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SND1-Targeted Gene Therapy for Hepatocellular Carcinoma

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SND1-TARGETED GENE THERAPY FOR HEPATOCELLULAR CARCINOMA

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science Virginia Commonwealth University, 2018

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LIST OF ABBREVIATIONS AND SYMBOLS

Ad.	Adenovirus
AFP	α-fetoprotein
CSC	Cancer Stem Cell
DNA	Deoxyribonucleic Acid
E-Cadherin	Epithelial Cadherin
ECM	Extracellular Matrix
EMT	Epithelial Mesenchymal Transition
FBS	Fetal Bovine Serum
HCC	Hepatocellular Carcinoma
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
GFP	Green Fluorescence Protein
miRNA	Micro RNA
MOI	Multiplicity of Infection
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
N-Cadherin	Neural Cadherin
RNA	Ribonucleic Acid
RNAi	RNA interference
qRT-PCR	Quantitative Real Time PCR
SCID	Severe Combined Immunodeficiency
SHARP	Study of Heart and Renal Protection
shRNA	Short Hairpin RNA
siRNA	Small Interfering RNA
SND1	Staphylococcal nuclease and tudor domain-containing 1
snRPN	Small Nuclear Ribonucleoproteins

TGF-β	Transforming Growth Factor Beta
TIC	Tumor Initiating Cells
UTR	Untranslated Region

ABSTRACT

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Staphylococcal nuclease and tudor-domain containing 1 (SND1) is an oncogene for a wide variety of cancers, including hepatocellular carcinoma (HCC). SND1 is a multifunctional protein regulating gene expression of proto-oncogenes and tumor suppressor genes, making SND1 a prime target for developing cancer therapeutics. This notion is especially attributed to HCC as most patients are diagnosed in advanced stages and the therapeutic options available for these patients are severely limited. In this study, we evaluated the therapeutic potential of a replication-defective adenovirus vector delivering SND1 shRNA (Ad.SND1sh) to human HCC cell lines, HepG3, HuH-7, and Hep3B. Adenovirus infection in HCC cells was confirmed by Western blotting and immunofluorescence. The efficacy of Ad.SND1sh to knockdown SND1 expression was confirmed via Western blot, qRT-PCR, and immunofluorescence. Ad.SND1sh did not significantly affect proliferation of the three human HCC cells but significantly inhibited their invasive and migratory capacities, as determined by wound healing and Matrigel invasion assays, respectively. As a corollary, Ad.SND1sh treatment resulted in a decrease in mesenchymal markers, such as N-cadherin, Twist, Snail, and Slug, without

affecting levels of epithelial marker E-Cadherin, indicating that SND1 knockdown induces mesenchymal conversion in HCC cells. Additionally, reductions in liver cancer stem cell marker CD133 and HCC marker α-fetoprotein (AFP) were observed with SND1 knockdown. HCC cells with aberrant expression of these markers are associated with tumor initiation, recurrence, and multi-drug resistance. Our findings indicate that Ad.SND1sh may potentially be an effective therapy for advanced HCC and needs to be studied further for its clinical application.

CHAPTER 1

INTRODUCTION

HEPATOCELLULAR CARCINOMA

Hepatocellular Carcinoma (HCC) is characterized as the development of malignant tumors from liver hepatocytes. HCC is the most common form of primary liver cancer and is the second most common leading cause of cancer related death worldwide, with nearly half a million new cases being diagnosed annually[1]. According to GLOBOCAN 2012 HCC is the fifth most common cancer in men, globally, and the seventh most common among women.

HCC INCIDENCE

The global incidence rates of patients diagnosed with HCC vary depending on geographical location. Regions with endemic HBV infection rates (prevalence ≥8%), such as Sub-Saharan Africa and Eastern Asia, have incident rates as high as 20 per 100,000 individuals being diagnosed with HCC annually[2].

The number of patients diagnosed with HCC appears to be on the rise in the United States (US). An analysis of Surveillance Epidemiology and End Results (SEER) registries based on population data showed that the annual age-adjusted incidence rates of HCC have doubled from 1977 to 2007.[3] With an overall 5-year survival rate of <12%, HCC is quickly becoming one leading causes of cancer-related death in the US[2].

HCC RISC FACTORS

Development of HCC has been shown to be comorbid with liver cirrhosis, as 80% to 90% of HCC cases occur in a cirrhotic liver setting[4]. Liver cirrhosis is defined as the

progressive loss of proliferative capacity in hepatocytes and the development of fibrotic scar tissue (fibrosis) that ultimately leads to portal hypertension and end-stage liver disease[5].

50% of HCC cases occur in individuals infected with Hepatitis B Virus (HBV), making it the leading risk factor for HCC worldwide[6]. HBV is hepatotropic, enveloped, partially double-stranded DNA virus belonging to the *Hepadnaviridae* family. HBV is characterized as an oncovirus primarily due to its ability to integrate its genome into host DNA leading to the development of mutations, causing chromosomal instability, and alterations to host gene expression[7]. Additionally, chronic HBV infection induces a sustained inflammatory response[8], which has a high probability of causing cirrhosis and leading to the development of HCC[9]. However, HBV infection has been shown to directly correlate with HCC development in the absence of cirrhosis[10].

Hepatitis C virus (HCV) infection is another risk factor associated with HCC development and accounts for up 25% of HCC cases in Asia and Africa, and 60% of the cases in the US[11]. HCV is an enveloped RNA virus which is incapable of host genome integration[7]. Hence, HCV infection leads to HCC development exclusively in a setting of liver cirrhosis. HCV infection produces core viral proteins in liver hepatocytes that promote tumorigenesis via interaction with host cell factors involved in apoptosis, DNA replication, DNA repair, cell cycle progression, and angiogenesis[12]. Both HBV and HCV are involved in the initiation and progression of HCC through alterations in DNA repair systems, centrosome duplication mechanisms, and disruption of gene expression and signaling pathways[9].

Chronic alcohol consumption represents another HCC risk factor. Alcohol consumption has been shown to lead to severe liver damage and disease via oxidative stress and inflammation[13]. Chronic alcohol consumption can also be a cofactor in other HCC risk factors to increase likelihood of HCC development. In one study, patients with HBV-induced cirrhosis, heavy drinking was found to triple their risk of HCC development[14].

Another HCC risk factor is Non-Alcoholic Fatty Liver Disease (NAFLD), which is the process by which fatty tissue accumulates in the liver, leading to cirrhosis. NAFLD is currently the leading cause of chronic liver disease in the US[2]. A population study analyzing SEER-Medicare database revealed a statistically significant correlation between HCC and metabolic syndrome, a disease which shares similar phenotypes with NAFLD[15]. Aflatoxin exposure is another risk factor of HCC[16].

HCC TREATMENTS

Liver transplantation and resection are the two most common treatments for HCC[17]. Liver Transplantation being the optimum choice for patients with advanced cirrhosis[18], and liver resection is best for patients with noncirrhotic functional livers[19]. Local ablation using radiofrequency is currently the standard form of care for patients with early stage tumors (<4cm) and has been shown to provide higher recurrence-free survival rates compared to that of percutaneous ethanol ablation[20]. Transcatheter arterial chemoembolization (TACE) involves the administration of a chemotherapeutic drug and embolization agent directly to a liver tumor through a catheter, with the goal of restricting tumor blood supply and increasing tumor exposure to chemotherapeutics[17]. TACE treatment is typically reserved for patients with HCC liver that lacks vascular invasion and is deemed unresectable[17]. Currently, Sorafenib is the only FDA approved

first line systemic treatment for HCC[21]. Sorafenib is a monoclonal antibody that acts as a tyrosine kinase inhibitor, targeting a multitude of cell surface receptors and downstream signaling modulators involved in tumor progression. In the Phase III Sorafenib HCC Assessment Randomized Protocol (SHARP) study, 602 patients with advanced HCC were randomized and administered either 400 mg of sorafenib or placebo[22]. The median overall survival of the sorafenib group in the SHARP trial was 10.7 months, which is a significant increase when compared to 7.9 months of the placebo group[22]. Like most other chemotherapeutics, Sorafenib treatment causes some negative side effects in patients. Adverse effects noted in the SHARP study include: diarrhea, fatigue, hand-foot skin reaction, rash, alopecia, hypophosphatemia, thrombocytopenia, and hypertension[22]. Regorafenib, a multi-kinase inhibitor, and nivolumab, a PD-1 check point inhibitor, are FDA approved monoclonal antibodies used as second line treatments for HCC after sorafenib progression[23,24]. The increasing global incidence of HCC combined with the lack of FDA approved mid-to-late stage treatment options makes the identification of novel therapeutic targets in HCC of critical concern.

SND1 STRUCTURE AND FUNCTIONS

In humans the *SND1* gene is located on chromosome *7q31.3*, with this region having been shown to undergo amplification in a variety of cancers[25-27]. The protein translated from this gene, Staphylococcal nuclease and tudor-domain containing 1 protein (SND1), contains four tandemly repeated staphylococcal nuclease (SN) domains and a fifth domain with tudor and nuclease (TSN) fusion activity[28]. Like most oligonucleotide/oligosaccharide binding (OB) fold proteins which lack enzymatic activity,

the SN domains of SND1 lack the amino acid residues required to facilitate the calcium-dependent thermonuclease activity that is commonly observed in SN domains[29-31]. OB fold proteins, like SND1, are reported to be involved in a multitude of cellular processes, such as transcriptional activation or repression, DNA repair, and chromatin modification[29]. As for the tudor domain, its presence has been well documented in proteins that interact with DNA, with specific functions related to epigenetic regulation, gene expression, and the biogenesis of regulatory RNA molecules (snRNP, miRNA, and siRNA)[31]. SND1 has been shown to be highly overexpressed in a large percentage of HCC patients, and is used as a marker to denote tumor stage progression[32]. The correlation between SND1 protein abundance and tumor progression appears to be a result of its ability to regulate gene expression of proto-oncogenes and tumor suppressor genes[33].

SND1 plays an important role in transcriptional regulation, as it was initially identified as a transcriptional co-activator in a study attempting to identify proteins that interact with Epstein-Barr nuclear antigen, where it was shown to directly interact with EBNA2[34]. As a transcriptional co-activator, SND1 has been shown to interact with transcription factors (TFs) such as STATs[35,36] and SMAD[37] family proteins which, respectively, leads to an increase in Janus kinases (JAK) and Transforming Growth Factor Beta (TGFβ) downstream signaling[35-37].

