CONTACTIN-1 AS A POTENTIAL ONCOGENIC FACTOR IN CLEAR CELL RENAL CARCINOMA

CONTACTIN-1 AS A POTENTIAL ONCOGENIC FACTOR IN CLEAR CELL RENAL CARCINOMA

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Abstract

Renal cell carcinoma (RCC), following prostate and bladder tumours, is the third most prevalent genitourinary malignancy. Clear cell renal cell carcinoma makes up the bulk of RCC cases (ccRCC). Despite the fact that ccRCC is the most aggressive type of RCC, our understanding of its pathophysiology is limited. Previous research in our laboratory revealed important oncogenic roles of contactin 1 (CNTN1), a neuronal cell adhesion protein, in prostate cancer. CNTN1 is involved in a number of signalling pathways that are often changed in cancer, including the VEGFC-VEGF receptor 3 (VEFGR3)/fms-related tyrosine kinase 4 (Flt4) axis, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) axis, and the Notch signalling system. Collectively, evidence suggests that CNTN1 facilitates ccRCC. To examine this possibility, I have established stable ccRCC 786-O and A498 cell lines expressing either empty vector (EV) or CNTN1. In comparison to the respective EV lines, ectopic expression of CNTN1 enhances colony formation and cell proliferation. In comparison to A498 EV cells, A498 CNTN1 cells seems to possess enhanced migration ability based on wound healing assay. Taken together, my research provides in vitro evidence supporting CNTN1 in facilitating ccRCC pathogenesis. Future research will be required to investigate this concept using in vivo systems and primary ccRCC tumor tissues.

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AKT	Protein Kinase B
ccRCC	Clear Cell Renal Cell Carcinoma
CNTN-1	Contactin-1
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
HIF1α	Hypoxia-inducible factor α
IHC	Immunohistochemistry
IFN-α	Interferon alpha
IgSF	Immunoglobin Super Family
IL2	Interleukin 2
mTOR	Mammalian target of rapamycin
PBS	Phosphate buffer saline
PCSC	Prostate Cancer Stem Cells
PD-1	Programmed Death 1
PI3K	Phosphatidylinositol-3 kinase
pRCC	Papillary renal cell carcinoma
RCC	Renal cell carcinoma
VEGF	Vascular endothelial growth factor
VHL	von hippel-lindau
VSV-G	Vesicular stomatitis virus G protein

DECLARATION OF ACADEMIC ACHIEVEMENT

I, Riad Houha, declare this thesis to be my own work. I am the sole author of this document. No part of this work has been published or submitted for publication or for a higher degree at another institution.

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I. Introduction

1.1 Overview of Renal Cell Carcinoma

Renal cancer impacts 7,900 Canadians annually and it is responsible for taking 2,000 lives in Canada every year (North et al. 2015). Renal cancer has a higher incidence in males than in females, and the median age for disease manifestation is 60 years old (Valerie et al., 2018). Wilms tumour, primary neoplasms of the renal pelvis/ureter, and renal cell carcinoma are among the several types of kidney cancer. Wilms tumour has an incidence rate of 1 in 10,000 births and possesses a cure rate upwards of 80% (Kutluk et al, 2006). Renal cell carcinoma, or RCC, is the third most common genitourinary cancer after prostate and bladder cancer (Hsieh et al., 2017). This accounts for 3% and 4% of newly diagnosed malignancies in adult females and males, respectively (Bray, 2018). RCC consists of several kidney epithelial neoplasms, as clear cell RCC (ccRCC, 80%), papillary RCC (pRCC, 15%), and chromophobe RCC (chRCC, 5%) are the three major subtypes (Hsieh et al., 2017). The most hostile type of RCC is clear cell RCC. About 40-45% of ccRCC patients at the point of diagnosis have locally advanced or metastatic cancer and 30% of initially organ-confined cases will develop metastases (Cairns, 2010). Typical clinical features of RCC include hematuria, flank pain, or a palpable abdominal mass are signs of ccRCC (Cohen et al., 2019). CcRCC patients may also experience systemic effects due to secreted proteins, including parathyroid hormone-related proteins that trigger hypercalcaemia, renin that causes hypertension, or erthyropoietin that results in erythrocytosis (Le et al., 2018). Diagnosis is commonly based on medical imaging procedures, including CT scans, MRI and ultrasound. Despite the numerous advancements in imaging diagnosis over the past two decades, however, small renal tumour masses (SRM) remain very difficult to detect and treat, as the distinction between benign and malignant masses remains blurred (Cohen et al., 2019). The

prognosis for ccRCC depends primarily on the clinical stage and the nuclear classification (Hsieh et al., 2017). Five and 10-year survival rates are approximately 95% for stage 1, 81-88% for stage 2, 43-59% for stage 3, and 14-20% for stage 4 disease (Feng et al., 2019).

1.2 Treatment of RCC

Surgery is the first choice for curative therapy by either radical or partial nephrectomy if ccRCC is identified at an early stage (Hsieh et al., 2017). Even in this group of patients with good prognosis, approximately 20-40% patients will have relapse (Yan et al., 2020); these recurrent tumors are associated with increased risk of metastasis (Yan et al., 2020). The common metastatic sites for ccRCC include lungs, liver, lymph node and bone (Cassell et al., 2019; Cohen et al., 2019). Patients with metastasized ccRCC have a poor prognosis. For these patients, systemic treatments are available. Immunotherapy agents such as interleukin 2 (IL2) and interferon alpha (IFN-alpha) have been used with minimal effectiveness but associate with extreme side effects (North et al., 2015). Latest progress enables metastatic ccRCC patients to undergo targeted therapies. The dependency of tumour cells on biological pathways is the theory behind selective therapies. The vascular endothelial growth factor (VEGF) pathway is one of the most frequent pathways attacked. VEGF is a potent growth factor that plays an important role in angiogenesis. The VEGF pathway is inhibited by two main approaches. One is to use tyrosine kinase inhibitors, including sunitinib, sorafenib, axitinib, and pazopanib, to block the tyrosine kinase function of the VEGF receptor (Hsieh et al., 2017). These drugs are widely used in treating metastatic ccRCC (Hsieh et al., 2017). Another strategy is to avoid VEGF from binding to its receptor by neutralising VEGF using monoclonal antibodies. Bevacizumab is one such drug as the mTOR pathway is often targeted (Hsieh et al., 2017).

MTOR is a kinase of serine/threonine which regulates angiogenesis and survival of cells. Rapamycin, temsirolimus, and everolimus are the compounds used to block the mTOR pathway (Saxton et al., 2017). In clinical trials, these medicines have been and are still being studied. A randomised phase III trial compared sunitinib with IF-alpha as the first line of therapy for advanced RCC revealed that the sunitinib arm had a response rate of 31 percent versus an IF-alpha response rate of 6 percent (Larkin et al., 2009). Progression-free mean survival was 11 months for sunitinib and 5 months for IF-alpha (Larkin et al., 2009). Despite these advancements in targeted treatments, metastatic ccRCC is still an incurable condition.

