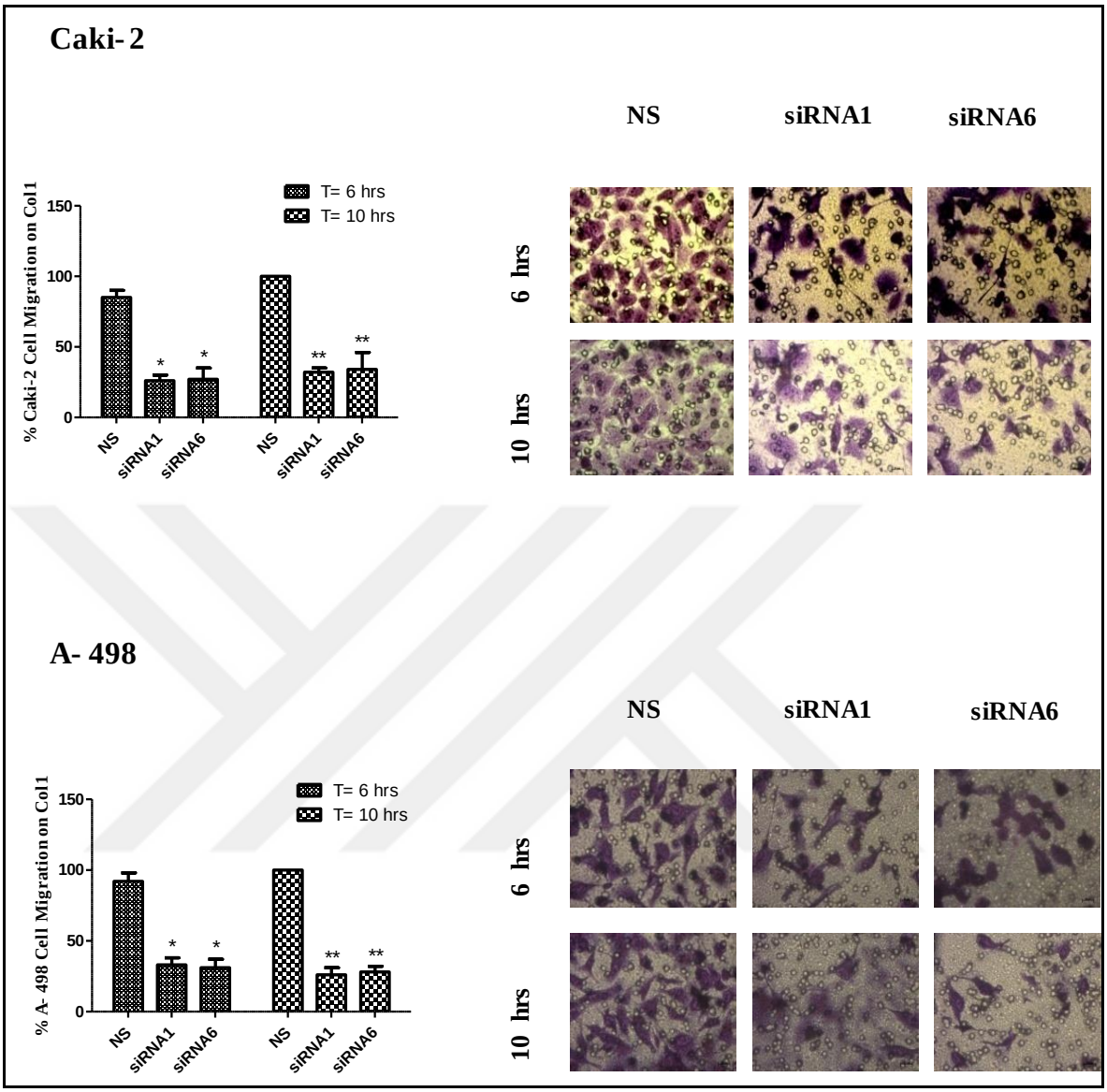

Figure 4.21. Determination the effect of TG2 on the invasive potentials of primary RCC cell lines Caki-2 and A-498 on ITGB1 ligand Col1 at T= 6 hours and T= 10 hours. The results are representative of three independent experiments. *P < 0.05 shows statistically significant difference versus corresponding control T= 6 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 10 hrs. Bar, 20µm

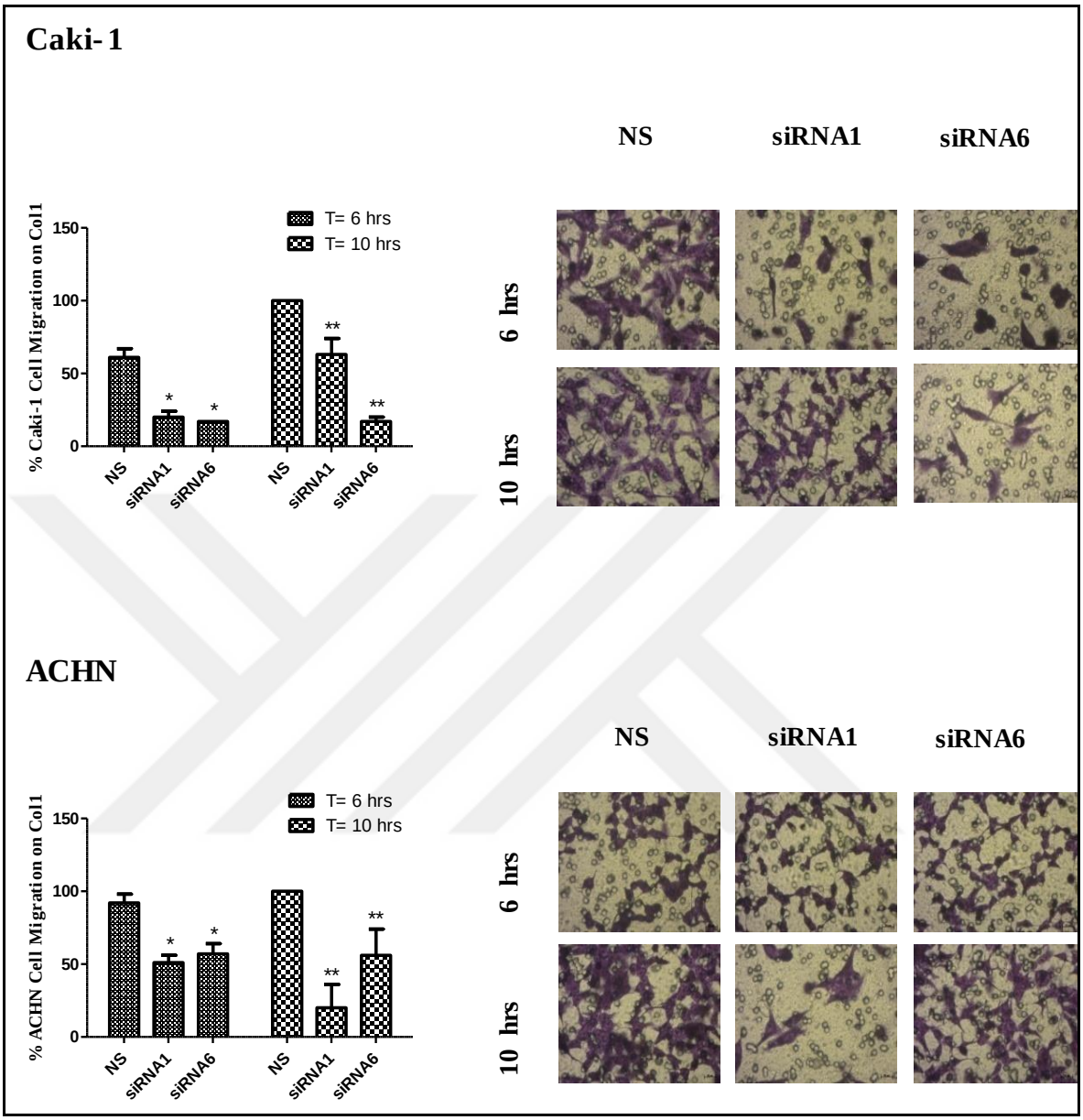

Figure 4.22. Determination the effect of TG2 on the invasive potentials of metastatic RCC cell lines Caki-2 and ACHN on ITGB1 ligand Col1 at T= 6 hours and T= 10 hours. The results are representative of three independent experiments. *P < 0.05 shows statistically significant difference versus corresponding control T= 6 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 10 hrs. Bar, 20µm