SND1 IN HCC

SND1 associates with Astrocyte Elevated Gene-1 (AEG1), another HCC oncogene, and other proteins to form a stable RNA-induced silencing complex (RISC) where SND1 functions as a nuclease. RISC interacts with either a single strand of small interfering

RNA (siRNA) or micro RNA (miRNA) and uses them as templates for targeting and suppressing complementary mRNA. This interaction facilitates the role of SND1 in modulating post-transcriptional gene expression.[32,38]

It has been shown that SND1 can interact directly with mRNA in a manner that is independent of its role in RISC. In one study, it was revealed that SND1 stabilizes mRNA transcripts of angiotensin II type 1 receptor (AT1R) by binding to its 3'UTR, leading to increased AT1R protein production and downstream signaling[39]. Additionally, we showed that stable knockdown of SND1 in human HCC cell lines resulted in a reduction of AT1R expression leading to decreased expression of TGFβ and its downstream signaling genes[40]. In the same study, SND1 was also stably over expressed in HCC cells leading to increased TGFβ signaling and activation of Plasminogen Activator Inhibitor- 1 (PAI-1), which promotes tumor migration and invasion[41].

In vitro studies have shown that SND1 plays an integral role in the development of HCC by affecting cellular proliferation, migration, invasion, angiogenesis, and epithelial-mesenchymal transition (Fig. 1). These results lead to the development of a transgenic mouse model (Alb/SND1) expressing Myc-tagged human SND1 under an albumin promoter with a B6CBAF1 background. The goal of this model was to understand how overexpression of SND1 in liver hepatocytes influenced HCC development *in vivo*[41]. To stimulate liver carcinogenesis, the mice received a single intraperitoneal injection of N-nitroso-diethylamine (DEN) at a dosage of 10 µg DEN/gram of body weight at 14 days of age, with their livers being harvested at 32 weeks of age[41]. The results of this study corroborated that Alb/SND1 mice injected with DEN developed robust tumorigenic

responses in the liver at 32 weeks of age, when compared to WT littermates injected with DEN[41]. An increase in mRNA levels of TNFα and c-myc, key HCC drivers, was detected as early as 2 months of age, with increased IL-6 levels at 12 months of age[41]. Western blot analysis using anti-SND1 and anti-Myc-Tag antibodies were used to confirm overexpression of SND1 in Alb/SND1 compared to WT littermates[41]. AFP and CD36 (HCC markers), CD31 (angiogenesis marker), and PCNA (proliferation marker) were also detected at higher levels in DEN-Alb/SND1 mouse livers compared to DEN-WT littermates[41]. An increase in activated NFκB, ERK, Akt, and GSK3β was established in both Alb/SND1 and DEN-Alb/SND1 mice, indicating that these genes are constitutively active in SND1 overexpressing liver tissue[41]. Hepatocytes isolated from Alb/SND1 mouse livers were able to form proliferative spheres when cultured in ultralow attachment plates and expressed high levels of Tumor Initiator Cell (TIC) markers EpCAM, CD44, and CD133[41]. Treatment with the drug pdTp, which binds the SN domains of SND1 inhibiting its enzymatic activity, resulted in significantly reduced sphere formation and TIC marker protein levels in both Alb/SND1 and WT hepatocytes compared to vehicle treated controls. This indicates that SND1 enzymatic activity is necessary to facilitate TIC expansion in liver hepatocytes[41]. Additionally, inhibition of NFκB and Akt phosphorylation was observed in pdTp treated Alb/SND1 hepatocytes, with ERK phosphorylation being unaffeceted[41]. It was found that pdTp treatment did not reduce AT1R mRNA levels, which was previously found to stimulate TGFβ signaling leading to ERK activation[40, 41]. Demonstrating that SND1 binds the 3'-UTR of mRNA transcripts independent of its enzymatic activity[41]. This data stands to further validate the novelty of SND1 targeted therapy as a means for potentially treating HCC.

EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-Mesenchymal Transition (EMT) is a complex biological process by which epithelial cells, which are characterized by interaction with the basement membrane via the basal surface of its polarized membrane, undergo a wide array of molecular and cellular changes which enable them to transition to a mesenchymal phenotype. Phenotypic traits of mesenchymal cells include enhanced motility, increased invasive capacity, resistance to apoptosis, and the production of ECM components[42]. Cells progressing through EMT are characterized by the down-regulation of epithelial markers like E-cadherin and claudin-1, and the up-regulation of mesenchymal markers such as N-cadherin and vimentin[43]. EMT is under strict regulation by several gene products, with the most well characterized being Snail, Slug, Twist1, ZEB1, and ZEB2[44]. Like many other carcinomas, EMT contributes to drug resistance and the metastatic nature of tumors in patients diagnosed with HCC.

EMT TRANSCRIPTION FACTORS

Snail and Slug are zinc-finger proteins belonging to the Snail family of transcription factors[45]. Snail family transcription factors are able to directly bind directly to the promoter region of the *CDH1* gene, which codes for E-cadherin, and repress its expression, while simultaneously up-regulating the expression of pro-invasive genes such as vimentin, fibrinogen, and matrix metalloproteinases[46]. Snail and Slug expression can be induced by hypoxic conditions and Transforming Growth Factor-β (TGF-β) activity, both of which are usually enhanced during EMT[47].

Twist1 is a basic helix-loop-helix transcription factor. The basic/helix-loop-helix domain of Twist1 transcription factor allows it to bind DNA directly and facilitates its ability to enhance expression of N-cadherin and repress the expression of E-cadherin during EMT[48]. Up-stream and down-stream signaling pathways related to Twist1 expression are not fully understood, however, there are studies that report that Twist1 expression is enhanced by classical EMT-inducing pathways during fetal development, inflammation, and carcinogenesis. As an example, TGF-β/Ras activated MAPKs have been shown to significantly increase Ser68 phosphorylation of Twist1, leading to increased stabilization and higher Twist1 protein levels in breast cancer cells[49]. Twist1 expression in cancer correlates with increased invasive capacity, metastasis, tumor aggressiveness, recurrence, and poor survival outcomes[45].

ZEB1 and ZEB2 are members of the zinc finger transcription factor family of proteins. ZEB1 is structurally composed of 7 zinc finger domains and one homeodomain, with ZEB2 having an additional zinc finger domain[50]. The homeodomains of these transcription factor proteins allow them to bind to specific regulatory regions of target genes, such as E-cadherin and vimentin, and either repress or enhance gene expression[51, 52]. ZEB1 and ZEB2 are induced by innate EMT factors like hypoxic conditions, inflammatory cytokines, and the TGF-β signaling pathway[53].

CANCER STEMNESS

The cancer stem cell theory proposes that tumors are composed of an organized hierarchy of heterogeneous cell populations and, like somatic cells, a specific subpopulation known as cancer stem cells (CSC) are responsible for tumor formation, growth, and maintence[54]. The stochastic model of tumor development contrasts with the

CSC model, and theorizes that somatic cell populations acquire multiple mutations over many generations culminating in tumor heterogeneity, and that all mutant cells are capable of self-renewal and tumorigenesis[55] (Fig2).

CSCs are thought to be transformed progenitor cells and all non-CSCs are called the derived population cells (DC) which are the result of multi-lineage differentiation from CSCs[56]. The main phenotypic traits possessed by CSCs are limited differentiation (via asymmetric division), self-renewal (via symmetric division), and altered proliferative capacity (mostly quiescent, but can rapidly divide when proper microenvironment stimuli are present) [57-59]. In addition to tumor initiation and maintenance, CSCs are believed to play significant roles in cancer cell metastasis, multi-drug resistance (MDR), and tumor recurrence[60]. In the case of HCC, tumors develop from mutant primary liver hepatocytes, not progenitor cells, and the cell population responsible for tumor formation and maintenance are known as the tumor initiator cells (TIC).

Development of therapies that are cable of reducing stemness or even eliminating the CSC populations are currently underway. However, targeting CSCs has proven to be a difficult and complex task. This is because CSCs activate signaling pathways and display cell surface markers, such as CD133, CD44, and EpCAM, similar to normal somatic stem cells. This makes it somewhat difficult to develop novel therapies against the CSC population that do not disrupt or kill somatic stem cells and non-dividing cells[61].

VIRUS VECTORS IN GENE THERAPY

Gene therapy is described as the experimental technique of transplanting genetic material into cells with the goal of replacing missing genes or reducing aberrant

expression of genes, in order to alter a disease phenotype. The first successful application of gene therapy was in a clinical trial for children with severe combined immunodeficiency (SCID) in 1990[62]. Since then, it has grown increasingly popular with the technique having been refined over time. Currently, viral vectors are employed as a viable application for treating single gene hereditary diseases, infections, and cancers[63].

Human adenoviruses are DNA viruses belonging to the genus *Mastadenovirus*, with 51 genetically distinct serotypes[64]. Currently, adenovirus vectors used in therapeutic settings are replication deficient, having had E1 (replication) and E3 (immune response evasion) proteins removed during manufacturing and attenuation[65]. Adenovirus is one of the most common vectors used for gene transfer. This is mainly due to their unique tropism which allows them to infect many different human tissues, ability to infect both dividing and non-dividing cells, high titer acquisition, large transgene capacity, and efficient transgene expression[66]. Additionally, adenovirus vectors, unlike retrovirus vectors, do not integrate their DNA into host cell genome upon infection, preventing mutagenesis and the development of genomic irregularities during treatment[67].

CHAPTER 2

MATERIALS & METHODS

Cell culture conditions

Hep3B cells were obtained from American Type Culture Collection (Manassas, VA); HepG3 and HuH-7 cells were kindly provided by Dr. Paul Dent. HuH-7 and HepG3 were grown in Dulbecco's Modified Eagle Media (DMEM) containing 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. Human HCC cell line Hep3B was grown in Minimum Essential Media (MEM) alpha containing 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, 1% non-essential amino acids, and 1% sodium pyruvate. All cells cultures were maintained at 37^{o}C with 5% CO_2.