Currently, immune checkpoint inhibitors are increasingly becoming standard of care in the treatment of a variety of cancers, including ccRCC, where one drug, nivolumab, an anti-programmed death-1 (PD-1) monoclonal antibody (mAb), is approved for patients with ccRCC (Goodman et al., 2016). RCC has tumor-associated PD-L1 expression, which is associated with a poor prognosis. PD-L1 is heavily expressed in tumor cells and tumor-infiltrating lymphocytes. In renal cancer, the presence of PD-L1 on cancer cells in primary tumors is linked to a poor prognosis (Taube et al., 2014). PD-L1 appears to be the main ligand in solid tumors, while PD-1's replacement ligand, PD-L2, appears to be the main ligand in B-cell lymphoma. PD-1 is more widely expressed than CTLA-4, and it is seen on other activated non-T-lymphocyte subsets including B cells and NK cells, decreasing their lytic potential (Taube et al., 2014). PD-1, like CTLA-4, is not found on memory T cells but is expressed during antigen detection and TCR interaction. In many solid tumours, including ccRCC, targeted therapies against the PD-1 receptor and its ligand PD-L1have shown excellent reaction rates with reduced toxicity.

1.3 RCC Genetics

CcRCC results from the aggregation of mutations. The Von Hippel Lindau (VHL) tumour suppressor gene is most frequently mutated in ccRCC (Hsieh, 2017). Changes in the MET and the fumarate hydratase (FH) gene are associated with papillary type 1 and type 2 RCC respectively. The defects in the Birt Hogg Dube (BHD) gene are also associated with Chromophobe RCC (5%) and oncocytoma (5%) (Linehan et al., 2004). In patients with ccRCC, mutations in tumour suppressor genes found in three unique regions on chromosome 3p have been identified. The t (3;8) translocation includes 3p12-p14, a region that is aligned with inherited ccRCC and comprises the FHIT (fragile histidine triad) gene, a putative tumour suppressor gene. In multiple tumours, including kidney and lung cancer, the deletion of 3p21.2-p21.3 happens regularly (Moore et al., 2011). 3p25-p26 containing the VHL tumour suppressor gene is the third altered region. VHL gene mutation is an early phenomenon of ccRCC pathogenesis (Moore et al., 2011). Individuals suffer from VHL disease with one wild form VHL and one inactivated VHL allele (Moore et al., 2011). The VHL disease involves multiple tissues, the predominant one being the kidneys. Owing to the inactivation of the wild type VHL allele, people with VHL disease are at risk for the formation of renal cysts. Such cysts can progress to ccRCC (Wolf et al., 2020). In nearly all families of VHL disease, germ line mutations of the VHL gene have been identified, causing it to become the most common cause of inherited ccRCC, whereas two thirds of sporadic ccRCC cases are impacted by pVHL loss of function (Haas et al., 2015). The hypoxia inducible factor 1-aph (HIF1-aph) transcription factor for ubiquitin-mediated degradation is the target of the VHL protein complex. Loss of VHL contributes to HIF1-alpha stabilisation, which transactivates several target genes, including VEGF, glucose transporter (GLUT 1), growth factor derived from platelets (PDGF), growth

factor-β transform (TGF-β) and erythropoietin (EPO) (Linehan et al., 2004). By boosting angiogenesis, cell survival and cell proliferation, these proteins facilitate tumorigenesis. In addition to the genes found on chromosome 3p that inhibit the tumour, abnormalities also contribute to ccRCC tumorigenesis of other chromosome areas, including the gain of chromosome 5p and chromosome 8p, 9p and 14q loss (Moore et al., 2011).

1.4 Contactin-1

Contactin-1 (CNTN1) was the first of the six CNTNs to be discovered, and the one that has been studied the most extensively for its involvement in diseases. CNTN1 is made up of six Immunoglobulin (Ig) C2 domains at the N-terminus, four fibronectin type III (FNIII) repeats at the C-terminus, and a hydrophobic C-terminal amino acid chain (Yan et al., 2016). CNTN1 is predominantly detected in the brain and cortical tissues in humans, with only minimal expression in other organs. CNTN1 is mainly an axonal glycoprotein that helps in axonal growth and neurite outgrowth, but it also has important roles in other neuronal developmental processes such as glial cell differentiation and production, myelination, and synaptogenesis (Chen et al., 2015). CNTN1 binds to extracellular matrix molecules in a variety of ways. The interaction of CNTN1 with tenascin-C, tenascin-R, and receptor protein tyrosine phosphatase (RPTP) facilitates axonal development and fasciculation, two essential aspects of neuron-glial integration (Lamprianou et al., 2011). The transmembrane protein tyrosine receptor type Z (PTPRZ) on the glial cell surface interacts with CNTN1 on the axon surface, facilitating neurite outgrowth and glial adhesion. Via its interactions with PTPRZ and the Notch receptor, CNTN1 regulates the maturation and proliferation of oligodendrocyte precursor cells (OPCs) (Bouyain et al., 2010; Sakurai et al., 1997). Interestingly, while CNTN1 interacts with the RPTPß substrate to activate DRG sensory

axon extension, it interacts with the Tenascin-R substrate to inhibit the cerebellar granule neuron receptor, suggesting that CNTN1's functions may vary based on the cellular and molecular context (Haenisch et al., 2005). Clinically, CNTN1 expression is significantly decreased in patients with age-related memory loss, implying that CNTN1 plays a part in memory-related processes (Haenisch et al., 2005). A lethal type of congenital myopathy was previously identified, which was thought to be caused by the loss of CNTN1 in the neuromuscular junction due to a familial mutation (Haenisch et al., 2005). These results indicate that CNTN1's adhesion mechanisms play a significant role in the formation of the central nervous system. Adhesion is a critical mechanism in tumorigenesis and cancer development, and it is worth revisiting the evidence for CNTN1's oncogenic roles.

1.5 Contactin-1 Tumorigenesis

The IgSF's (immunoglobin superfamily) neural cell adhesion molecules, which include CNTNs, neural cell adhesion molecule, L1, and neuron-glia associated (Nr)-CAM, are complex membrane-anchored proteins that mediate molecular interactions. It's becoming apparent that improvements in cancer cells' adhesive properties, such as cell adhesion and adhesion to the extracellular matrix, play a role in tumor growth (Haenisch et al., 2005). Previous research has suggested that some members of the Ig superfamily may play a role in tumor invasion and spread. NCAM is the first CAM discovered, and it has five Ig-like and two fibronectin type III repeats, as well as two main transmembrane isoforms and a GPI-linked isoform (Lehembre et al., 2008). According to research, NCAM is generally upregulated during EMT and facilitates an adhesion transition that is related to cancer invasion. The reciprocal staining of E-cadherin and NCAM from the RipTag2 mouse model of pancreatic cancer and aberrant persistence of E-

cadherin expression resulting from NCAM deficiency indicate that forced expression of NCAM promotes mesenchymal-like properties.