4.5.3. Invasion of RCC Cell Lines on Laminin

After first 10 hours, it was analyzed that 588 Caki-2 cells, 434 A-498 cells, 681 Caki-1 cells and 397 ACHN cells reached the bottom of the transwell by degrading the laminin (LM) matrix. The most invasive potential on ITGB1 ligand LM at 10 hours was demonstrated by metastatic Caki-1 cells. At second time point (T= 14 hours) it is quantified that the number of migrated cells increased to 810, 511, 732, and 529, respectively (Figure 4.23.)

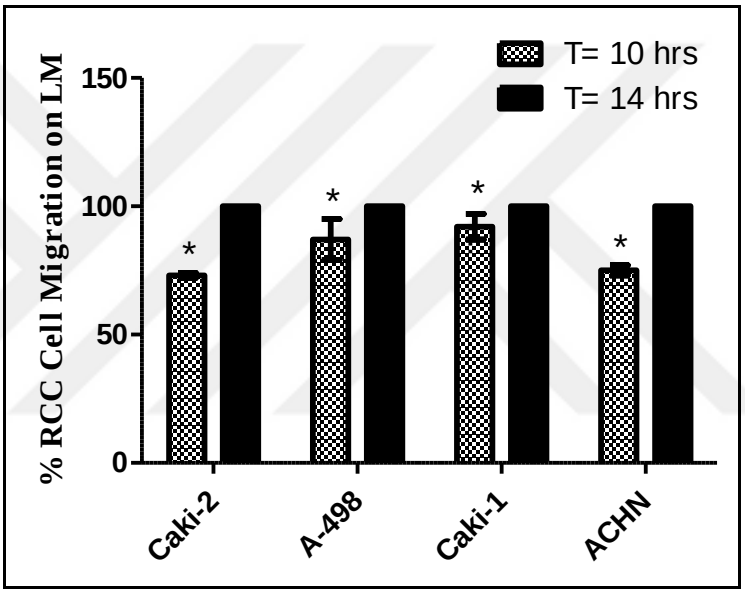

Figure 4.23. Determination of the invasive potentials of RCC cell lines on Laminin at T= 10 hours and T= 14 hours. The results are representative of three independent experiments. *P < 0.05 shows the statistically significance between two different time points recorded for each cell line

Caki-2 cells showed similar (27 % and 26 % of NS control) migratory potential at the early time points (T=10 hours) when transfected with siRNA1 and siRNA6. At the end of late time point (T=14 hours), siRNA1 transfected Caki-2 cells displayed 31% invasive potential and siRNA6 treated cells demonstrated 41% migratory potential on LM, when compared to Ns control. Primary Caki-2 cells showed the lowest invasive potential on LM among other RCC cell lines (Figure 4.24.).

Primary A-498 cells displayed similar migration potential (52% and 51% of NS control) on LM at both time points when transfected with siRNA1. The invasive potential was recorded as 40% and 39% for both time points (T=10 hours and T= 14 hours) when A-498 cells were treated with siRNA6 (Figure 4.24.).

Down-regulation of TG2 led to a reduction in the migratory potential of Caki-1 cells on LM by an average of 59% at T=10 hours and 62% at T=14 hours when the cells were transfected with siRNA1. siRNA6 transfected metastatic Caki-1 cells showed 58% and 60% decrease in the transmigration of LM in early and late time points, respectively (Figure 4.25.).

siRNA1 transfected metastatic ACHN cells demonstrated a 29% reduction at T=10 hours and a 45% reduction after 14 hours on LM. The results indicated that 42% of ACHN cells could migrate through LM after 10 hours when transfected with siRNA6. The invasive potential of siRNA6 transfected ACHN was drastically reduced to 64% on LM at the late time point (T= 14 hours) (Figure 4.25.).