Adenovirus Infection

Adenovirus vectors were purchased from Vector Biolabs. All adenovirus vectors were human adenovirus serotype 5 with attenuation of E1 and E3 proteins. Adenovirus-CMV-RNAi-GFP (Ad.RNAi) encodes a scrambled RNAi sequence under the U6 promoter with an eGFP-expression cassette under the CMV promoter. Adenovirus-GFP-U6-SND1-shRNA (Ad.SND1sh) encodes human SND1 shRNA under the U6 promoter with an eGFP-expression cassette under the CMV promoter. Adenovirus containing media for infection was made by diluting concentrated adenovirus vector stocks in serum-free DMEM containing 1% penicillin/streptomycin, to the desired multiplicity of infection (MOI) based on the number of cells being infected. Adenovirus in media solution was administered to and infected for two hours at 37^{o}C with 5% CO_2. The adenovirus solution being removed and DMEM with 10% FBS and 1% penicillin/streptomycin was

added to the plate/well, and cells were incubated at 37^{o}C with 5% CO_2 for 24 hours (experimental assays) or 48 hours (lysate collection).

Western blotting

Cells were washed twice with PBS and lysed with 250 ul of 1.5% n-dodecyl-D-maltoside (DDM) lysis buffer. Cellular debris was removed by centrifugation at 15,000 rpm for 15 minutes at 4^{o}C. Protein concentrations were determined by Bradford protein assay. Lysates containing equal amounts of protein were resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 hour at room temperature (RT) in blocking buffer (5% nonfat milk powder in TBST: 10 mm TRIS-HCL (pH 8.0), 150 mm NaCl, 0.05% Tween 20), incubated overnight at 4^{o}C with respective primary antibodies diluted in blocking buffer. Membranes were washed three times with TBST (10 min, RT), incubated in respective secondary antibodies for 1 hour at RT, and then washed with TBST thrice more (10 min, RT). Enhanced Chemiluminescence (ECL) detection reagents were used to detect proteins of interest, with GAPDH being used as an internal control.

qRT-PCR

Purified RNA was extracted from cells with the Zymo Research Direct-zol RNA MiniPrep Plus kit. 2 µg sample of extracted RNA was added to master mix (2 ul 10x buffer, 2 ul random primers, 0.8 ul dNTP, 1 ul RNase out, and 1 ul of Reverse Transcriptase) to synthesize cDNA. 2.5 ul of cDNA sample was then added to 25 ul of Taqman master mix, 20 ul of RNase free H_2O, and 2.5 ul of probe. Two 20 ul aliquots of this mixture

then underwent PCR. SND1 and GAPDH probes were used, with the latter serving as an internal control.

Immunofluorescence

For Immunofluorescence assay, cells were seeded on Millicell EZ slide (Millipore) with a density of $2.5x10^4$ cells/mL in each chamber. Cells were infected with adenovirus, as described above, and incubated for 24 hours at 37^{o}C with 5% CO_2. Cells were then fixed with 4% paraformaldehyde for 20 minutes at RT, washed thrice with PBS, permeabilized with 0.1% Triton in PBS for 5 minutes at RT, and washed with PBS three more times. Next, the cells were blocked with 200 ul of blocking buffer (1% Bovine Serum Albumin and 10% Goat serum in PBS) for 2 hours at RT and incubated with primary antibody diluted in blocking buffer overnight at 4^{o}C. After washing with PBS, cells were then incubated with fluorochrome conjugated secondary antibodies (1:400 dilution). Images were captured using a confocal microscope (LSM700) provided by MCV microscopy core. Uninfected cells processed according to the above-mentioned protocol, with the exception of primary antibody incubation, were used as a negative control in this assay.

MTT Assays

2000 cells were plated in a 96-well plate and infected 24 hours later. At 48, 96, and 144 hours post infection 10% (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) in media was administered to the cells. After 4 hours of incubation, an equal volume of 10% sodium dodecyl sulphate (SDS) in H_2O was added. The plates were incubated overnight. Absorbance was measure at 600 nm.

In vitro Wound Healing Assays

A wound healing scratch assay was used to evaluate cell migration. Two-well silicon inserts (IBIDI, Martinsried, Germany) were individually placed inside the chambers of a 12-well plate. Cells were infected with adenovirus, as described above, and incubated for 24 hours. 70 µls of a suspension containing $5x10^5$ cells/ml were seeded into each side of the insert and incubated overnight at 3 7°C with 5% CO_2. After the cells have adhered, the insert was removed, forming a 500 µm lesion in between the cells. The scratched monolayer images were captured at 0, 24, and 48 hours after wounding. Wound healing was measured and quantified using MetaVi Labs Wound Healing analysis software (IBIDI, Martinsried, Germany).

Invasion Assays

BioCoat cell culture inserts with an 8-µ-porosity polyetyleneterapthalate membrane coated with Matrigel basement membrane matrix (100 µg/cm^2) were used to measure the invasive capacity of cells. Inserts were rehydrated in 24-well plates with serum free DMEM (0% FBS) for 2 hours at 37°C with 5% CO_2. The lower chamber (well) in the 24-well plate was filled with 0.75 ml of 10% FBS in DMEM. Cells were infected with adenovirus, as described above, an incubated for 24 hours. $2.5x10^4$ cells were suspended in 0.5 mL of serum free DMEM (0% FBS) and seeded in the upper compartment (6.25 mm membrane size) of the insert. The inserts were placed in a 24-well plate and incubated for 22 hours at 37°C with 5% CO_2. Inserts were then removed, fixed, and stained using the Diff-Quick staining kit. Non-invasive cells were scrubbed away using a cotton swab, and invasion was determined by counting the number of cells that migrated to the lower side of the filter with a microscope at 100x magnification.

Statistical Analysis

Results were checked for statistical significance using the student T-test with a one-tailed hypothesis, the one-way ANOVA test, and the two-way ANOVA test. A *P* value of <0.05 was considered as statistically significant.

CHAPTER 3

RESULTS

Adenovirus vectors can efficiently infect Human HCC cells

HCC cell lines, HepG3, HuH-7, and Hep3B, were either uninfected (control), infected with 100 MOI of Ad.RNAi (virus control), or infected with 100 MOI of Ad.SND1sh (treatment). Both adenoviruses contain an eGFP-expression cassette under the CMV promoter incorporated into their vector constructs. GFP fluorescence was examined in control and adenovirus-infected cells from all three HCC cell lines (Fig. 3a). No GFP expression was observed in control cells. GFP expression was observed in more than 90% of cells observed in both Ad.RNAi- and Ad.SND1sh-infected groups indicating that these adenovirus vectors can effectively deliver transgenes to HCC cells when inoculated at 100 MOI. A Western blot was performed to quantify the levels of GFP being produced in control, Ad.RNAi-, and Ad.SND1sh-infected HCC cells (Fig. 3b). The GFP levels showed variability in the 3 HCC cell lines, with HepG3 showing the highest expression indicating a high degree of adenovirus tropism in this cell line. This data shows that the adenovirus vectors are capable of infecting human HCC cells, however, the rate of the viral infection appears to differ depending on the cell line used.

Ad.SND1sh infection reduces SND1 in HCC cells

Ad.SND1sh-mediated knockdown of SND1 was confirmed via immunofluorescence (IF) in HuH-7 cells, and by Western blot and qRT-PCR in all 3 HCC cell lines. IF of HuH-7 cells showed that Ad.SND1sh-infected cells expressing high GFP levels (top and bottom arrows of Ad.SND1sh row) also expressed reduced levels of SND1, which

indicated that Ad.SND1sh-infection can effectively knockdown SND1 in HCC cells (Fig. 4a). There was no observale difference is SND1 levels in uninfected cells (bottom arrow of Ad.RNAi row) or cells infected with Ad.RNAi (top arrow of Ad.RNAi row) (Fig. 4a). Western blot of protein lysates collected from control and adenovirus-infected HCC cells showed a noticeable reduction in SND1 protein levels in cells infected with Ad.SND1sh compared to Ad.RNAi-infected cells (Fig. 4b). These effects were shown to be statistically significant upon quantification of image densitometry (Fig. 4c). The results of qRT-PCR using RNA from control, Ad.RNAi-, and Ad.SND1sh-infected cells showed a reduction of about 95% in SND1 mRNA levels in Ad.SND1sh-infected cells compared to control and Ad.RNAi-infected cells (Fig. 4d).

Infection with Ad.SND1 does not affect proliferation in HCC

MTT assay was performed on control and adenovirus-infected HCC cells to determine if SND1 knockdown affected cellular proliferation. of HCC cells inoculated at 100 MOI showed potential cytotoxic effects in cells after an extended period of exposure (>72 hours) to adenovirus vectors (Fig 5a, b, c). No consistent statistically significant changes in cellular proliferation were observed in Ad.SND1sh-infected cells when compared to Ad.RNAi-infected cells.

Ad.SND1sh-infection inhibits migration in HCC cells

In vitro wound healing assay was performed on control and adenovirus-infected HCC cells to determine if SND1 knockdown affected cellular migration. HepG3 cells inoculated with 50 MOI of Ad.SND1sh showed reduced migratory capacity at 24 hours and at 48 hours compared to Ad.RNAi-infected cells (Fig. 6a). HuH-7 cells inoculated

with 50 MOI of Ad.SND1sh showed reduced migratory capacity at 24 hours and 48 hours compared to Ad.RNAi-infected cells (Fig. 6b). Hep3B cells inoculated with 25 MOI of Ad.SND1sh showed reduced migratory capacity at 24 hours and 48 hours compared to Ad.RNAi-infected cells (Fig. 6c). Using a two-way ANOVA test with unweighted mean analysis showed that in HepG3 and Hep3B cells treatment ($p<0.0001$), independent of time ($p=0.2779$ and $p=0.9204$, respectively), was statistically significant in reducing cellular migration (Fig. 6d, f). In HuH-7 cells, both treatment and time were found to be statistically significant ($p=0.0113$) in reducing cellular migration (Fig. 6e).

Ad.SND1sh-infection inhibits invasion in HCC cells

A Matrigel invasion assay was performed on control and adenovirus-infected HCC cells to determine if SND1 knockdown affected cellular invasion. After fixing and staining of Matrigel inserts, reductions in the number of cells per field were observed in Ad.SND1sh-infected HCC cells compared to Ad.RNAi-infected cells (Fig. 7a,b). Quantification and statistical analysis using a one-way ANOVA for repeated measurements showed that HepG3 ($p= 0.00873$) and HuH-7 ($p= 0.00474$) cells infected with Ad.SND1sh had a significant reduction in the number of invading cells compared to Ad.RNAi-infected cells (Fig. 7 c,d).