CNTN1 has structural and functional similarities to NCAM, but its role outside of the nervous system has been relatively unknown until recently. Low levels of CNTN1 transcripts have been found in many tissues, including the pancreas, liver, kidney, and skeletal muscles, but how CNTN1 acts in these tissues is unclear (Berflund et al., 1994). Nevertheless, since the central nervous system was the only organ in CNTN1 deficient mice that was abnormal, the protein did not seem to play a significant role in the development of other organs in mice. The CNTN1 gene is found on chromosome 12q11-q12, and it is the only IgSF gene that is found there (Pollerberg et al., 2013). Incidentally, several other genes that are mapped between 12q11 and q13 have all been linked to human cancers. This involve the proto-oncogenes erb-b3 and int-1, as well as the homeobox protein HOX3, the integrin 5 subunit, collagen type II, and the integrin 5 subunit (Pollerberg et al., 2013). The near proximity of the CNTN1 gene locus to a tumor breakpoint suggests that CNTN1 may function in tumour development and progression. CNTN1 expression has been shown to be upregulated in many cancers, including lung, gastric prostate, and breast cancer, as interest in its cancer-related functions has grown.

1.6 Contactin-1 Across Cancers

The oncogenesis of Contactin-1 and its impact on tumorigenesis has been studied across a wide variety of cancers, which has helped advance our understanding of its role. Su et al. used a genome-wide cDNA microarray study to look for new regulatory genes involved in cancer invasion and metastasis and discovered CNTN1 as a metastasis-promoting oncogene in lung cancer. The vascular endothelial growth factor (VEGF)C-VEFGR3/Flt4 caused invasion and

metastasis in lung cancer through the Src/p38 MAPK-mediated C/EBP signaling pathway, and CNTN1 was discovered as an immediate downstream effector (Wang et al., 2016). The activation of the VEGF-C/Flt4 axis significantly increased CNTN1, resulting in the rearrangement of F-actin-containing microfilament bundles essential for cell motility, increased cell invasion in vitro, and increased lung metastasis development in vivo (Wang et al., 2016). In lung adenocarcinoma patients, CNTN1 expression was shown to be linked to Flt expression, tumor level, lymph node metastasis, and patient survival. Via promoting angiogenesis and lymphangiogenesis, the vascular endothelial growth factor (VEGF)/VEGF-receptor signaling pathway is involved in the growth, invasion, and metastasis of carcinomas (Wang et al., 2016). During lymph node metastasis, tumor cells may travel through freshly developed lymphatic capillaries or pre-existing afferent lymphatic vessels. Extracellular VEGFC acts as a ligand, cell membrane VEGFR3 (Flt4) acts as a receptor, and extracellular or intracellular pathway-related molecules act as executors in the VEGFC-VEFGR3/Flt4 biochemical axis (Kaushal et al., 2005). The behavior of CNTN1 in this regard can be mediated via the facilitation of the epithelial-to-mesenchymal transfer (EMT). During production, epithelial cells rely on a finely tuned and closely controlled EMT to convert to a mesenchymal state. EMT gives cells properties including increased migratory and invasive abilities, increased apoptosis resistance, and increased development of extracellular matrix components, all of which help cancer spread (Zhang et al., 2018). Local cancer epithelial cells hijack the evolutionarily conserved EMT pathway in tumor cells, weakening their polarity and cell-cell connectivity and inducing abrupt cytoskeletal remodeling to acquire an invasive, well-defined mesenchymal phenotype that involves E-cadherin loss and overexpression of mesenchymal proteins (Lamouille et al., 2014; Zhang et al., 2018). Although CNTN1 knockdown did not affect lung cancer cell proliferation, it did

significantly reduce cancer cell invasive abilities in vitro and in xenograft models, according to Yan et al. CNTN1 specifically plays this metastasis-promoting role by inhibiting E-cadherin. Upstream and downstream phosphatases, PTEN and PHLPP, regulate AKT activity. AKT activation decreases E-cadherin expression and is a central feature of EMT in cancer cell invasion and metastasis (Molina et al., 2016). CNTN1 induces EMT in lung cancer cells, which facilitates chemo-resistance. BCT-100 is an anti-cancer drug that treats arginine auxotrophic tumors such as small cell lung cancer (Xu et al., 2012). CNTN1 was upregulated and induced an EMT phenotype in resistant cells by targeting of the AKT pathways in a gene chip assay comparing BCT-100-resistant cells to parent cells. CNTN1 silencing re-sensitized resistant cells to BCT-100 therapy and reduced EMT phenotypes in resistant cell lines, according to further quantitative research (Xu et al., 2018). CNTN1 expression was also higher in multidrug-resistant (MDR) A549/cisplatin (A549/DDP) cells than in their progenitor A549 lung cancer cells and silencing of CNTN1 increased cisplatin sensitivity and upregulated cisplatin-induced apoptosis, inhibiting tumor invasion and metastasis (Zhang et al., 2015). This CNTN1-enhanced chemoresistance of lung cancer cells was also related to PI3K/AKT-mediated EMT enhancement. Indeed, CNTN1 downregulation successfully hindered cisplatin resistance and malignant development by partly inactivating the EMT system by regulating the PI3K/AKT pathway (Zhang et al., 2015).

1.7 Contactin-1 in Urinary Tract Disorders

CNTN-1 plays a role in genitourinary malignancy. Our laboratory has previously studied the various impacts of CNTN-1 in prostate cancer and substantial conclusions were drawn. This research is relevant to my project, as both kidney and prostate cancer belong to the same family

of urinary tract disorders. By using DU145 and other prostate cancer cells, the contributions of CNTN1 to cell proliferation, metastasis and cancer progression have been investigated. It was found that ectopic CNTN1 increased AKT activation in DU145 CNTN1 and LNCaP C4-2 CNTN1 cells in response to serum stimulation when compared to their respective EV counterparts, despite ectopic CNTN1 having no effect on the basal levels of AKT activation in both lines (Yan et al., 2016). CNTN1 knockdown in DU145 cell-derived prostate cancer stem cells (PCSCs), on the other hand, inhibited AKT activation (Yan et al., 2016). AKT activation was shown to be higher in DU145 CNTN1 monolayer cell–produced xenograft tumours than in DU145 EV cell–derived xenograft tumours, however the differences were not significant, which might have been due to a variety of reasons. However, AKT activity was significantly reduced in xenograft tumours generated by CNTN1-knockdown DU145 spherical cells (Yan et al., 2016). In conclusion, these findings support the theory that CNTN1 is involved in AKT activation in prostate cancer cells. The capacity of cancer stem cells (CSCs) to induce cancer in immunocompromised animals is what distinguishes them. The presence of CNTN1 in DU145-derived PCSCs, as well as its involvement in boosting prostate cancer cell invasion and AKT activation, all point to a role for CNTN1 in DU145 PCSC-associated tumour initiation. In DU145 spherical (PCSCs) cells, knockdown of CNTN1 did not affect the cell's capacity to produce spheres (Yan et al, 2016). However, CNTN1 knockdown substantially decreased the potential of DU145 sphere cells to develop tumours when compared to DU145 Ctrl shRNA sphere cells (Yan et al., 2016). In xenograft tumours generated by CNTN1 knockdown spherical cells, CNTN1 expression was substantially reduced (Yan et al., 2016). Furthermore, overexpression of CNTN1 in DU145 monolayer cells increased the development of xenograft tumours. CNTN1 levels in DU145 CNTN1 cell–produced xenograft tumours remained higher