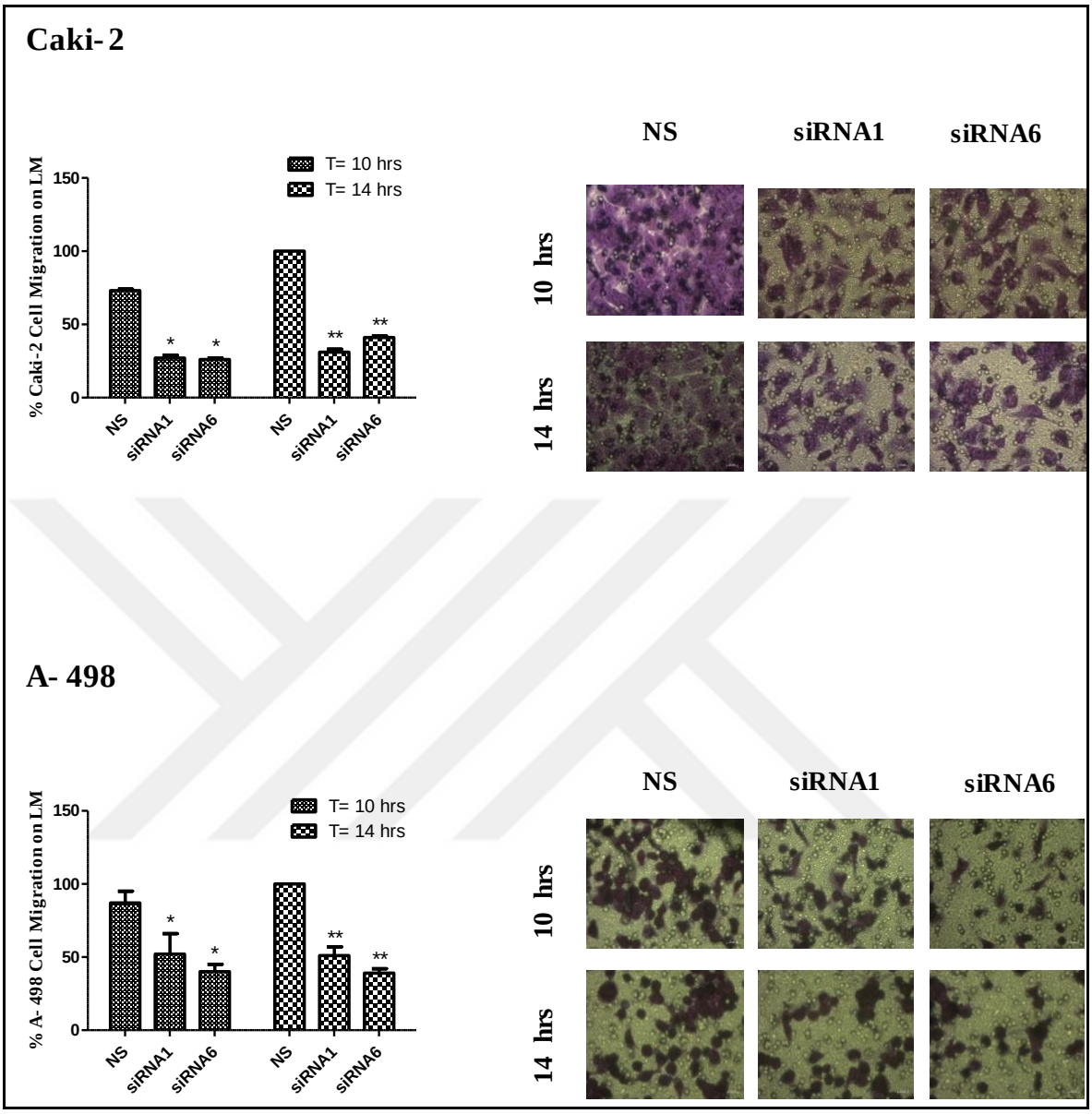

Figure 4.24. Determination the effect of TG2 on the invasive potentials of primary RCC cell lines Caki-2 and A-498 on ITGB1 ligand LM at T= 10 hours and T= 14 hours. The results are representative of three independent experiments. *P < 0.05 shows statistically significant difference versus corresponding control T= 10 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 14 hrs. Bar, 20μm

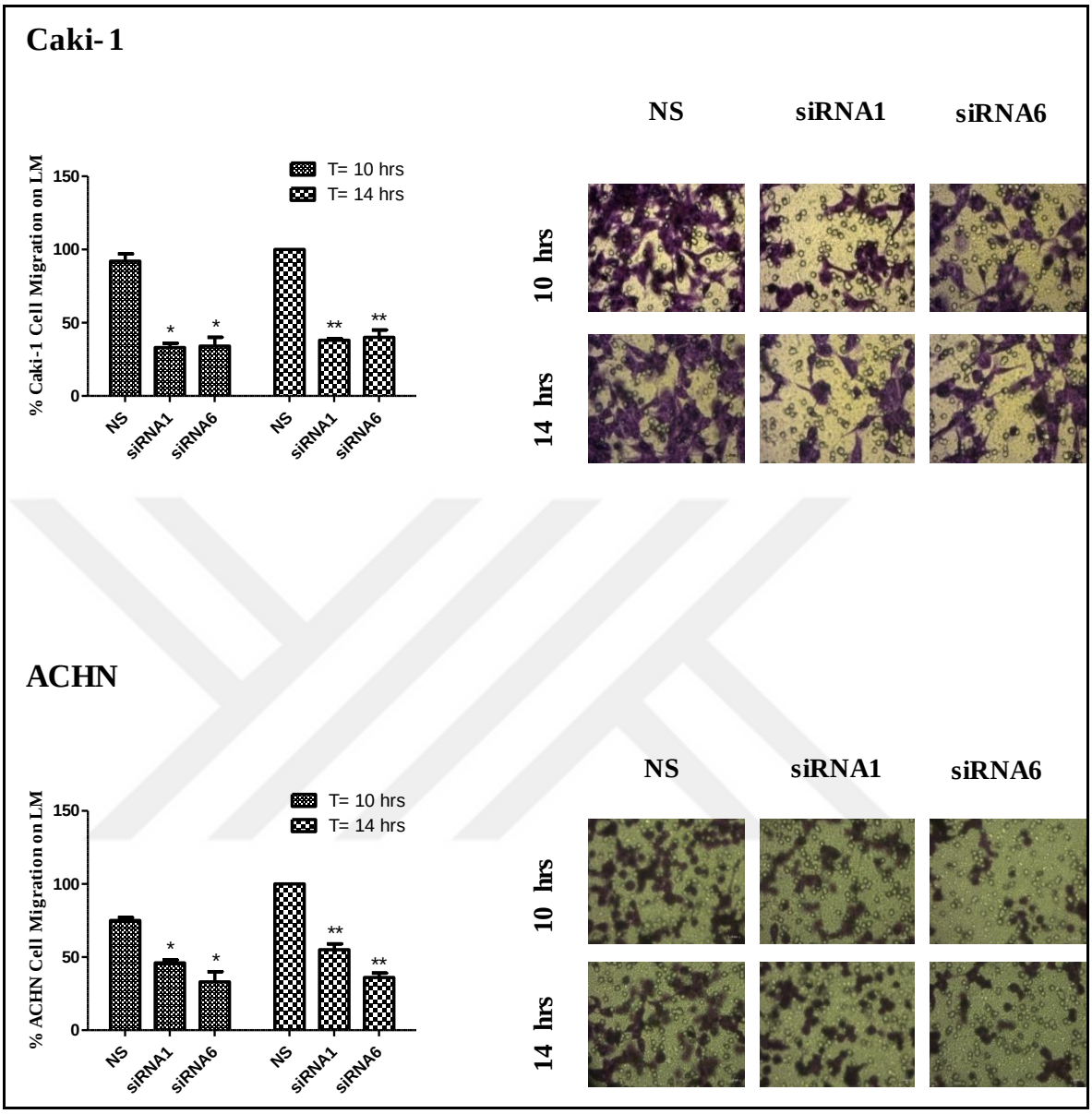

Figure 4.25. Determination the effect of TG2 on the invasive potentials of metastatic RCC cell lines Caki-1 and ACHN on ITGB1 ligand LM at T= 10 hours and T= 14 hours. The results are representative of three independent experiments. *P < 0.05 shows statistically significant difference versus corresponding control T= 10 hrs. ** represents the statistical significant difference (P < 0.05) versus corresponding control at T= 14 hrs. Bar, 20µm

5. CONCLUSION

In this thesis, the aim was to elucidate the role of TG2 on RCC cell adhesion and cell migration. It can be concluded that primary tumors may increase the transglutaminase II (TG2) expression level together with the expression level of β1 integrin (ITGB1) to support cell adhesion, cell migration, cell survival in order to have the invasive potential and provide metastasis. In support of this hypothesis, western blot analysis showed that TG2 protein expression was found to be higher in primary (Caki-2 and A-498) cell lines compared to metastatic ones. The result could be explained by the protein turn-over of TG2 together with ITGB1 in metastatic cell lines which will be studied in the future studies. The effect of TG2 on actin stress fiber formation in RCC cell lines demonstrated that, TG2, rather than its transamidating activity, might act a novel cell adhesion protein together with ITGB1. With this respect, down-regulation of TG2 affected the actin stress fiber formation of Caki-2 and Caki-1 cells the most. Wound scratch assay results indicated that while the fastest migration potential was found to be in metastatic Caki-1 and ACHN, silencing of TG2 decreased the migration potential by %50. Finally transwell assay was performed in RCC cell lines with β-1 integrin ligands fibronectin (FN), collagen type-I (Col1) and laminin (LM). While Caki-2 cells were showing the highest migration potential on FN, down-regulation of TG2 decreased the invasiveness of Caki-2 at most. Metastatic RCC cell lines ACHN and Caki-1 demonstrated the highest migration on Col1, the migration potential of ACHN and Caki-1 through Col1 decreased by 50% whereas the primary RCC cells affected by the silencing of TG2 the most. The most invasive potential on ITGB1 ligand LM was demonstrated by metastatic Caki-1 cells. Down-regulation of TG2 affected the migration potential of metastatic Caki-1 the most by decreasing 60%. LM is the one of the main basement membrane component. Hence, metastatic Caki-1 and ACHN invasion potential on LM are being effected most by TG2 down-regulation, this might suggest that TG2 is not only important in cell migration but also in intravasation (the invasion of cancer cells through the basal membrane into a lymphatic or blood vessel) and extravasation (refers to cancer cells exiting the capillaries and entering organs in the case of malignant cancer metastasis) stages of metastasis.