Ad.SND1sh-infection reduces expression of mesenchymal markers in HCC cells

Western blot was performed to determine the levels of EMT markers in control and adenovirus-infected HCC cells (Fig. 8a). Changes in EMT marker expression were shown to be statistically significant upon quantification of image densitometry (Fig. 8b). Ad.SND1sh-infected HepG3 and HuH-7 cells showed decreased levels of mesenchymal

marker N-Cadherin when compared to Ad.RNAi-infected cells. No change in epithelial marker E-Cadherin observed in Ad.SND1sh-infected cell compared to Ad.RNAi-infected cells. The levels of transcription factors known to regulate EMT, such as Twist, Snail, and Slug, were also probed (Fig. 8a). Twist and Snail levels were significantly reduced in Ad.SND1sh-infected HuH-7 cells, while Slug was significantly reduced in HepG3 and HuH-7 cell lines infected with Ad.SND1sh compared to Ad.RNAi-infected cells (Fig. 8b). Hep3B appears to display reduced levels of Slug protein, however, statistical analysis comparing Ad.RNAi- and Ad.SND1sh-infected Hep3b cells determined the p value to be 0.063, which is extremely close to the threshold of statistical significance (<0.05) used in this study. No expression of Snail was detected in Hep3B cells.

Ad.SND1sh-infection reduces AFP and CD133 expression in HCC cells

Western blot was performed to check the levels of HCC marker α-fetoprotein (AFP) and tumor initiating cell marker CD133 in control and adenovirus-infected HCC cells (Fig. 9a). Changes in AFP and CD133 protein levels were shown to be statistically significant upon quantification of image densitometry (Fig. 9b). Ad.SND1sh-infected cells from all three HCC cell lines showed a significant reductions in protein levels of AFP and CD133 when compared to Ad.RNAi-infected cells (Fig. 9b).

CHAPTER 4

FIGURES

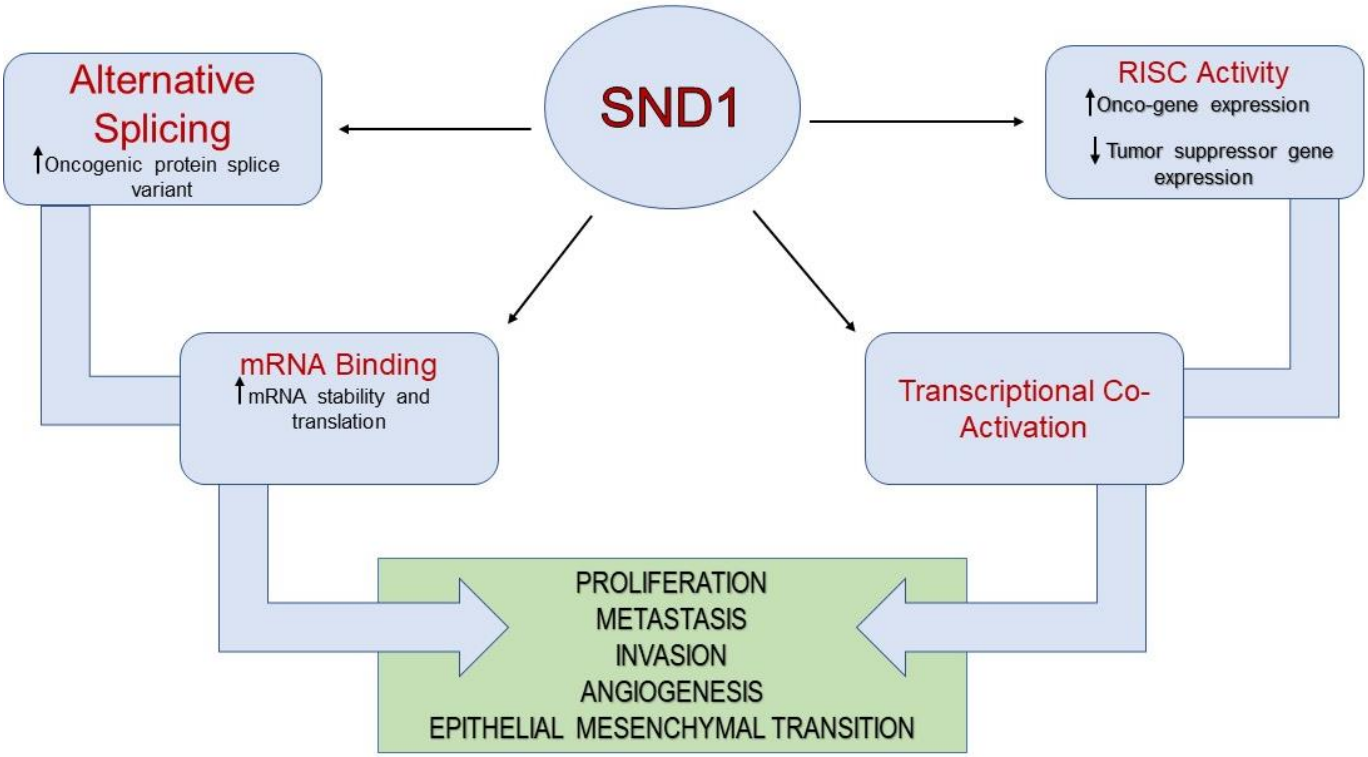


Figure 1: A schematic detailing the various functions of SND1 and how it contributes to oncogenesis.

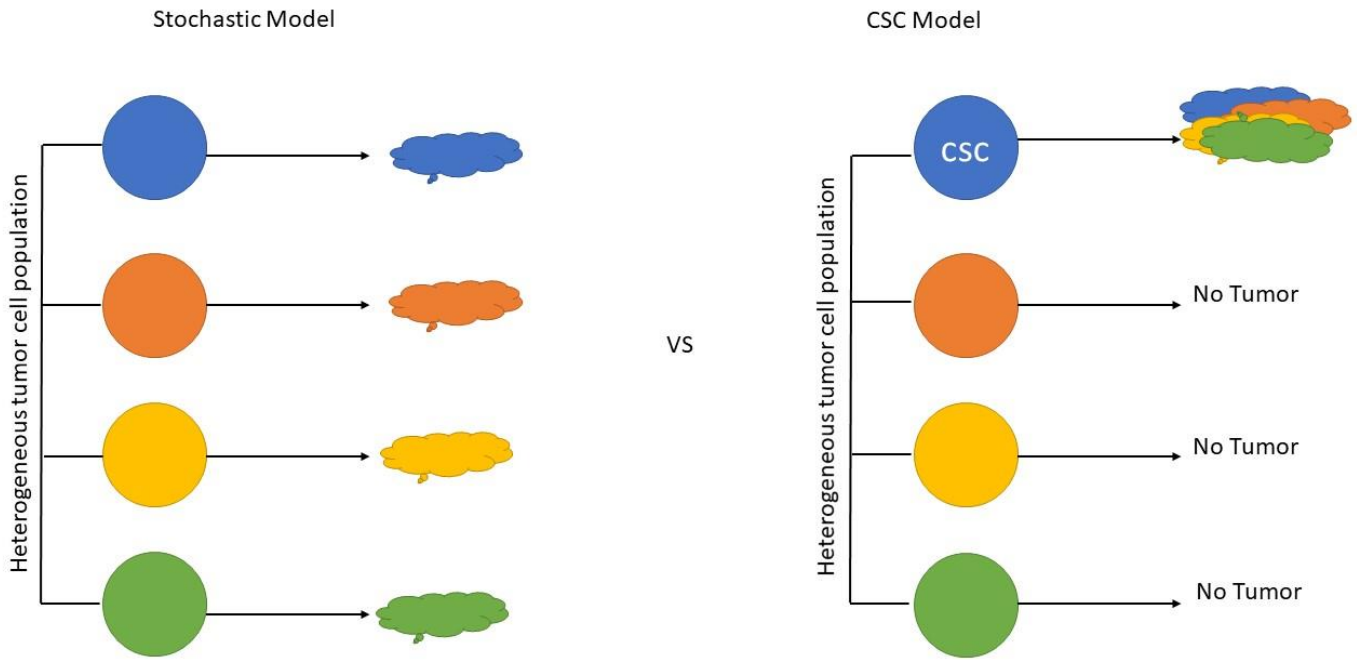


Figure 2: A schematic representation of the stochastic and cancer stem cell model theories of tumor development.