than in DU145 EV cell–derived tumours (Yan et al., 2016). DU145 CNTN1 cells produced xenograft tumours, hence CNTN1 was clearly visible on the cell surface. Despite the variability in CNTN1 expression in xenografts, the findings show that CNTN1 plays an essential role in prostate tumour development. My peers looked at the expression of CNTN1 in primary prostate cancer tissues to learn more about its function in prostate cancer carcinogenesis. In IHC staining, an anti-CNTN1 antibody identified tumor associated CNTN1 with no detectable staining with control IgG, and the positive signals were competed off with a CNTN1 peptide (Yan et al., 2016). CNTN1 intensity was found to be either negative or very low in normal prostate glands and high in advanced prostate carcinomas (Gleason 8-10) in a small number of patients from our cohort, which included three samples with Gleason Score 6 to 7, six 8 to 10, three pairs of local and lymph node metastases, and nine bone metastases (Yan et al., 2016).

Collectively, cell invasion and AKT activation are enhanced by CNTN1, but E-cadherin expression is reduced. Furthermore, CNTN1 promotes xenograft tumour formation and lung metastasis by upregulating AKT activation and downregulating E-cadherin at the same time. CNTN1 is found in high-risk primary prostate cancer as well as lymph node and bone metastases in prostate cancer tissues, and it is linked to prostate cancer development. As a result, these findings are the first to show that the neuronal CAM CNTN1 promotes prostate cancer growth and metastasis. The results demonstrated by the work of Yan et al. presents a strong case for similar investigations to be undertaken on CNTN-1 in different urinary tract disorders. Hence, previous research on prostate cancer has allowed us to conceptualize and ponder the same questions regarding kidney cancer; and providing strong clinical relevance to justify my research project.

II. Hypothesis

Based on the current knowledge of CNTN1's contributions to oncogenesis in multiple cancer types, particularly its role in promoting prostate cancer. I hypothesize CNTN1 facilitating ccRCC pathogenesis. The main objectives are to examine the impact of CNTN1 on a set of in vitro oncogenic events in two ccRCC cell lines: 786-O and A498. Specifically, both cell lines will be engineered to express empty vector (EV) or CNTN1, followed by analyzing cell's abilities of colony formation, proliferation, and migration.

III. MATERIALS AND METHODS

3.1. Reagents

Ampicillin, ammonium per sulphate (APS), calcium chloride (CaCl2), crystal violet dimethyl sulfoxide (DMSO), ethidium bromide, EDTA, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), β-glycerophosphate, Triton X-100, Tween 20, βmecaptoethanol, puromycin, bromophenol blue, ammonium per sulphate (APS), iodacetamide, sodium bicarbonate (CHNaO3), Carbonate (Na2CO3), puromyocin, potassium dihydrogen orthophosphate (KH2PO4), disodium hydrogen orthophosphate (Na2HPO4), glycerol, thiourea and GenElute Plasmid Miniprep kit were purchased from Sigma, Oakville, ON. Agarose, bovine serum albumin (BSA), Tris base, glycine, sodium dodecyl sulphate (SDS), sodium citrate, sodium chloride (NaCl), TEMED were purchased from Bioshop Burlington, ON. Trypsin-EDTA was purchased from Invitrogen, Carlsbad, CA. Methanol and isopropyl alcohol were purchased from Caledon Laboratories, Georgetown, ON and reagent alcohol was purchased from Fisher Scientific, Ottawa, ON. Anhydrous Ethyl Alcohol was purchased from Commercial Alcohols, Brampton, ON. Ultraclean 15 DNA purification kit was purchased from MoBio Laboratories Carlsbad, CA. 30% Bis Acrylamide solution, Biolytes 3/10, Urea, CHAPS and IPG strips was purchased from Bio-Rad, Mississauga, ON.

3.2. Cell Lines

293T human embryonic kidney cells, human ccRCC cell lines 786-O and A498 were purchased from American Type Culture Collection (ATCC, Manassas VA) and cultured in RPMI 1640, MEM, DMEM media supplemented with 10% fetal bovine serum and 1%

Penicillin-Streptomycin (Invitrogen, Burlington ON). The cells were grown in a 37C and 5% CO2 tissue culture incubator.

3.3. Protein Analysis

Cell lysates were prepared by washing the cells twice with phosphate buffered saline (PBS) on ice (PBS: 1.36 M NaCl, 14.7 mM KH2PO4, 80 mM Na2HPO4, 26.8mM KCl pH 7.2), harvesting cells in PBS using a cell scraper, and centrifugation at 2000rpm at 4^{o}C for 5 minutes. PBS was aspirated and the cell pellet was resuspended in lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate (Na3VO4), 1 mM PMSF, 2 µg/ml leupeptin and 10 µg/ml aprotinin for 30 minutes on ice. The lysates were then centrifuged at 13,000rpm at 4^{o}C for 5 minutes. The supernatant was collected, and protein concentration was determined using a Bradford assay (Bio-Rad, Mississauga, ON). Absorbance was measured at 595nm.