6. DISCUSSION

Renal cell carcinoma (RCC) is the most widespread type of kidney cancer seen at a rate of 90% worldwide among all different types of kidney cancer. The disadvantage of poor prognosis results in RCC to be diagnosed at the metastatic stage. Cell invasion mechanism of RCC tumors is not completely known yet and this gap has been bringing a mortality of ~100,000 deaths/year [12, 13]. The focus of this thesis is to elucidate the role of TG2 in RCC cell adhesion, migration and invasion at a molecular level.

TG2 is a cross-linking protein, when deposited to ECM, it is responsible from stabilizing the ECM by catalyzing intermolecular cross-linkings composed of ε(γ-glutamyl) lysine bridges in the presence of Ca^{2+}. TG2 is mainly found in intracellular spaces of the tissue with its Ca^{2+} dependent cross-linking activity being tightly regulated by GTP/GDP; however, TG2 can also be found on the cell surface and at ECM [80, 81]. The role of extracellular TG2 on cell surface and in ECM was established in 2002, demonstrating that TG2 can both act as a novel cell adhesion protein on the cell surface and as an extracellular scaffold protein involved in matrix stabilization through its transamidating activity [80]. The first function can occur when TG2 takes role as a co-receptor for binding of β-1 integrins to FN by forming a high affinity-bounded heterocomplex with FN when deposited into ECM [41, 82]. Studies showed that TG2 can shape the ECM by binding tightly to both FN through the gelatin-binding domain and integrins on the cell surface. In complex with FN, TG2 enzyme becomes more resistant against proteolytic degradation and additionally its transamidating activity becomes reduced [51, 83]. Interactions of TG2 with the cell surface receptors promote cell adhesion, migration, signaling and survival by implication [49, 51, 57, 60, 68].

Pioneer work indicated that when TG2 is over expressed with SDC4 and ITGB1, TG2 loses its transamidating activity and no longer acts as a matrix stabilizer but takes role as a novel cell adhesion protein [49]. In this work, TG2 activity assay performed on RCC tumor lysates, primary cell lines and control RPTEC (renal proximal epithelial cell) cells demonstrated that TG2 activity was found to be high in control tissues in comparison with the metastatic tumor tissues and RCC cell lines. A study on breast cancer also found a decrease in the TG2 expression and activity in primary tumors and suggested that the TG2 levels/activity must have been lowered selectively in these primary tumors to generate a

more destabilized ECM which is necessary for the primary tumor growth [78, 84]. Interestingly, in metastatic tumors with high TG2 mRNA level, the TG2 activity was found to be low when these tumors had the over expression of TG2 together with SDC4 and ITGB1. This result suggested that TG2 might have lost its transamidating activity due to its interaction with the cell surface receptors SDC4 and ITGB1 [79, 85]. In support of this hypothesis, when TG2 is bound to FN, it is recognized by SDC4 as a cell adhesion protein and regulates RDG-independent cell adhesion, signaling cooperatively with ITGB1 [61, 79]. Other studies demonstrated that increased expression of TG2 together with the increased ITGB1 has been proven to cause drug resistance and increased metastatic potential of tumor cells found in melanoma pancreatic adenocarcinoma, breast and ovarian cancer [23, 49, 60, 78, 86, and 87]. For example, Mehta et al. showed that the up-regulation of TG2 can be used as a prognostic marker for metastatic and drug-resistant breast cancer tumors which could protect cancer cells from the stress-induced apoptosis and chemotherapy [87]. Although studies demonstrated the importance of TG2 in drug resistance and metastasis of tumors originated from epithelial tissues, pioneer study from our group was the first to demonstrate the importance of TG2 on RCC [79]. In this thesis, as continuation of our previous study, primary (A-498 and Caki-2) and metastatic (Caki-1 and ACHN) RCC cell lines, expressing different levels of TG2, were used as a model to elucidate the role of TG2 in RCC cell adhesion and migration.

Our group found decreased TG2 expression levels in the primary (A-498 and Caki-2) RCC cells suggesting that the environment might be favorable to have destabilized ECM for the growth of RCC tumor itself [79]. Similar results were found in breast cancer cell study demonstrating up-regulated TG2 protein expression in drug resistant and metastatic breast cancer cells [87]. In order to check TG2 and ITGB1 protein levels in the RCC cell lines, Western Blot Analysis was firstly performed on RCC cell lines. Data indicated that A-498 and Caki-2 (primary RCC cell lines) showed higher TG2 protein level compared to ACHN and Caki-1 (metastatic RCC cell lines. On the contrary, Erdem et al. reported that TG2 mRNA expression level was found to be lower in primary RCC cell lines (A-498 and Caki-2) when compared to the metastatic ones [79]. Results from both studies suggest that higher TG2 protein level found in primary RCC cell lines could be accompanied by the high transamidase activity of TG2 and TG2 might still act as ECM stabilizer. In other words, although TG2 mRNA was found to be lower in the primary RCC cells; once TG2

mRNA is translated to protein, it might be deposited to the ECM in primary RCC cell lines and thereby escape from the high protein turn-over seen in the cell and cell surface [83]. In support of this hypothesis, Erdem et al. demonstrated that the primary RCC cell lines Caki-2 and A-498 has higher TG2 activity on the cell surface and ECM compared to metastatic RCC cell lines Caki-1 and ACHN [79]. Although metastatic RCC cell lines possess higher TG2 expression levels [79], TG2 protein levels were found lower in metastatic RCC cells compared to primary cells suggesting a possible high protein-turnover for TG2 in these metastatic cell lines. Therefore, TG2 in the metastatic RCC cell lines might act as a novel cell adhesion protein and associate with SDC4 and induce integrin trafficking process. In support of this hypothesis, Bass et al. also demonstrated that SDC4 induces the adhesive strength of the fibroblast cells via triggering the α5β1-integrin endocytosis during this process. Thus, TG2 might be taken into endosomes and get degraded leading to a high protein turnover rate [52].