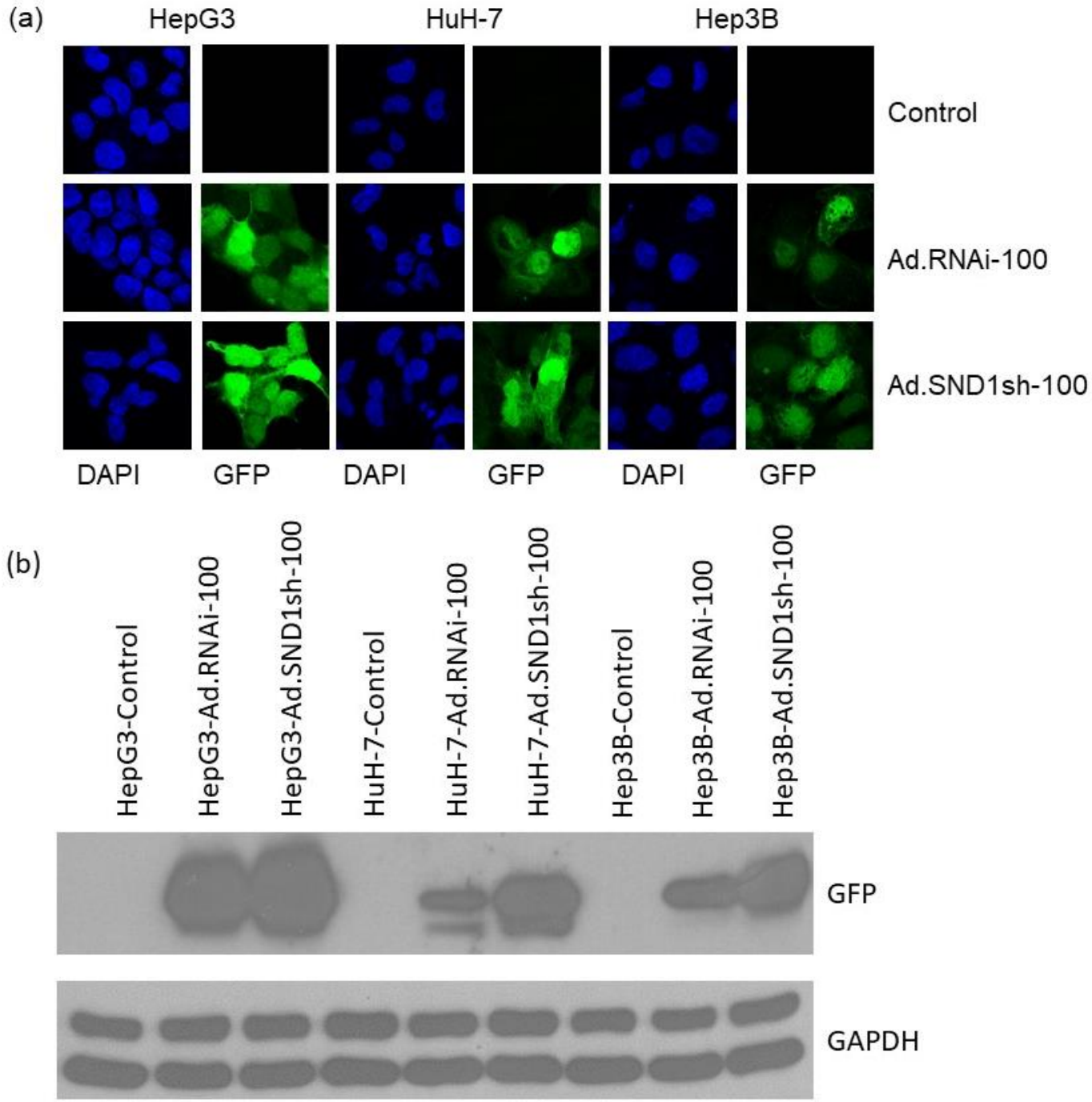
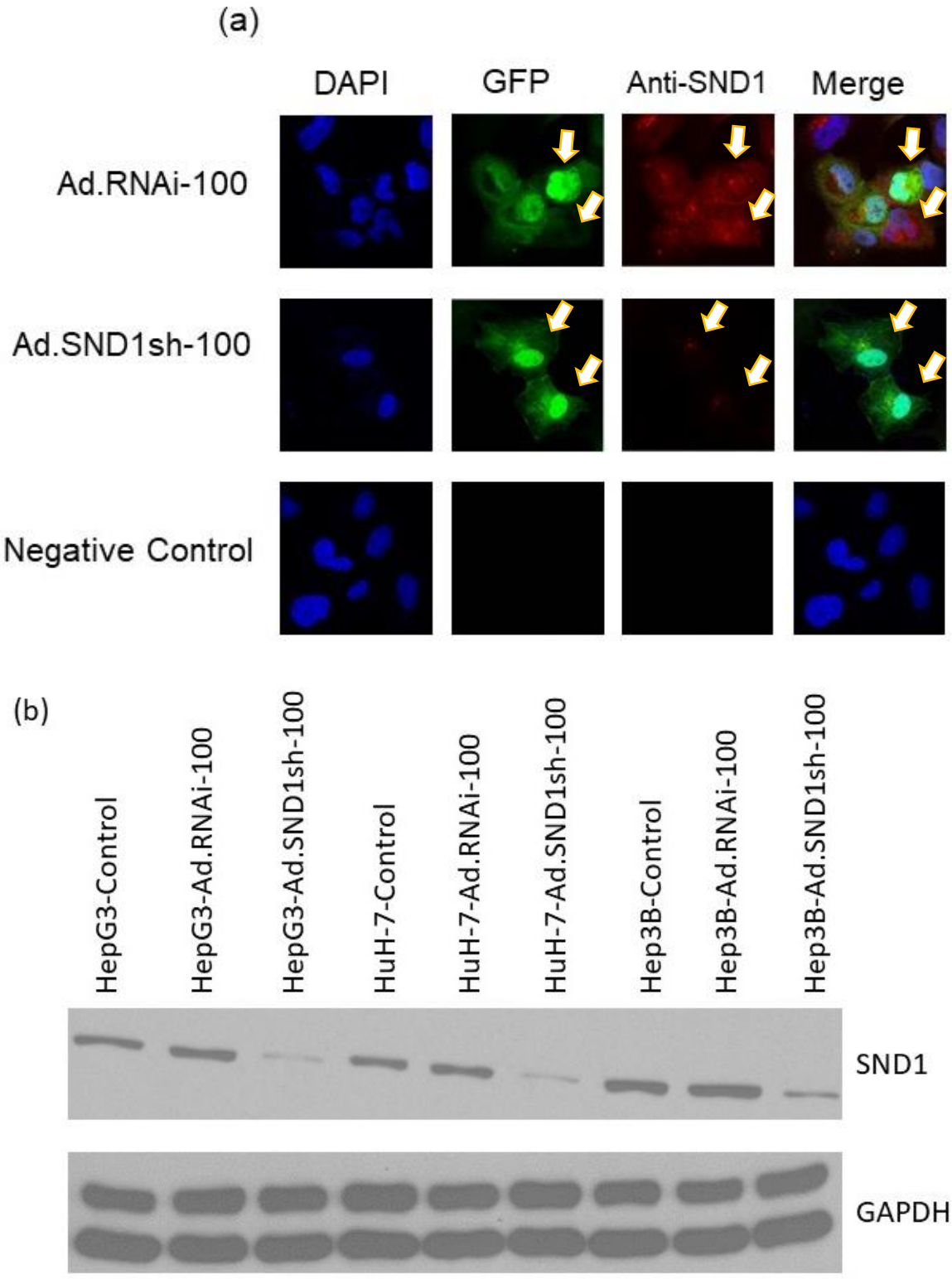


Figure 3: Adenovirus vectors can efficiently infect human HCC cells. Adenovirus infection is detectable via eGFP-cassette expression. (a) A set of representative fluorescent confocal micrographs and (b) a western blot of control, Ad.RNAi, and Ad.SND1sh-infected cells, where eGFP expression is only observed in adenovirus-infected cells.


(a)
DAPI
GFP
Anti-SND1
Merge
Ad.RNAi-100
Ad.SND1sh-100
Negative Control
(b)
HepG3-Control
HepG3-Ad.RNAi-100
HepG3-Ad.SND1sh-100
HuH-7-Control
HuH-7-Ad.RNAi-100
HuH-7-Ad.SND1sh-100
Hep3B-Control
Hep3B-Ad.RNAi-100
Hep3B-Ad.SND1sh-100
SND1
GAPDH

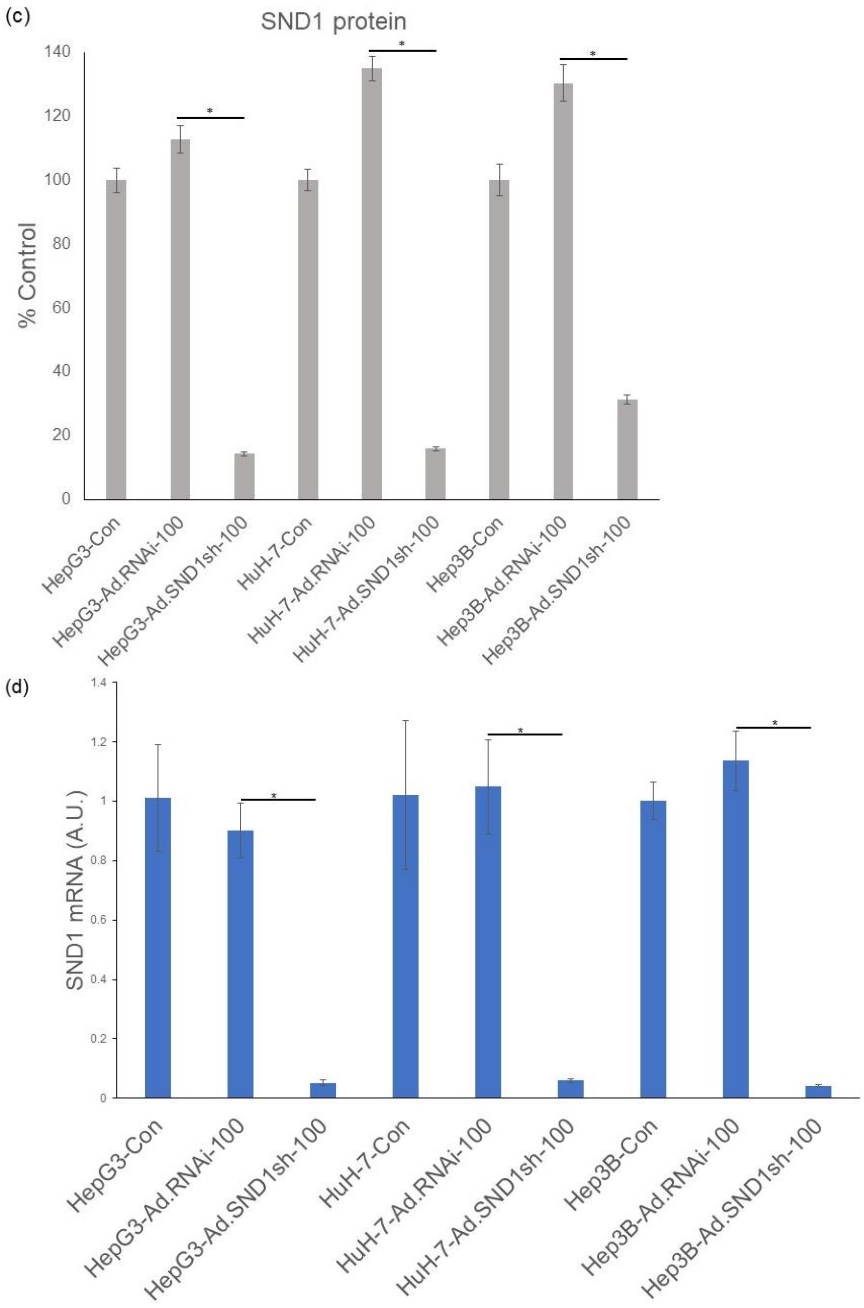


Figure 4: Ad.SND1sh reduces SND1 in HCC cell lines.
(a) A set of representative fluorescent confocal micrographs from an immunofluorescence assay probing for SND1 expression in Ad.RNAi-, Ad.SND1sh-infected, and negative control HuH-7 cells. The top arrow in Ad.RNAi indicates a GFP-positive (infected) cell and the lower arrow shows a GFP-negative (uninfected) cell. The arrows in Ad.SND1sh indicate two GFP-positive (infected) cells. (b) A western blot probing for SND1 protein in control and adenovirus-infected HCC cells. (c) A representative bar graph of SND1 blot densitometry in HCC cells. (d) mRNA expression levels of SND1 quantified by q-RT-PCR in control and adenovirus-infected infected HCC cells. *P< 0.05, Student's t test.