3.4. Western Blot Analysis

50 µg of total lysate protein (for cell and tissue lysates) was diluted in 5xPSB (Protein sample buffer, 0.1 mM Tris pH 6.8, 5% SDS, 50% glycerol, 2% βmercaptoethanol, 0.02% bromophenol blue and add ddH2O to a final concentration of 1XPSB). The protein was denatured by boiling for three minutes at 100^{o}C in a heat block. The samples were separated on a 10% SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel with a 3% stacking gel at 50 mA in running buffer (20 mM Tris-HCl, 192 mM glycine, 1% SDS and ddH2O to a final concentration of 1x running buffer), followed by transfer in transfer buffer (25

mM Tris-HCl, 192 mM glycine, 20% methanol) onto Immobilon-P membranes (Millipore, Billerica MA) at 260 mA for 80 minutes. Membranes were blocked with 5% skim milk for one hour while shaking at 55 rpm and then washed with 1xTBST (Tris buffered saline + 0.1% Tween 20) at 150 rpm three times. Membranes were incubated at 4oC over night with the indicated antibodies prepared in 5% bovine serum albumin solution in 1x TBST. The appropriate secondary antibodies were prepared in 5% skim milk and incubated for an hour at 55rpm. The membranes were then washed with 1xTBST three times for 10 minutes each. Signals were detected using an ECL Western Blotting Kit (Amersham, Pittsburg PA). Excess ECL solution was absorbed using a paper towel before exposure onto Kodak X-OMAT X ray film. Primary antibodies and concentrations used were anti-contactin 1:1000 (Santa Cruz, Santa Cruz CA), anti-actin 1:1000 (Santa Cruz, Santa Cruz CA), anti-phospho AKT 1:500 (Cell Signaling, Danvers MA), anti-AKT 1:1000 (Santa Cruz, Santa Cruz CA), anti-phospho ERK 1:1000 (Cell Signaling, Danvers MA), anti ERK 1:1000 (Santa Cruz, Santa Cruz CA). Western blot images were analyzed using imageJ software (National Institute of Health, USA).

3.5. Generation of Stable Line

Packaging retrovirus was performed using 293T embryonic cells. 10 µg of pVPackVSV-G, and 10 µg of pVPack-GP [Agilent Technologies (Mississauga, ON)] plus 10 µg of the retroviral vector (pBabe CNTN1 and pBabe EV) were used for a calcium phosphate transfection into 293T cells. The calcium phosphate transfection cocktail included 50 µL of 2.5M $CaCl_2$, H_2O and the required vectors for a total volume of 500 µl in a 13 ml tube. In between the addition of each component of the cocktail the tube was gently vortexed to mix the solution. Subsequently, another tube with 500 µl of 2 x HeBS (HeBS, 0.28 M NaCl, 0.05 M hepes, 1.5 mM Na2HPO4,

pH 7.1) was gently vortexed as the solution from the first tube was added drop wise using a glass pipette, followed by 30 seconds of vortexing. This solution was incubated at room temperature for 20 minutes. Next, using a Pasteur pipette the transfection solution was mixed several times and added drop wise to the entire plate of 293T cells, the plate was mixed in a figure eight configuration to allow for proper distribution of calcium and DNA precipitate. The plate was incubated for 10 hours at 37ºC and 5% CO_2. Media was then changed for fresh complete DMEM media. Cells were incubated for another 48 hours. At this point, the supernatant containing virus particles was obtained and filtered through a 0.45 μm filter, 3 mL of supernatant was incubated with 786O and A498 cells, which were seeded the night before infection. 786-O or A498 cells with the virus supernatant was swirled in the tissue culture plate every 20 minutes for two hours. Virus media was then replaced with fresh media and incubated for another 24-48 hours at which point the cells were selected by the addition of 1μg/ml of puromycin.

3.6. Colony Formation Assay

786O/A498 EV and 786O/A498 CNTN1 cells were seeded at 100, 500, or 1000 cells per well, followed by culture in MEM/RPMI media in the presence of 1 μg/ml puromyocin for approximately two weeks until the colonies being clearly visible. Media was changed every 3 days. At the end of the two weeks, media was aspirated, and the plate was washed twice with 1xPBS. The cells were then fixed with fixing solution (2% formaldehyde and 0.2% glutaraldehyde in PBS) for 20 minutes, washed with PBS and stained for 20 minutes with 0.3% crystal violet. Excess staining including background stain was reduced by dipping the plates into water multiple times. This experiment was repeated 3 times.

3.7. Proliferation Assay

For the proliferation assay, cells were seeded, and cell numbers were then counted using a hemocytometer every day for a period of 7 days. The formula used to calculate the number of cells in culture was as followed: [(# of cells in 4 quadrants/4) x 10,000] x volume of solution added.

3.8. Wound Healing Assay

A 6 well plate was seeded with 100,000 786O CNTN1/EV cells and 50,000 A498 CNTN1/EV cells were cultured for 2 days in a 37oC incubator until cells were 100% confluent. A wound was then introduced by using a 1ml blue pipette tip to create a vertical scratch through the cells. The media was then aspirated to remove the cells that had been scraped off. This was followed by the addition of 2 ml of MEM/RPMI media to each well. Images of the vertical scratch (wound) were taken at three different locations, top, middle and bottom of the line. Images were taken at 2.5x magnification at 0, 4 hours, 8 hours and 24 hours.

IV. RESULTS

4.1. Establishing CNTN-1 Stable ccRCC Cell Lines

786-O EV, 786-O CNTN1, 498 EV, and 498 CNTN1 lines were using pBabe-based retrovirus, pBabe and pBabe CNTN1. Specifically, the retrovirus was packaged into embryonic 293T cells, which were used to infect A498 and 786O cells. Stable lines were selected using puromycin, the antibiotic selection marker for pBabe. The stable expression of CNTN1 in the respective cell lines was confirmed by Western blot analysis (Fig 1).

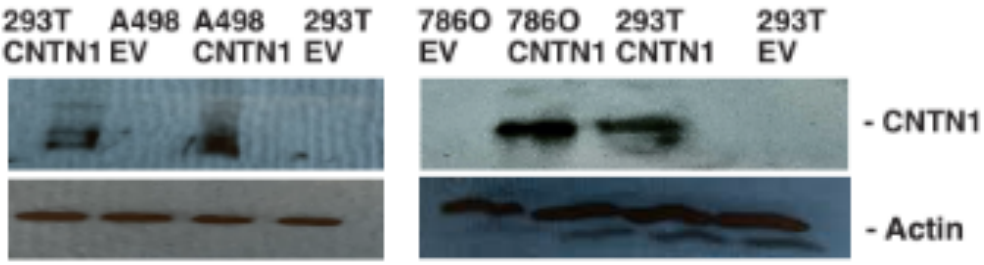

Figure 1 – Construction of CNTN1 stable lines. 786-O and A498 ccRCC cells were stably expressed with empty vector (EV) and CNTN1. Western blot analyses for these cell lines and 293T cells transiently transfected with EV and CNTN1 were performed for the indicated proteins and used as a positive control.

4.2 Enhancement of Colony Formation by CNTN1

Using the stable cell lines established, the impact of CNTN1 on cell's ability for forming colonies was first examined. A colony formation helps demonstrate a single cell's ability to form a colony. Hence, 100, 500 and 1000 cells were seeded in a 6 well plate. The cells were then monitored for growth period of 13 days. In comparison to their respective EV stable line cells, A498 CNTN1 and 786-O CNTN1 cells formed significantly more colonies (Fig 2).