Western Blot results indicated that ITGB1 protein level was found to be the highest in metastatic Caki-1 and ACHN cells, respectively compared to primary RCC cell lines. Supporting these results Erdem et al. also demonstrated a simultaneous increase in the expression of TG2 with ITGB1 and SDC4 in metastatic RCC tumors suggesting that the co-overexpression of TG2 together with ITGB1 and SDC4 might enhance the invasive potential of primary tumors resulting in the metastatic behavior [79]. In other words, primary RCC cell lines might increase TG2 expression independent of transamidating activity together with SDC4 and ITGB1 to support both cell adhesion and migration which leads cell survival necessary for the metastatic potential [79]. Results suggest that increased ITGB1 in metastatic cell lines together with TG2 may allow TG2 act as a novel cell adhesion protein on the cell surface rather than behaving as a transamidating enzyme and facilitate metastatic potential and drug resistance. It was therefore desirable to down-regulate TG2 expression levels both in primary and metastatic RCC cell lines using siRNA technology and elucidate the role of TG2 on cell adhesion and migration in these RCC cell lines. For this purpose, each RCC cell line was transfected with siRNA1 and siRNA6 independently against TG2. Results showed that siRNA1 and siRNA6 could effectively down-regulate the TG2 expression in Caki-2, A-498, Caki-1, and ACHN cells. Therefore, using siRNA silencing, the effect of TG2 on cell adhesion was investigated by analyzing

actin stress fiber formation and cell migration was determined performing wound scratch assay and transwell assays in RCC.

In cell adhesion, TG2 plays a key role by functioning as an integrin-binding co-receptor for FN and a stabilizer of ECM proteins [49]. Reorganization of actin cytoskeleton and additionally the activation of integrins are required for cell adhesion and spreading on ECM providing the formation of actin stress fibers and focal adhesions for stable cell adhesion [88]. Combinated signaling from both syndecans and integrins after attachment to FN is crucial for the organization of the actin cytoskeleton and subsequent activation of the MAPKs such as FAK [65, 89]. TG2-FN complex binds to SDC4 and this binding activates PKCα. Activated PKCα, then associates with ITGB1 and reinforces the actin stress fiber organization and formation with the activation of MAPK pathway [49]. Moreover, it has been shown that the association of integrins with TG2 on the cell surface mediates the FAK/SRc, p190RhoGAP activities leading the upregulation of the activation levels of RhoA GTPase and also its target, ROCK. This activation pathway promotes the increased focal adhesion formation, actin stress fiber formation and additionally increased actomyosin contractility in the TG2 expressing cells [41]. Recent studies showed the importance of interaction of TG2-FN complex with ITGB1 and SDC4 in organization of actin stress fibers and cell adhesion. RGD-containing peptide is used to impair the integrin-FN binding of fibroblasts, however when these cells were seeded on TG2 containing matrix. Cell adhesion was restored suggesting a novel RGD-independent cell adhesion process where binding of TG2-FN complex to SDC4 activated PKCα resulting in the activation of ITGB1 which in turn promotes actin stress fiber formation together with MAPK pathway activation [49]. Another study demonstrated the effect of TG2 together with Syndecan2 (SDC2) and SDC4 in cell adhesion enhancing actin cytoskeletal formation in osteoblasts in the presence of RGD peptides. TG2 over-expressing human osteoblast cells were used and SDC2 and SDC4 were silenced to see the effect on TG2-FN heterocomplex in cell adhesion and actin cytoskeletal formation where TG2 was the functional component. The importance of SDC2 and SDC4 in the regulation of TG-FN induced cell adhesion and actin cytoskeletal formation was found using siRNA silencing in osteoblast cell. TG2-SDC4 and TG2-SDC2 interactions together with α5β1 integrin association were indicated using inhibitory peptides and performing immunoprecipitation assay. In this respect, the molecular mechanism of TG2 in osteoblast cell adhesion process

was shown [50]. In order to assess if TG2 has an effect in the formation of actin stress fibers in RCC cell lines, TG2 was down-regulated using siRNA technology and the formation of actin stress fibers was analyzed using FITC- phalloidin. For this purpose, RCC cells were transfected with siRNA1 and siRNA6 independently and RCC cells were allowed to attach on the surface at optimized time intervals. Silencing TG2 expression caused the loss of actin stress fiber formation in all RCC cell lines. Primary RCC cell line Caki-2 appeared to be more sensitive to TG2 down regulation compared to the other RCC cell lines. siRNA silencing of TG2 affected the actin stress fiber formation in metastatic Caki-2 cells the most, followed by Caki-1, A-498, and ACHN, respectively. Results suggested that decreased expression level of TG2 in RCC cell lines might weaken the interaction between TG2 and ITGB1. The low interaction between TG2 and ITGB1 might lead a distinct reduction in the formation of stress fibers in RCC cell lines. Results suggest that TG2 may have a significant role in actin stress fiber formation in RCC cell lines hence the silencing of TG2 could be promising therapeutic approach in the treatment of metastatic RCC.

Although TG2 is mostly found in cytosol, it can also found to be placed in nuclear membranes and plasma. Moreover, cell surface TG2 takes a role as a co-receptor for FN as mentioned before [68]. Studies showed that cells secreting TG2, have enhanced cell attachment to Fibronectin (FN) in either cross-linking of FN [58, 90] or cross-linking independent association of TG2 with FN matrix [91, 92]. The way that TG2 functions on the cell surface is that it associates with β1 and β3 integrins when it also simultaneously binds to FN through the gelatin-binding domain [60]. So that, TG2 plays role in wound healing, fibrosis, inflammation, and tumor metastasis [80]. Reports demonstrate that TG2 has an important role in tissue injury and wound repair [93, 94]. Verderio et al. showed that RGD-independent cell adhesion to TG-FN complex did not require transamidating activity whereas it requires induced the formation of focal adhesion contacts, the assembly of associated actin stress fibers, and FAK phosphorylation [51]. As TG2 is a novel cell adhesion protein, in 2005 Gambetti et al reported the interaction between TG2 and heparan sulfates (HS) [71]. In 2009, Scarpellini et al for the first time demonstrated data about the affinity of TG2 for HS. Depending on the results, TG2 affinity for HS is strong as comparable as the affinity of TG2 to FN [95]. More studies showed that extracellular TG2 was catalytically inactive under normal conditions; catalytic activity of extracellular TG2

was studied under normal and stressed conditions with WI-38 fibroblasts and MDA-MB-231 cells [96]. Another study showed that any injury case could lead a conformational change in extracellular TG2 protein resulting in the activation of TG2 whereas intracellular TG2 (Ca^{+2} level is low) stays in inactive form during wound repair system. On the other hand, it is thought that extracellular TG2 stays inactive as well as it plays a role as a cell adhesion proteins when it binds to ITGB1 and FN in ECM [96]. In this thesis, the effect of TG2 on cell migration was determined performing wound scratch assay and transwell assay in RCC cell lines, using siRNA silencing against TG2. Wound scratch assay was performed for 24 and 48 hrs. At the end of 48 hrs, it was found that all four RCC cell lines completely closed the wound area by migrating to each other. Wound scratch results indicated that after 24 hrs of migration, metastatic Caki-1 and ACHN cells had the highest migration potential among four cell lines, respectively. Results showed that the metastatic cell lines had the highest potential to close the wound area compared to the primary cell lines. Data demonstrated that metastatic RCC cells express more ITGB1 suggesting that higher ITGB1 expression level found in metastatic RCC cell lines together with TG2 might let TG2 act as a novel cell adhesion protein on the cell surface rather than behaving as a transamidating enzyme which could cause metastasis and drug resistance. Primary RCC cell line A-498 had the slowest migratory potential in wound scratch assay. To see the effect of TG2 in RCC cell migration, TG2 was down regulated using siRNA1 and siRNA6. Silencing of TG2 led a great decrease of the migration potential of both primary and metastatic RCC cell lines. Data demonstrated that the down-regulation of TG2 led Caki-1, A-498, and Caki-2 close half of the wound area after 24 hrs whereas ACHN cells could only closed 35% of the scratch area suggesting that the RCC cell migration potential might be reduced with decreasing TG2 expression which in turn led to an aberration in TG2 induced ITGB1 activation. It was reported that the TG2 activity was found to be decreased in metastatic tumors [79]. Supporting this evidence, TG2 protein in metastatic RCC cells might act as a novel cell adhesion protein rather than transamidating enzyme activity which may result in metastasis and drug resistance in cancer cells.