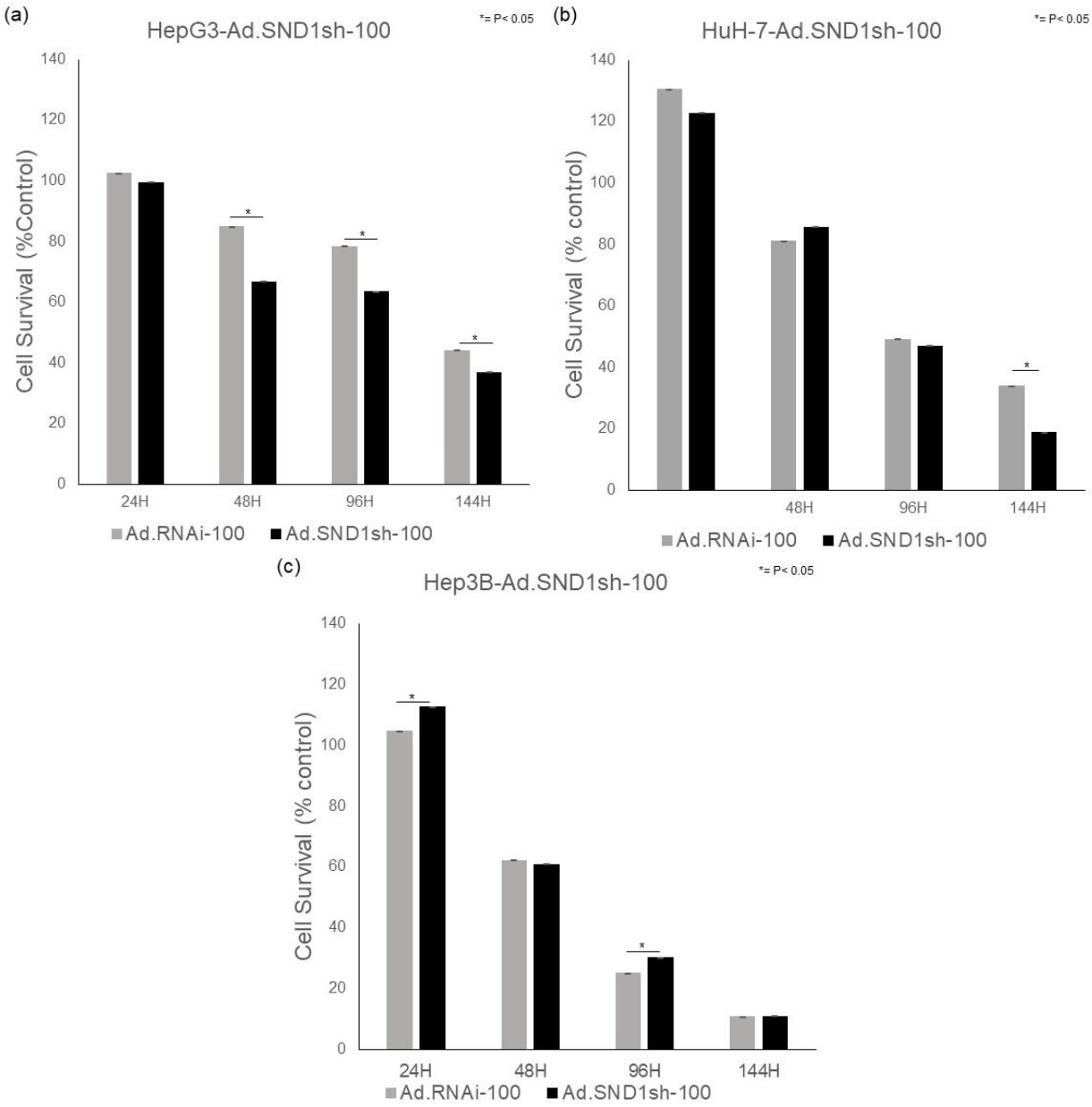


Figure 5: Ad.SND1sh does not affect proliferation in HCC cells.
Bar graphs representing the quantification of cell survival data from MTT assays of control and adenovirus-infected (a) HepG3 cells (b) HuH-7 cells (c) and Hep3B cells at 24, 48, 96, and 144 hours post infection. *P< 0.05, Student's t test.

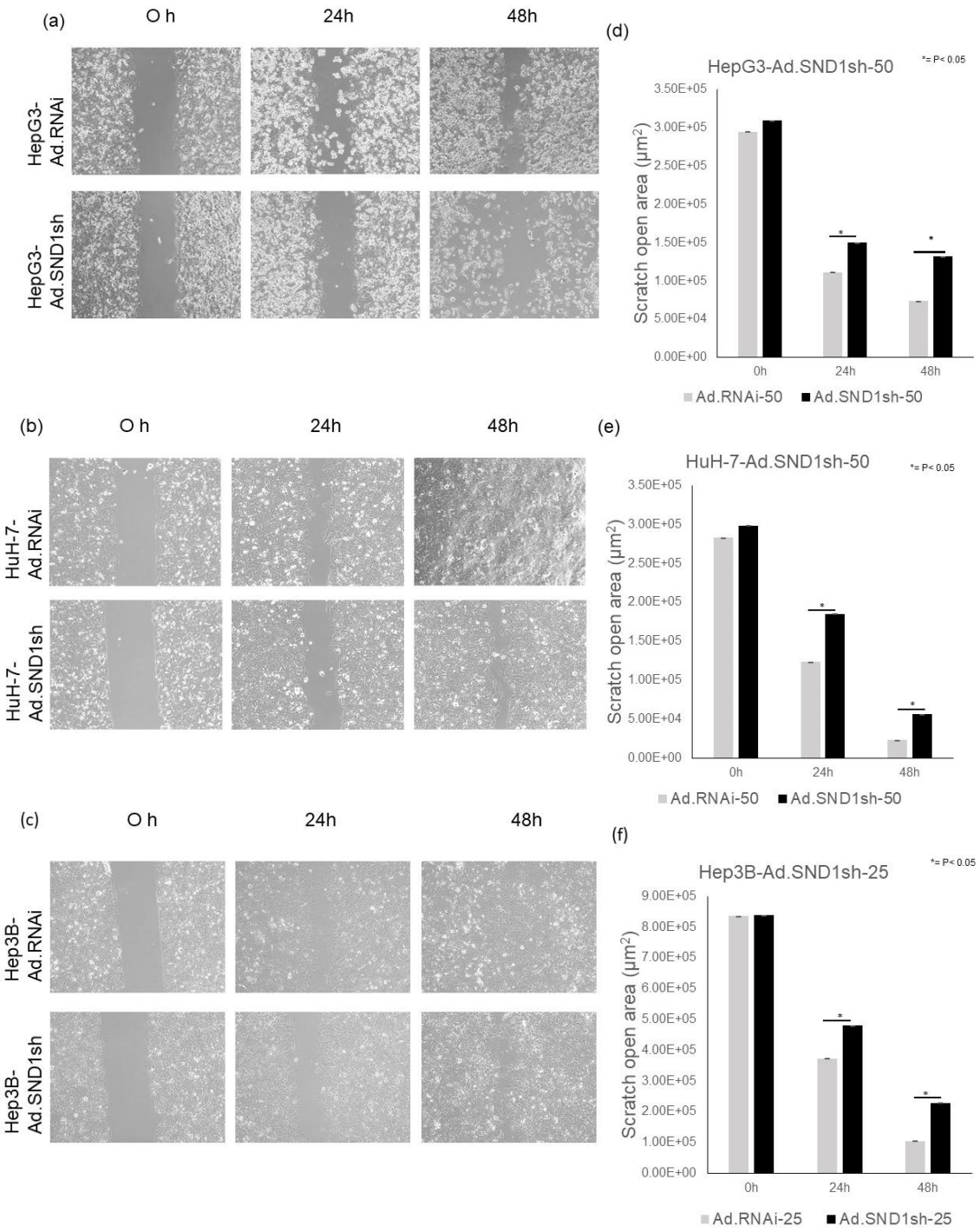


Figure 6: Ad.SND1sh-infection inhibits migration in HCC cells.
Pictomicrographs of *in vitro* wound healing assay in adenovirus-infected (a) HepG3 (b) Huh-7 and (c) Hep3B cells at 0, 24, and 48 hours post wounding. Bar graphs representing measurements of the scratch open area (lesion width) observed in pictomicrographs of adenovirus-infected (d) HepG3 (e) Huh-7 and (f) Hep3B cells. *$P<$ 0.05, Two-Way ANOVA Test-Unweighted.