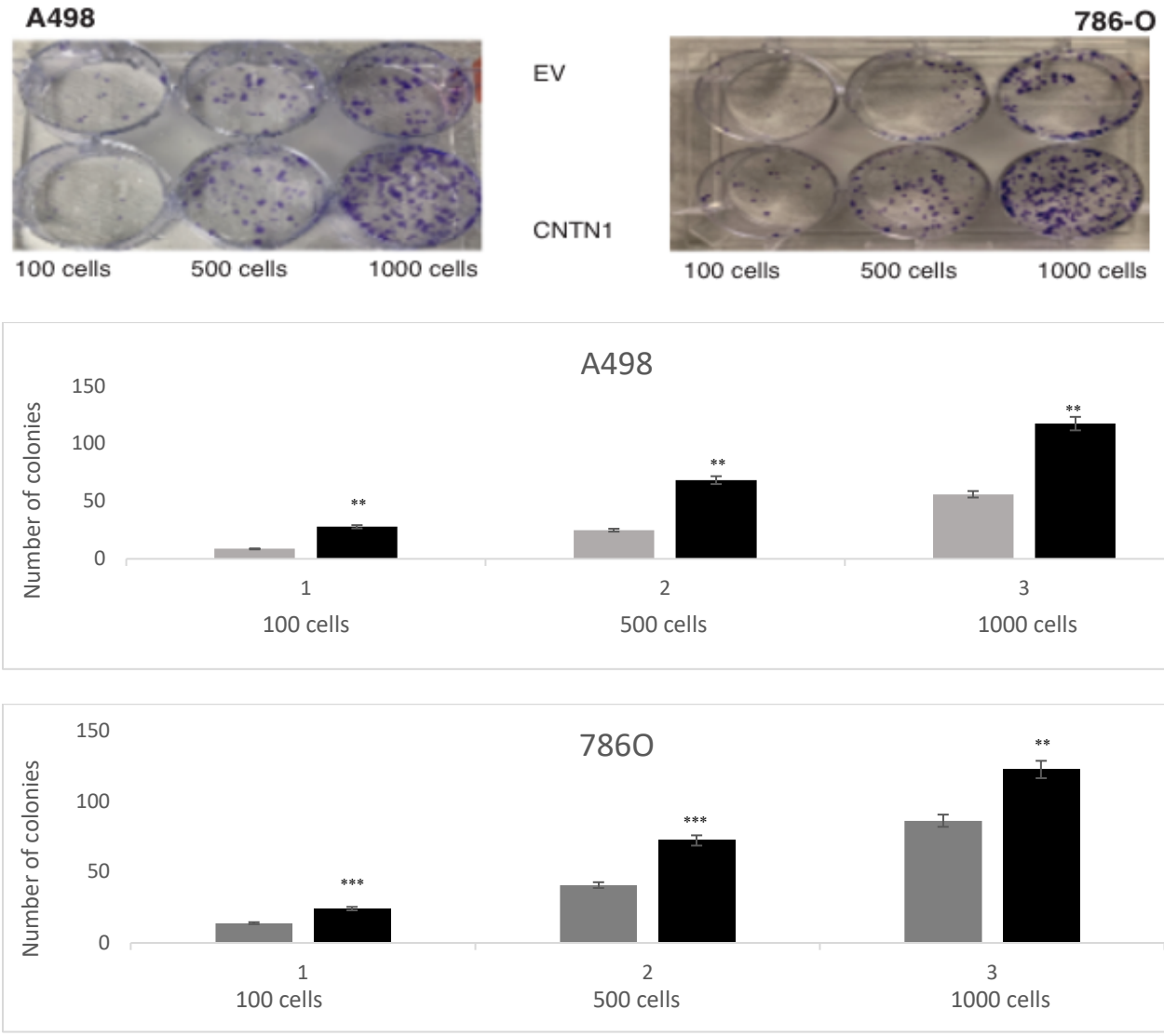

Figure 2. – Colony formation assay. A498 EV, A498 CNTN1, 786-O EV, and 786-O CNTN1 cells were seeded at the indicated densities. Cells were cultured until colonies were visible. Colonies were stained with crystal violet and number of colonies were counted. Experiments were repeated 3 times. Typical images are presented (top panel). Colony numbers are graphed (bottom panel). ** $p<0.01$; *** $p<0.001$ in comparison to the respective EV cells by 2-tailed Student's t-test.

4.3 CNTN1 promoting Cell Proliferation

The evidence above supports the theory that CNTN1 plays a role in facilitating ccRCC cell proliferation. To further support this claim, we carried out a proliferation assay over a period

of 7 days, as the cells were seeded in a 6-well plate, with approximately 10,000 cells on day 1. The cells were then counted using a hemocytometer and trypan blue to exclude dead cells. The data was then analyzed using a two-factor ANOVA test and graphed. Upon analysis, it is evident that compared to EV cells, A498 CNTN1 and 786-O CNTN1 cells proliferate at a quicker rate, as seen in Figure 3.