ECM is a complex mixture of proteins secreted by cells and providing attachment sides for cell surface receptors. Adhesive proteins such as vitronectin, laminin (LM), fibronectin (FN), and collagen (Col) are some of the ECM proteins [84]. Integrinα1β1, integrinα2β1 and integrinα7β1 recognize both collagen type I (Col1) and LM, integrinα5β1 binds simply

to FN, integrinα6β1 recognizes LM, whereas integrin α3β1 binds FN, Col1, and LM [97]. The major integrin binding site to ECM proteins such as FN, Col1 and LM is the RGD region consisting Asparagine-Glycine and Arginine providing integrin receptors to recognize β1-integrin ligands [98]. Reports indicated that there is an interaction between TG2 and β1-integrin to enhance the cell adhesion [60, 99]. With respect to the interaction between TG2 and ITGB1, our hypothesis states that TG2 might also have a role to regulate the RCC cell migration. Therefore, the effect of TG2 silencing on the migration of RCC cell lines through β1-integrin substrates such as FN, Col1 and LM as a supportive agent for cell attachment was analyzed. Mehta et al. showed that increased TG2 expression rendered ovarian cancer resistant against paclitaxel and transwell assays indicated that TG2 provided the cell invasion through Matrigel. Silencing of TG2 via siRNA technology led ovarian cells to gain more sensitivity against chemotherapy [100]. In parallel with this study, another group demonstrated that increased TG2 expression together with FN elevate the cell migration potential in ovarian cancer cells. In vivo experiments showed that TG2 demonstrates its function by interacting with β1 integrin, suggesting that TG2 might have a novel in ovarian cancer metastasis [101]. Another study, focused on the effect of TG2 on the cell migration of Human CD8+ T cells suggesting that tissue transglutaminase might mediate transendothelial migration of T cells.

In this thesis, migration potentials of RCC cell lines were analyzed at optimized time intervals specific to each β1-integrin ligands. Transwell analysis indicated that primary Caki-2 cells showed the highest invasive potential at early time point on FN. Metastatic ACHN cells demonstrated the highest migration potential on Col1 at both early and late time pints comparing to other RCC cell lines suggesting that activity of TG2 might be low due to its metastatic property and TG2 would act as a novel cell adhesion protein together with the increased ITGB1 protein level. The most invasive potential on β1-integrin ligand LM at early time point was demonstrated by Caki-1 cells parallel with its skin metastatic property.

Previous studies reported that overexpression of TG2 is related with the drug resistance and metastasis [86, 87]. Studies demonstrated that TG2 plays an important role in promoting of cell invasion and migration. In this thesis project, silencing of TG2

expression using siRNA1 and siRNA6 transfection resulted in a distinct reduction in both cell adhesion and cell migration of RCC cell lines *in vitro.*

7. FUTURE DIRECTIONS

Although studies demonstrated the importance of transglutaminase II (TG2) in drug resistance and metastasis of tumors originated from epithelial tissues, yet there is only one study showing the importance of TG2 on renal cell carcinoma [79]. As a continuous of this work, in this thesis project primary and metastatic RCC (Renal Cell Carcinoma) cell lines were used as a model to elucidate the role of TG2 in RCC cell adhesion and migration. Therefore, this thesis project is the first work to elucidate the role of TG2 in RCC cell adhesion, migration and invasion at a molecular level.

The future prospects of the project includes the investigation of the expression of syndecan 4 (SDC4) in the RCC cell lines used. In the following work, TG2 association with SDC4 and the interaction between TG2 and integrin-β1 (ITGB1) in RCC progression and metastasis can be investigated. For this purpose, co-immunoprecipitation assay might be performed to investigate the interaction level of TG2-SDC4 and TG2-ITGB1 in primary (A-498 and Caki-2) and metastatic (Caki-1 and ACHN) RCC cell lines and RCC tumors in order to suggest TG2 in association with SDC4 and ITGB1 as a marker to indicate the metastatic potential of RCC.

APPENDIX

Table A.1. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-2 at T=3 hrs on β1 Integrin Substrate FN

One-way analysis of variance						
P value	< 0.0001					
P value summary	***					
Are means signif. different? (P < 0.05)	Yes					
Number of groups	3					
F	424.5					
R square	0.993					
ANOVA Table	SS	df	MS			
Treatment (between columns)	3962	2	1981			
Residual (within columns)	28	6	4.667			
Total	3990	8				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff	
NS vs siRNA1	45	36.08	Yes	***	39.59 to 50.41	
NS vs siRNA6	44	35.28	Yes	***	38.59 to 49.41	
siRNA1 vs siRNA6	-1	0.8018	No	ns	-6.412 to 4.412	

Table A.2. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-2 at T=6 hrs on β1 Integrin Substrate FN

One-way analysis of variance						
P value	< 0.0001					
P value summary	***					
Are means signif. different? (P < 0.05)	Yes					
Number of groups	3					
F	490.5					
R square	0.9939					
ANOVA Table	SS	df	MS			
Treatment (between columns)	5886	2	2943			
Residual (within columns)	36	6	6			
Total	5922	8				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff	
NS vs siRNA1	57	40.31	Yes	***	50.86 to 63.14	
NS vs siRNA6	51	36.06	Yes	***	44.86 to 57.14	
siRNA1 vs siRNA6	-6	4.243	No	ns	-12.14 to 0.1363	

Table A.3. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-2 at T=6 hrs on β1 Integrin Substrate Col1

One-way analysis of variance						
P value	< 0.0001					
P value summary	***					
Are means signif. different? (P < 0.05)	Yes					
Number of groups	3					
F	97.8					
R square	0.9702					
ANOVA Table	SS	df	MS			
Treatment (between columns)	6846	2	3423			
Residual (within columns)	210	6	35			
Total	7056	8				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff	
NS vs siRNA1	59	17.27	Yes	***	44.18 to 73.82	
NS vs siRNA6	58	16.98	Yes	***	43.18 to 72.82	
siRNA1 vs siRNA6	-1	0.2928	No	ns	-15.82 to 13.82	

Table A.4. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-2 at T=10 hrs on β1 Integrin Substrate Col1

One-way analysis of variance						
P value	< 0.0001					
P value summary	***					
Are means signif. different? (P < 0.05)	Yes					
Number of groups	3					
F	88.08					
R square	0.9671					
ANOVA Table	SS	df	MS			
Treatment (between columns)	8984	2	4492			
Residual (within columns)	306	6	51			
Total	9290	8				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff	
NS vs siRNA1	68	16.49	Yes	***	50.11 to 85.89	
NS vs siRNA6	66	16.01	Yes	***	48.11 to 83.89	
siRNA1 vs siRNA6	-2	0.4851	No	ns	-19.89 to 15.89	

Table A.5. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-2 at T=10 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	1082				
R square	0.9972				
ANOVA Table	SS	df	MS		
Treatment (between columns)	4326	2	2163		
Residual (within columns)	12	6	2		
Total	4338	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	46	56.34	Yes	***	42.46 to 49.54
NS vs siRNA6	47	57.56	Yes	***	43.46 to 50.54
siRNA1 vs siRNA6	1	1.225	No	ns	-2.543 to 4.543