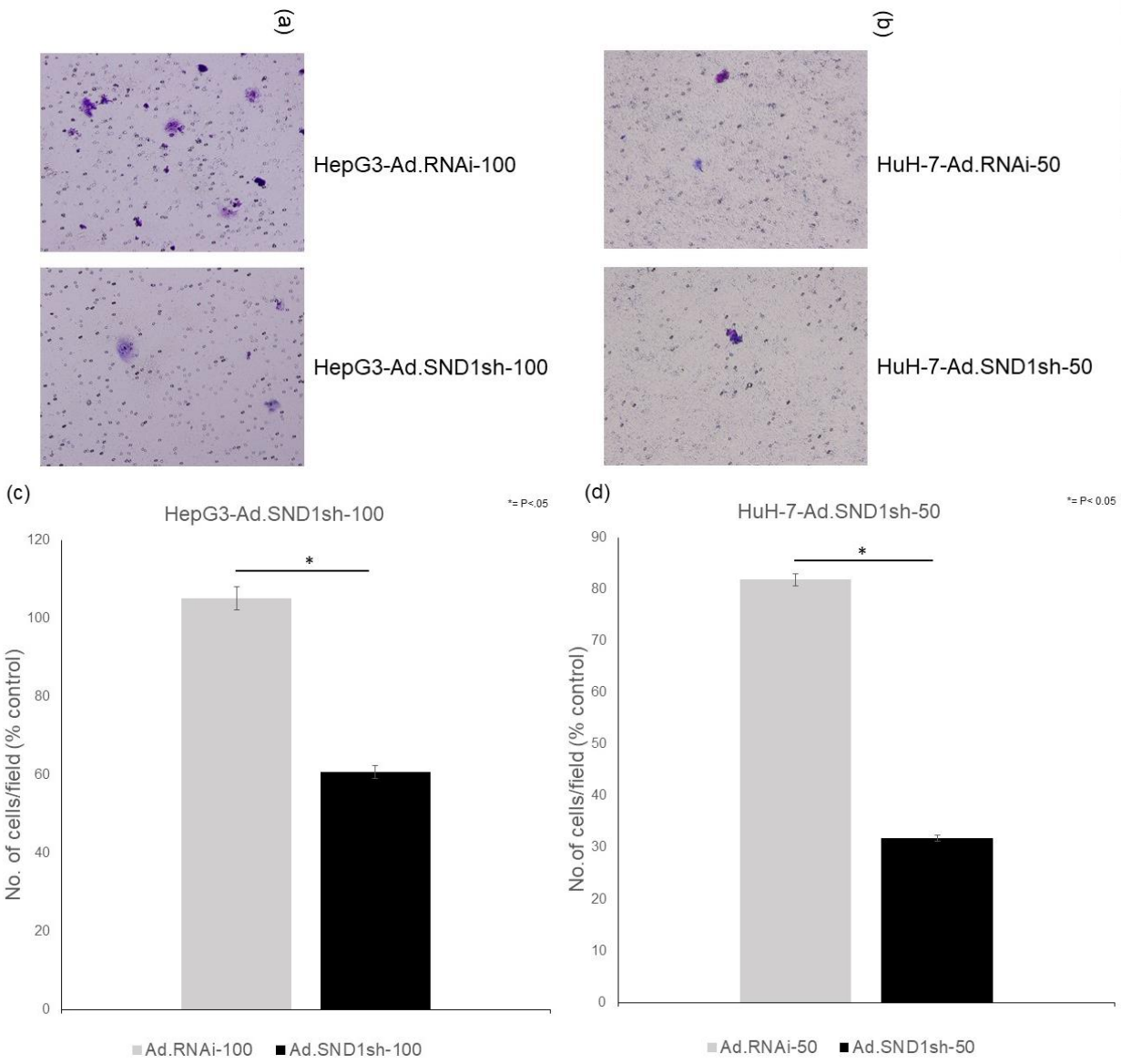
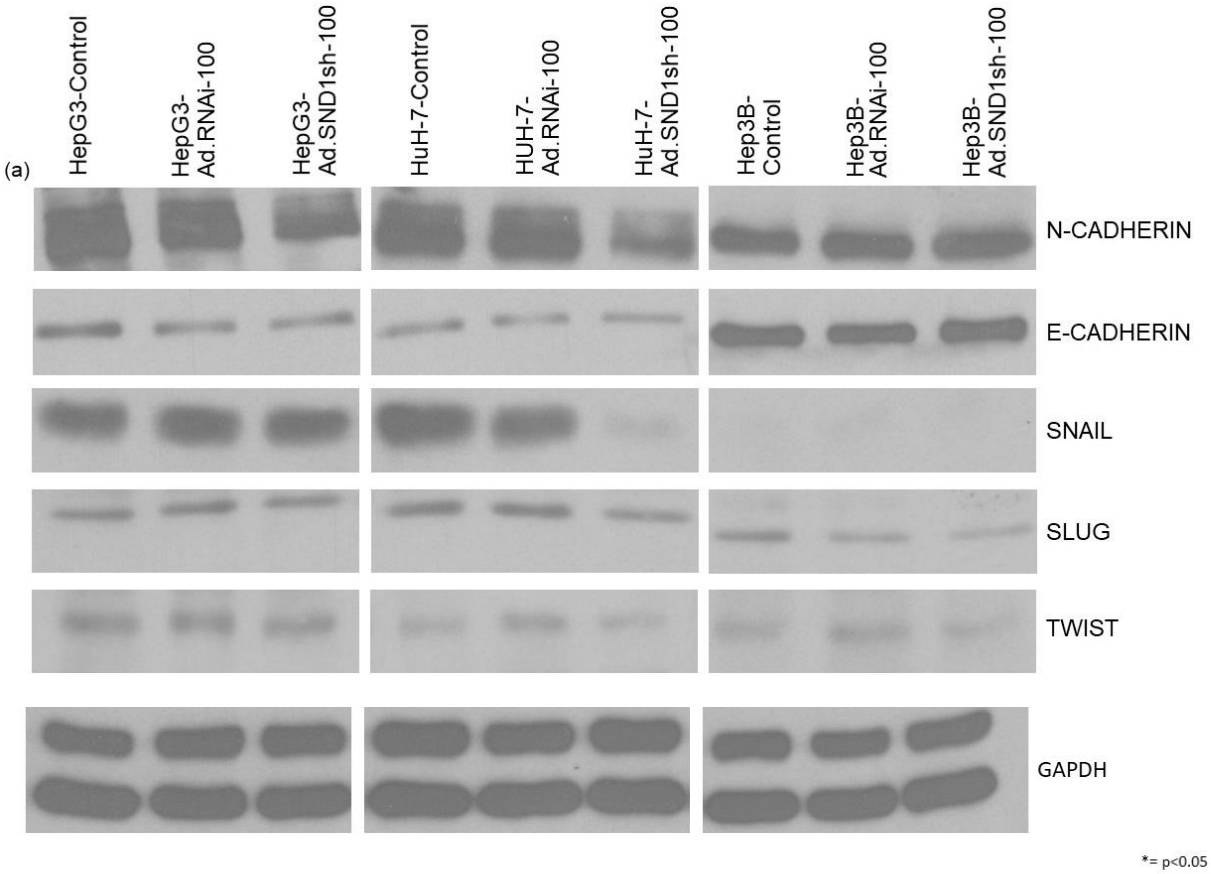
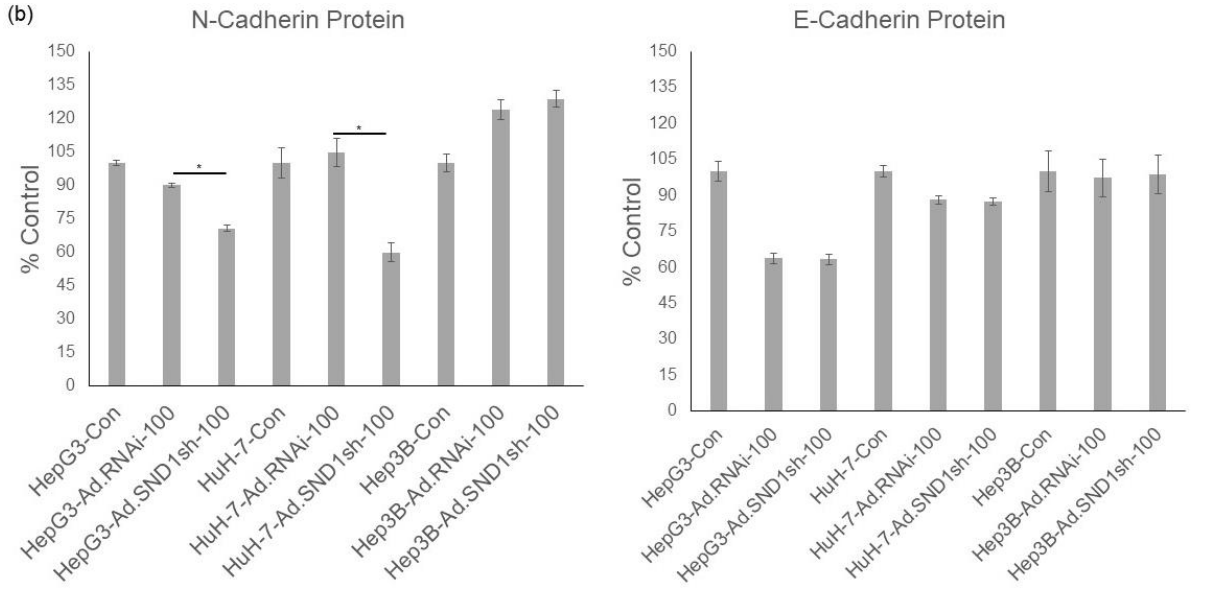


Figure 7: Ad.SND1sh-infection inhibits invasion in HCC cells.
Pictomicrographs of Matrigel invasion assay in adenovirus-infected (a) HepG3 and (b) Huh-7 cells. Bar graphs representing the number of stained invading cells per field observed in pictomicrographs of adenovirus infected (c) HepG3 and (d) HuH-7 cells. *P< 0.05, One-Way ANOVA for Repeated Measures.


(a)
HepG3-Control
HepG3-Ad.RNAi-100
HepG3-Ad.SND1sh-100
HuH-7-Control
HUH-7-Ad.RNAi-100
HuH-7-Ad.SND1sh-100
Hep3B-Control
Hep3B-Ad.RNAi-100
Hep3B-Ad.SND1sh-100
N-CADHERIN
E-CADHERIN
SNAIL
SLUG
TWIST
GAPDH
*= p<0.05


(b)
N-Cadherin Protein
% Control
150
135
120
105
90
75
60
45
30
15
0
HepG3-Con
HepG3-Ad.RNAi-100
HepG3-Ad.SND1sh-100
HuH-7-Con
HuH-7-Ad.RNAi-100
HuH-7-Ad.SND1sh-100
Hep3B-Con
Hep3B-Ad.RNAi-100
Hep3B-Ad.SND1sh-100
E-Cadherin Protein
% Control
HepG3-Con
HepG3-Ad.RNAi-100
HepG3-Ad.SND1sh-100
HuH-7-Con
HuH-7-Ad.RNAi-100
HuH-7-Ad.SND1sh-100
Hep3B-Con
Hep3B-Ad.RNAi-100
Hep3B-Ad.SND1sh-100