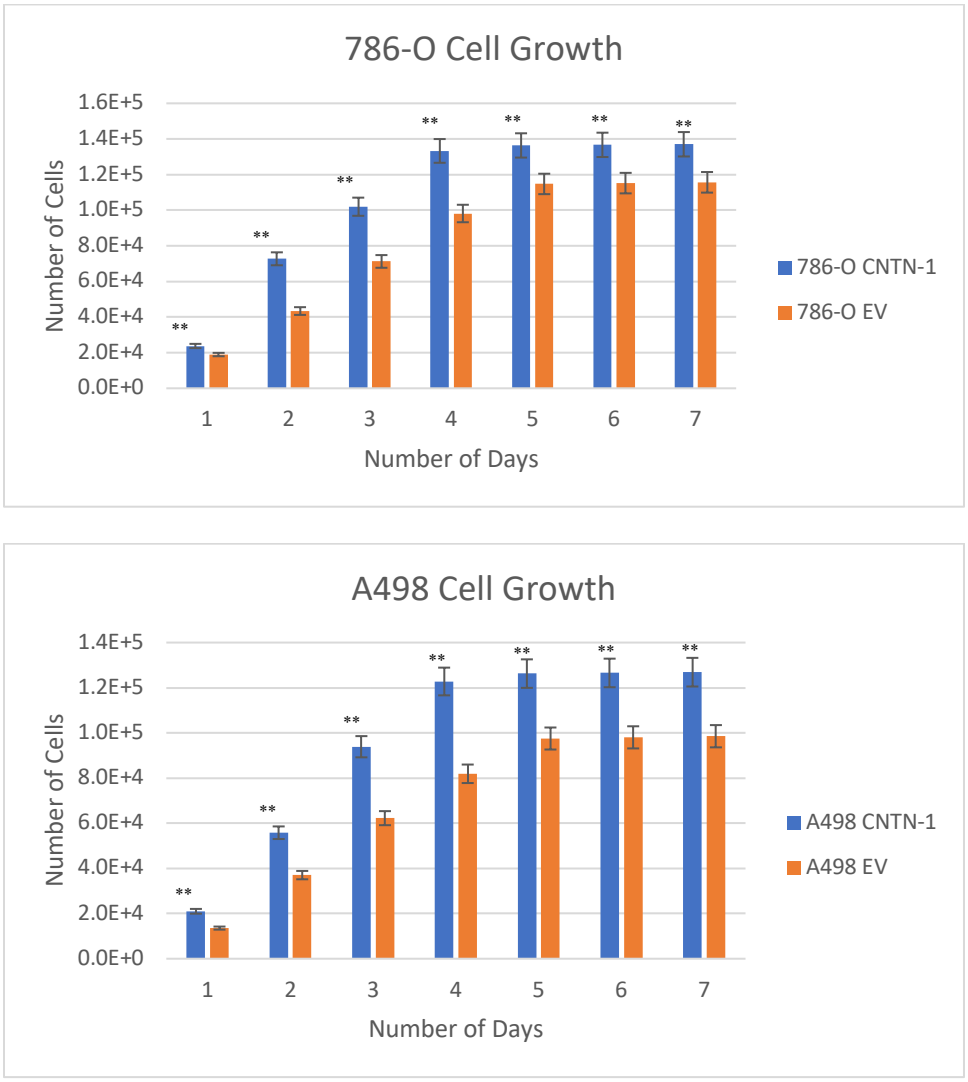

Figure 3.- CNTN1 enhances ccRCC cell proliferation **A)** Quantitative results obtained after completing a cell counting assay on A498 EV (red line) and A498 CNTN-1 (black line) comparing their proliferative abilities. B) Cell counting assay on 786-O EV (red line) and 786-O CNTN-1 (black line). ** $p<0.01$; *** $p<0.001$ (3 trials conducted).

4.4 The impact of CNTN1 on cell's ability to close gaps in wound healing assay

I subsequently examined the effects of CNTN1 on cell migration, a process facilitating cancer metastasis. For this purpose, we performed a wound healing assay. This involves growing the cells in a 6 well plate and creating a straight line wound from top to bottom using a pipette tip. Pictures were then taken at 4 different time points to observe the closing rate of the wound, as the cells migrate from each side of the border. Our experiment shows that the gaps were closed a slightly more rapidly in A498 CNTN1 cells compared to A498 EV cells (Figure 4). However, it is not clear whether CNTN1 facilitate gap closure in 786O cells (Figure 5).

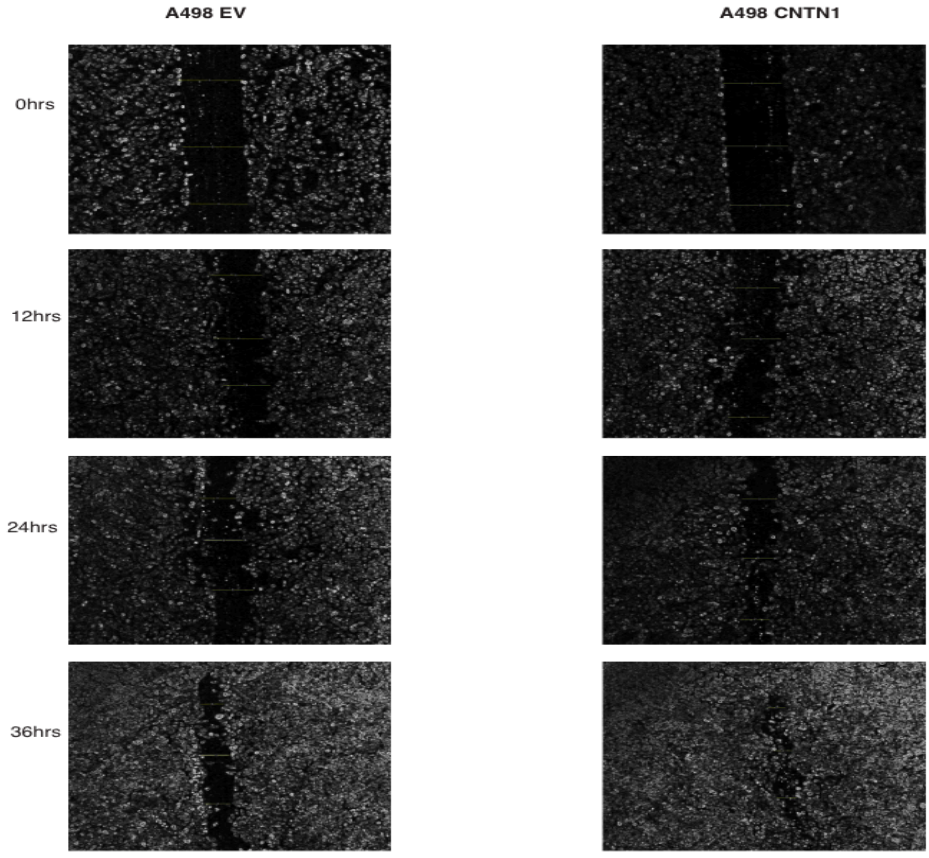

Figure 4. Wound healing assay performed on A498 EV (left) and A498 CNTN-1, at 4 different time points. (Experiments were performed twice.