Table A.6. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-2 at T=14 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	2503				
R square	0.9988				
ANOVA Table	SS	df	MS		
Treatment (between columns)	8342	2	4171		
Residual (within columns)	10	6	1.667		
Total	8352	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	69	92.57	Yes	***	65.77 to 72.23
NS vs siRNA6	59	79.16	Yes	***	55.77 to 62.23
siRNA1 vs siRNA6	-10	13.42	Yes	***	-13.23 to -6.766

Table A.7. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of A-498 at T=3 hrs on β1 Integrin Substrate FN

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	372.7				
R square	0.992				
ANOVA Table	SS	df	MS		
Treatment (between columns)	4472	2	2236		
Residual (within columns)	36	6	6		
Total	4508	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	44	31.11	Yes	***	37.86 to 50.14
NS vs siRNA6	50	35.36	Yes	***	43.86 to 56.14
siRNA1 vs siRNA6	6	4.243	No	ns	-0.1363 to 12.14

Table A.8. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of A-498 at T=6 hrs on β1 Integrin Substrate FN

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	183.6				
R square	0.9839				
ANOVA Table	SS	df	MS		
Treatment (between columns)	8322	2	4161		
Residual (within columns)	136	6	22.67		
Total	8458	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	65	23.65	Yes	***	53.07 to 76.93
NS vs siRNA6	64	23.28	Yes	***	52.07 to 75.93
siRNA1 vs siRNA6	-1	0.3638	No	ns	-12.93 to 10.93

Table A.9. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of A-498 at T=6 hrs on β1 Integrin Substrate Col1

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	111.4				
R square	0.9738				
ANOVA Table	SS	df	MS		
Treatment (between columns)	7206	2	3603		
Residual (within columns)	194	6	32.33		
Total	7400	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	59	17.97	Yes	***	44.76 to 73.24
NS vs siRNA6	61	18.58	Yes	***	46.76 to 75.24
siRNA1 vs siRNA6	2	0.6092	No	ns	-12.24 to 16.24

Table A.10. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of A-498 at T=10 hrs on β1 Integrin Substrate Col1

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	390.1				
R square	0.9924				
ANOVA Table	SS	df	MS		
Treatment (between columns)	10664	2	5332		
Residual (within columns)	82	6	13.67		
Total	10746	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	74	34.67	Yes	***	64.74 to 83.26
NS vs siRNA6	72	33.73	Yes	***	62.74 to 81.26
siRNA1 vs siRNA6	-2	0.937	No	ns	-11.26 to 7.261

Table A.11. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of A-498 at T=10 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	0.0026				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	18.83				
R square	0.8626				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3578	2	1789		
Residual (within columns)	570	6	95		
Total	4148	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	35	6.22	Yes	*	10.58 to 59.42
NS vs siRNA6	47	8.352	Yes	**	22.58 to 71.42
siRNA1 vs siRNA6	12	2.132	No	ns	-12.42 to 36.42

Table A.12. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of A-498 at T=14 hrs on β1 Integrin Substrate LM

One-way analysis of variance						
P value	< 0.0001					
P value summary	***					
Are means signif. different? (P < 0.05)	Yes					
Number of groups	3					
F	208.9					
R square	0.9858					
ANOVA Table	SS	df	MS			
Treatment (between columns)	6266	2	3133			
Residual (within columns)	90	6	15			
Total	6356	8				
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff	
NS vs siRNA1	49	21.91	Yes	***	39.30 to 58.70	
NS vs siRNA6	61	27.28	Yes	***	51.30 to 70.70	
siRNA1 vs siRNA6	12	5.367	Yes	*	2.298 to 21.70	

Table A.13. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-1 at T=3 hrs on β1 Integrin Substrate FN

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	136.3				
R square	0.9785				
ANOVA Table	SS	df	MS		
Treatment (between columns)	4634	2	2317		
Residual (within columns)	102	6	17		
Total	4736	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	41	17.22	Yes	***	30.67 to 51.33
NS vs siRNA6	53	22.26	Yes	***	42.67 to 63.33
siRNA1 vs siRNA6	12	5.041	Yes	*	1.671 to 22.33

Table A.14. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-1 at T=6 hrs on β1 Integrin Substrate FN

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	1264				
R square	0.9976				
ANOVA Table	SS	df	MS		
Treatment (between columns)	10958	2	5479		
Residual (within columns)	26	6	4.333		
Total	10984	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	73	60.74	Yes	***	67.79 to 78.21
NS vs siRNA6	75	62.4	Yes	***	69.79 to 80.21
siRNA1 vs siRNA6	2	1.664	No	ns	-3.215 to 7.215

Table A.15. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-1 at T=6 hrs on β1 Integrin Substrate Col1

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	104.6				
R square	0.9721				
ANOVA Table	SS	df	MS		
Treatment (between columns)	3626	2	1813		
Residual (within columns)	104	6	17.33		
Total	3730	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	41	17.06	Yes	***	30.57 to 51.43
NS vs siRNA6	44	18.31	Yes	***	33.57 to 54.43
siRNA1 vs siRNA6	3	1.248	No	ns	-7.430 to 13.43

Table A.16. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-1 at T=10 hrs on β1 Integrin Substrate Col1

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	119.7				
R square	0.9756				
ANOVA Table	SS	df	MS		
Treatment (between columns)	10374	2	5187		
Residual (within columns)	260	6	43.33		
Total	10634	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	37	9.735	Yes	**	20.51 to 53.49
NS vs siRNA6	83	21.84	Yes	***	66.51 to 99.49
siRNA1 vs siRNA6	46	12.1	Yes	***	29.51 to 62.49

Table A.17. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-1 at T=10 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	146.7				
R square	0.98				
ANOVA Table	SS	df	MS		
Treatment (between columns)	6846	2	3423		
Residual (within columns)	140	6	23.33		
Total	6986	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	59	21.16	Yes	***	46.90 to 71.10
NS vs siRNA6	58	20.8	Yes	***	45.90 to 70.10
siRNA1 vs siRNA6	-1	0.3586	No	ns	-13.10 to 11.10

Table A.18. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of Caki-1 at T=14 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	429.7				
R square	0.9931				
ANOVA Table	SS	df	MS		
Treatment (between columns)	7448	2	3724		
Residual (within columns)	52	6	8.667		
Total	7500	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	62	36.48	Yes	***	54.63 to 69.37
NS vs siRNA6	60	35.3	Yes	***	52.63 to 67.37
siRNA1 vs siRNA6	-2	1.177	No	ns	-9.375 to 5.375

Table A.19. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of ACHN at T=3 hrs on β1 Integrin Substrate FN

One-way analysis of variance					
P value	0.0004				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	38.21				
R square	0.9272				
ANOVA Table	SS	df	MS		
Treatment (between columns)	1554	2	777		
Residual (within columns)	122	6	20.33		
Total	1676	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	31	11.91	Yes	***	19.70 to 42.30
NS vs siRNA6	23	8.835	Yes	**	11.70 to 34.30
siRNA1 vs siRNA6	-8	3.073	No	ns	-19.30 to 3.296

Table A.20. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of ACHN at T=6 hrs on β1 Integrin Substrate FN