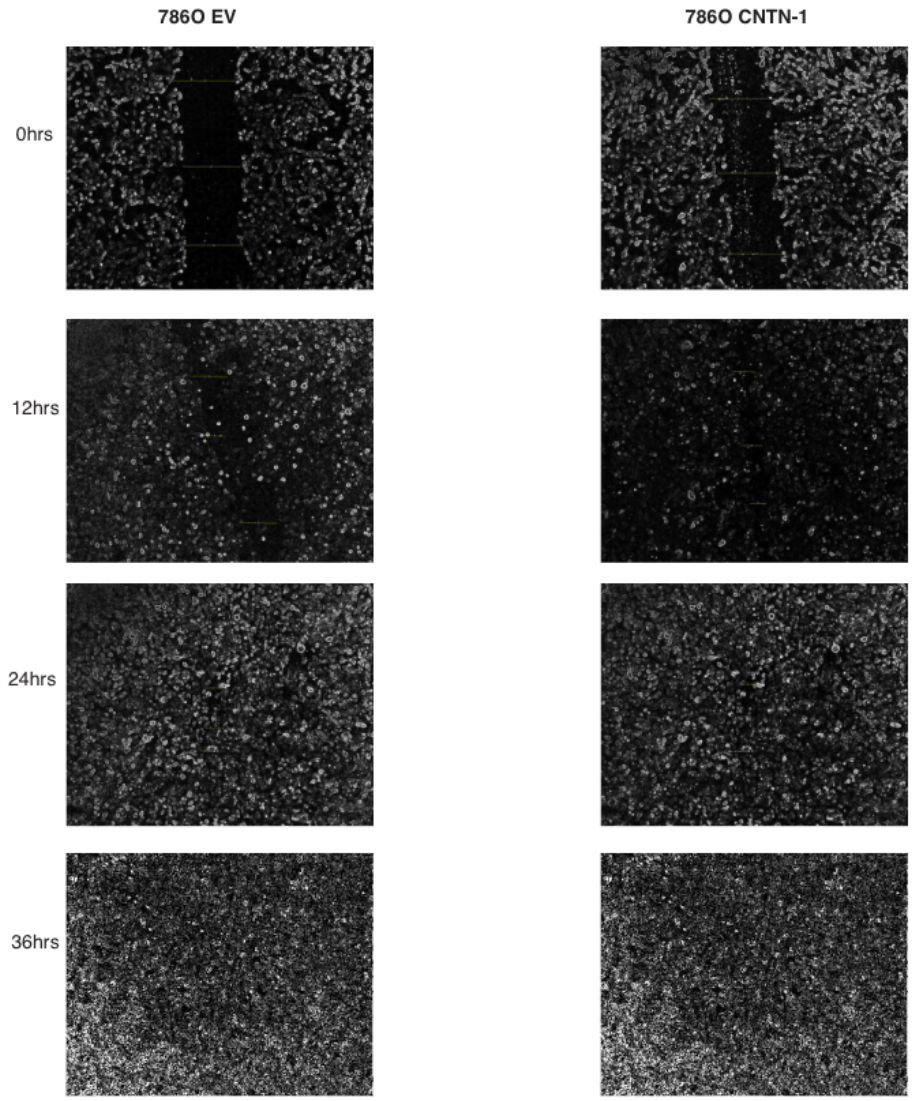

Figure 5. Wound healing assay performed on 786-O EV (left) and 786-O CNTN-1, at 4 different time points. Experiments were carried out twice.

V. DISCUSSION

Clear cell RCC is the most common kind of RCC, accounting for 75-80% of all cases and possesses an aggressive profile (Akhtar et al., 2019). Clear cell RCC has a significant risk of metastasis, and metastasized ccRCC remains incurable. While early identification is critical to preventing ccRCC from progressing, ccRCC is typically asymptomatic, making early diagnosis difficult (Akhtar et al., 2019). Our lack of understanding of ccRCC pathophysiology is primarily to blame for the current situation. This necessitates the discovery of novel oncogenic factors to obtain insight into the genesis of ccRCC. The oncogenic involvement of CNTN1 in ccRCC has not been reported, although evidence supports CNTN1 promotes tumorigenesis in numerous other cancer types, including prostate cancer (Yan et al., 2016). My research supports a potential oncogenic role of CNTN1 in ccRCC.

The above concept is supported by CNTN1's activities in increasing cell proliferation, colony formation, and migration in both 786-O and A498 cells at least under overexpression condition. I have not examined whether knockdown of CNTN1 will reduce these in vitro events, which was due to two factors, 1) low level of endogenous CNTN1 in both 786-O and A498 cells and 2) the on-going pandemic situation. Nonetheless, the impact of knockdown of CNTN1 on oncogenic processes of both cell lines should be investigated in future. As well, the potential mechanisms by which CNTN1 enhances 786-O and A498 cells proliferation, colony formation, and wound healing should be investigated in future; the past and current pandemic situation greatly impacted my ability to examine these mechanisms. Nonetheless, the collective impact of CNTN1 on a set of in vitro processes critical on oncogenesis supports CNTN1-mediated promotion of ccRCC. Metastasis is the dominant cause of cancer death, including fatalities

resulted from ccRCC; metastasis is facilitated via increases in cancer cell's migratory capacity. In this regard, my observation of CNTN1 promoting A498 ccRCC cell migration (Figure 4) indicates a role of CNTN1 in facilitating ccRCC metastasis. Furthermore, cancer cell's migration and invasion abilities, two essential processes of metastasis, are underpinned by AKT and EMT; CNTN1 has been reported to activate both processes during oncogenesis (Yan et al., 2016).

It is intriguing for CNTN1 not only promoting 786-0 and A498 ccRCC cell proliferation but also increasing their saturation density (Figure 3). A typical characteristic of epithelial cells is contact inhibition, a property that is partially deregulated during oncogenesis. Mechanistically, E-cadherin plays a major role in contact inhibition (Mendonsa et al., 2018). In this regard, CNTN1 enhances the oncogenic properties of both 786-0 and A498 ccRCC cells likely in part via further deregulating contact inhibition of 786-0 and A498 cells; this inference is in accordance with the reported activities of CNTN1 in downregulating E-cadherin in prostate cancer cells (Yan et al., 2016).

While detail mechanisms underpinning CNTN1's activities in promoting ccRCC cell proliferation, migration, and increasing ccRCC cell's saturation density remain to be illustrated, it is highly likely a network being involved. For instance, in a recent publication on CNTN1's oncogenic actions in prostate cancer, it was reported that CNTN1 promotes caner progression through a network action, including enrichment of the CREIGHTON ENDOCRINE THERAPY RESISTANCE 3 gene set (Gu et al., 2021). A multigene panel, consisting of 10 CNTN1-related genes, was constructed; the panel significantly predicts prostate cancer relapse (Gu et al., 2021). Intriguingly, this multigene panel also robustly predicts ccRCC fatality risk (Gu et al., 2021), providing further support for CNTN1 being an oncogenic factor in ccRCC. It will be of importance to direct analyze the expression of this panel's component gene in ccRCC in future.

Collectively, my research provides direct in vitro evidence supporting CNTN1's oncogenic contributions to ccRCC; my research is in accordance with CNTN1's actions in pathway important to tumorigenesis, including the VEGF/FLT4 pathway (Wu et al., 2012), AKT pathway (Yan et al., 2016), and EMT process (Yan et al., 2016).

While accumulative indirect evidence, as discussed above, supports CNTN1 as a potential oncogenic factor in ccRCC, the clinical relevance of CNTN1 in primary ccRCC needs to be studied in future. For instance, the expression levels of CNTN1 at both mRNA and protein levels in ccRCC tissues and adjacent normal kidney tissues should be determined. Upregulations in ccRCC tumor tissues would support CNTN1 as an oncogenic factor in ccRCC. Furthermore, whether CNTN1 expression is associated with ccRCC severities (high grade vs low grade) and metastasis should also be investigated. The potential biomarker values of CNTN1 should be studied; those values include association with cancer relapse and survival.

Finally, the functionality of CNTN1 in ccRCC pathogenesis can also be examined in future. This task can be carried out using multiple systems. 1) 786-O CNTN1 and 498 CNTN1 cells can be analyzed for tumorigenesis in immunocompromised mice in comparison to the respective EV cells. 2) Primary ccRCC tissues with and without CNTN1 upregulation can be used to produce patient-derived xenografts (PDX). 3) Transgenic mice with kidney tubular epithelial cell-specific expression of CNTN1 can be generated and used to determine the impact of CNTN1 on ccRCC formation. Should CNTN1 enhance oncogenic events in one or all the above three systems, it will reveal CNTN1 being functionally important in ccRCC.

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