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	158				
R square	0.9814				
ANOVA Table	SS	df	MS		
Treatment (between columns)	4214	2	2107		
Residual (within columns)	80	6	13.33		
Total	4294	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	49	23.24	Yes	***	39.85 to 58.15
NS vs siRNA6	42	19.92	Yes	***	32.85 to 51.15
siRNA1 vs siRNA6	-7	3.32	No	ns	-16.15 to 2.147

Table A.21. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of ACHN at T=6 hrs on β1 Integrin Substrate Col1

One-way analysis of variance					
P value	0.0012				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	25.36				
R square	0.8942				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2942	2	1471		
Residual (within columns)	348	6	58		
Total	3290	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	41	9.325	Yes	**	21.92 to 60.08
NS vs siRNA6	35	7.96	Yes	**	15.92 to 54.08
siRNA1 vs siRNA6	-6	1.365	No	ns	-25.08 to 13.08

Table A.22. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of ACHN at T=10 hrs on β1 Integrin Substrate Col1

One-way analysis of variance					
P value	0.0012				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	24.91				
R square	0.8925				
ANOVA Table	SS	df	MS		
Treatment (between columns)	9632	2	4816		
Residual (within columns)	1160	6	193.3		
Total	10792	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	80	9.965	Yes	***	45.17 to 114.8
NS vs siRNA6	44	5.481	Yes	*	9.168 to 78.83
siRNA1 vs siRNA6	-36	4.484	Yes	*	-70.83 to -1.168

Table A.23. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of ACHN at T=10 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	73				
R square	0.9605				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2774	2	1387		
Residual (within columns)	114	6	19		
Total	2888	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	29	11.52	Yes	***	18.08 to 39.92
NS vs siRNA6	42	16.69	Yes	***	31.08 to 52.92
siRNA1 vs siRNA6	13	5.166	Yes	*	2.080 to 23.92

Table A.24. One-way ANOVA Analysis with Tukey post test for the Transwell Assay of ACHN at T=14 hrs on β1 Integrin Substrate LM

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	388.9				
R square	0.9923				
ANOVA Table	SS	df	MS		
Treatment (between columns)	6482	2	3241		
Residual (within columns)	50	6	8.333		
Total	6532	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
NS vs siRNA1	45	27	Yes	***	37.77 to 52.23
NS vs siRNA6	64	38.4	Yes	***	56.77 to 71.23
siRNA1 vs siRNA6	19	11.4	Yes	***	11.77 to 26.23

Table A.25. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of Caki-2 at T=24 hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	212.1				
R square	0.9876				
ANOVA Table	SS	df	MS		
Treatment (between columns)	7953	3	2651		
Residual (within columns)	100	8	12.5		
Total	8053	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	6	2.939	No	ns	-3.245 to 15.24
C vs siRNA1	50	24.49	Yes	***	40.76 to 59.24
C vs siRNA6	58	28.41	Yes	***	48.76 to 67.24
NS vs siRNA1	44	21.56	Yes	***	34.76 to 53.24
NS vs siRNA6	52	25.47	Yes	***	42.76 to 61.24
siRNA1 vs siRNA6	8	3.919	No	ns	-1.245 to 17.24

Table A.26. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of Caki-2 at T=48 hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	2721				
R square	0.999				
ANOVA Table	SS	df	MS		
Treatment (between columns)	16328	3	5443		
Residual (within columns)	16	8	2		
Total	16344	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	0	0	No	ns	-3.698 to 3.698
C vs siRNA1	69	84.51	Yes	***	65.30 to 72.70
C vs siRNA6	78	95.53	Yes	***	74.30 to 81.70
NS vs siRNA1	69	84.51	Yes	***	65.30 to 72.70
NS vs siRNA6	78	95.53	Yes	***	74.30 to 81.70
siRNA1 vs siRNA6	9	11.02	Yes	***	5.302 to 12.70

Table A.27. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of A-498 at T=24 hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	128.9				
R square	0.9797				
ANOVA Table	SS	df	MS		
Treatment (between columns)	870	3	290		
Residual (within columns)	18	8	2.25		
Total	888	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	1	1.155	No	ns	-2.922 to 4.922
C vs siRNA1	18	20.78	Yes	***	14.08 to 21.92
C vs siRNA6	17	19.63	Yes	***	13.08 to 20.92
NS vs siRNA1	17	19.63	Yes	***	13.08 to 20.92
NS vs siRNA6	16	18.48	Yes	***	12.08 to 19.92
siRNA1 vs siRNA6	-1	1.155	No	ns	-4.922 to 2.922

Table A.28. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of Caki-1 at T=24hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	5144				
R square	0.9995				
ANOVA Table	SS	df	MS		
Treatment (between columns)	7716	3	2572		
Residual (within columns)	4	8	0.5		
Total	7720	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	0	0	No	ns	-1.849 to 1.849
C vs siRNA1	44	107.8	Yes	***	42.15 to 45.85
C vs siRNA6	56	137.2	Yes	***	54.15 to 57.85
NS vs siRNA1	44	107.8	Yes	***	42.15 to 45.85
NS vs siRNA6	56	137.2	Yes	***	54.15 to 57.85
siRNA1 vs siRNA6	12	29.39	Yes	***	10.15 to 13.85

Table A.29. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of Caki-1 at T=48hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	2308				
R square	0.9988				
ANOVA Table	SS	df	MS		
Treatment (between columns)	13850	3	4617		
Residual (within columns)	16	8	2		
Total	13866	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	0	0	No	ns	-3.698 to 3.698
C vs siRNA1	62	75.93	Yes	***	58.30 to 65.70
C vs siRNA6	73	89.41	Yes	***	69.30 to 76.70
NS vs siRNA1	62	75.93	Yes	***	58.30 to 65.70
NS vs siRNA6	73	89.41	Yes	***	69.30 to 76.70
siRNA1 vs siRNA6	11	13.47	Yes	***	7.302 to 14.70

Table A.30. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of ACHN at T=24hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	70.29				
R square	0.9634				
ANOVA Table	SS	df	MS		
Treatment (between columns)	369	3	123		
Residual (within columns)	14	8	1.75		
Total	383	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	0	0	No	ns	-3.459 to 3.459
C vs siRNA1	10	13.09	Yes	***	6.541 to 13.46
C vs siRNA6	12	15.71	Yes	***	8.541 to 15.46
NS vs siRNA1	10	13.09	Yes	***	6.541 to 13.46
NS vs siRNA6	12	15.71	Yes	***	8.541 to 15.46
siRNA1 vs siRNA6	2	2.619	No	ns	-1.459 to 5.459

Table A.31. One-way ANOVA Analysis with Tukey post test for the Wound Scratch Assay of ACHN at T=48hrs

One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	39.4				
R square	0.9366				
ANOVA Table	SS	df	MS		
Treatment (between columns)	5732	3	1911		
Residual (within columns)	388	8	48.5		
Total	6120	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant	Summary	95% CI of diff
C vs NS	6	1.492	No	ns	-12.21 to 24.21
C vs siRNA1	47	11.69	Yes	***	28.79 to 65.21
C vs siRNA6	46	11.44	Yes	***	27.79 to 64.21
NS vs siRNA1	41	10.2	Yes	***	22.79 to 59.21
NS vs siRNA6	40	9.948	Yes	***	21.79 to 58.21
siRNA1 vs siRNA6	-1	0.2487	No	ns	-19.21 to 17.21

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