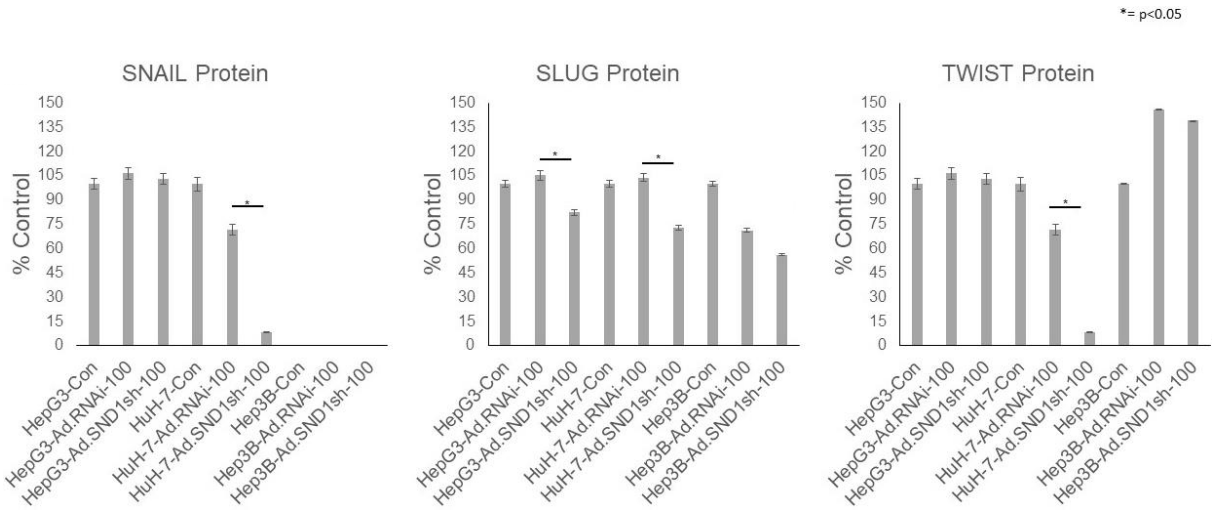


Figure 8: Ad.SND1sh-infection reduces expression of mesenchymal markers in HCC cells. (a) A western blot showing EMT markers N-Cadherin, E-cadherin, Twist, Snail, and Slug protein levels in control and adenovirus-infected HCC cell lines. (b) Bar graphs representing densitometry of mesenchymal markers from western blot of control and adenovirus-infected HCC cells. *P< 0.05, Student's t test.

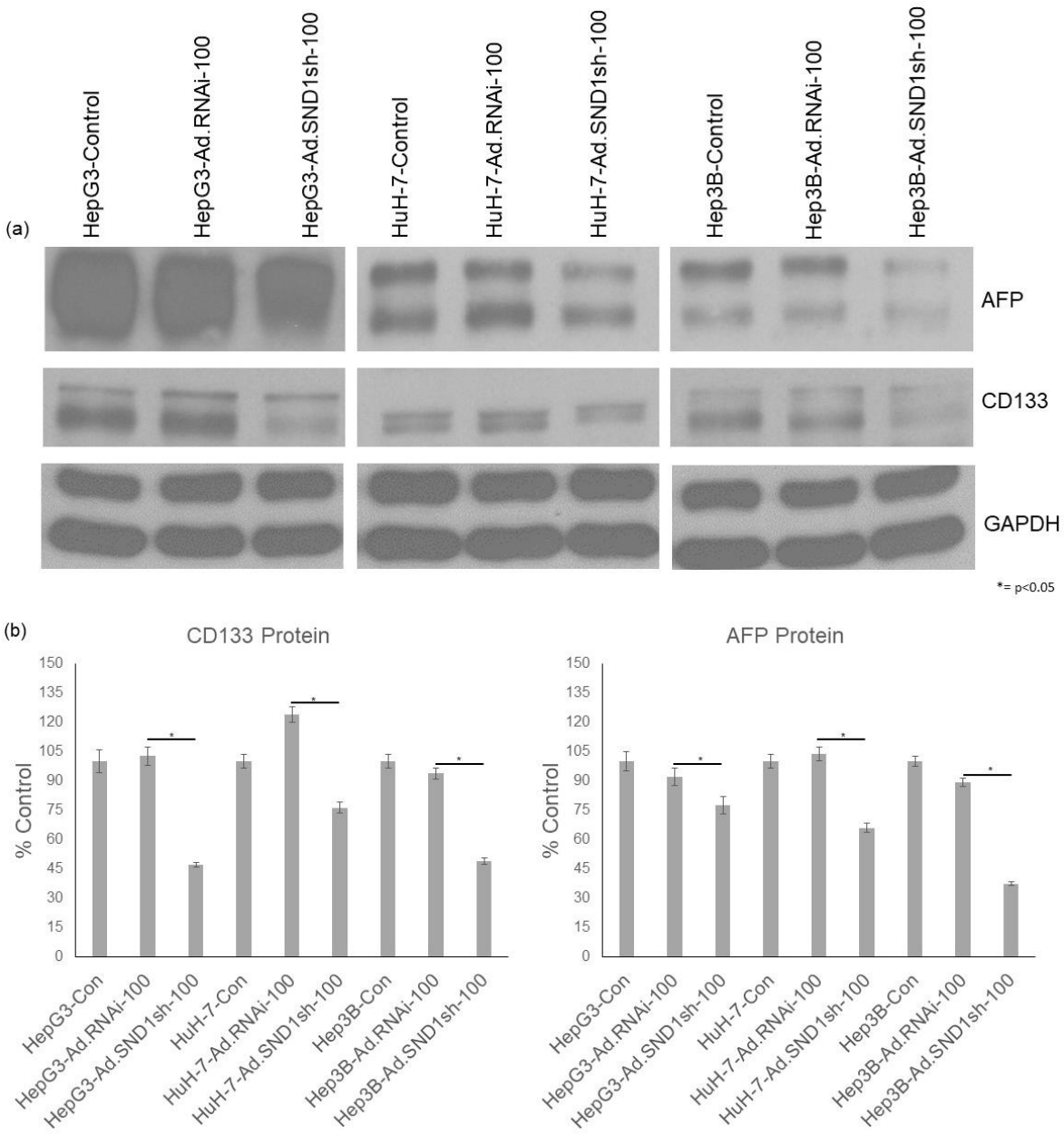


Figure 9: Infection with Ad.SND1sh reduces AFP and CD133 expression in HCC cells. A western blot showing tumor cell initiator marker CD133 and HCC marker Alpha-fetoprotein (AFP) levels in control and adenovirus-infected HCC cell lines. (b) Bar graphs representing densitometry of CD133 and AFP from western blot of control and adenovirus-infected HCC cells. *P< 0.05, Student's t test.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

SND1 is an established oncogene that has been shown to be overexpressed in many organ cancers including HCC. In HCC, SND1 is over expressed to such a significant extent that its protein level abundance has been seen to positively correlate with increased tumor progression and poor survival outcomes in patients. These findings indicate that SND1 inhibition might be a potential treatment strategy for advanced HCC. Therapeutic options available for treating advanced HCC are severely limited, especially in patients diagnosed with late stage development where liver resection is no longer a viable option. In fact, HCC is usually detected at late stages and currently Sorafenib and regorafenib are the only FDA approved chemotherapy drugs available to patients in this situation. Recently anti-PD-1 antibody, Nivolumab, has been approved for HCC treatment by FDA with limited therapeutic outcome. To that end, we are seeking to develop additional therapies that may potentially inhibit or reduce carcinogenesis in human HCC cells.

Recent studies involved in improving cancer treatment have been moving toward a “targeted gene therapy” approach, where the expression of oncogenes and tumor suppressor genes (TSGs) are altered in ways that reduce the malignancy of cancer cells. The present study indicates that targeting the expression of SND1 oncogene in human HCC cell lines holds promise as a potential HCC therapy. It was decided that an adenovirus vector would serve as the best delivery system for SND1 targeted gene therapy. This is because, unlike many viral vector systems, adenoviruses do not integrate their DNA into the host cell genome. Though inability to integrate means that the desired alterations to SND1 expression are robust, yet transient, it also means that

this system avoids induction of mutations/indels due to sporadic viral genome site integration. Transposon induced mutagenesis has been shown to impact EMT and chemotherapy resistance in HCC cells[68], so it is best to avoid integrative viruses if possible. Furthermore, attenuated human adenovirus vectors are capable of carrying large transgenes, infecting a wide-range of cells and can easily be propagated to a high titer. These features are what make adenovirus vectors a promising mode of delivery in our study. Additionally, three distinct immortalized HCC cell lines were used in this study to observe the potential of treatment variability associated with cellular heterogeneity. HepG3 cells are derived from a liver hepatocellular carcinoma of a 15-year-old Caucasian male, with wild type p53 expression, and stably express duck HBV transgene. HuH-7 cells are a well differentiated hepatocellular carcinoma cell line taken from a liver tumor in a 57-year-old Japanese male, express mutant p53, and have no viral background. Hep3B cells originate from an 8-year-old African American male, lack p53 expression, and the patient was determined to be HBV positive. Data from this study shows that adenovirus vectors effectively infect human HCC cells *in vitro* and that the therapeutic results of adenovirus-mediated knockdown of SND1 are consistent across heterogeneous cell populations.

shRNA knockdown of SND1 was highly effective, with roughly 95% of SND1 mRNA being reduced when cells were infected at 100 MOI. 100 MOI was determined to be the standard for capturing the effects of Ad.SND1sh infection of HCC cell lines. This is because significant changes at the protein level were seen via Western blot with SND1, but not with any of the other proteins of interest when using lysates from cells infected at lower MOIs. Additionally, a high f MOI was needed because prolonged exposure to

the virus (>72 hours) showed signs of cytotoxicity in HepG3 and Hep3B HCC cell lines. This may explain why the MOIs used for the wound healing and Matrigel invasion assays had to be altered in order to get accurate results that were independent of virus's toxic effects. Statistical analyses comparing data from MTT assay of control, Ad.RNAi-, and AdSND1sh-infected HCC cells showed that there was no consistent statistically significant differences in proliferation associated with adenovirus mediated of SND1 in these HCC cell lines.

Changes in cellular migration and invasion are phenotypic attributes associated with epithelial mesenchymal transition (EMT). EMT is as hallmark feature of carcinogenesis which grants cancer cells the ability to break down and migrate through the basement membrane, invade the blood stream or lymphatic system, and metastasize in distant tissues. To assess if knockdown of SND1 reverses EMT in infected human HCC cells, cell lysates were probed for EMT markers: N-Cadherin and E-Cadherin. A reduction in N-Cadherin at the protein level was observed in Ad.SND1sh-infected HepG3 and HuH-7 cells, yet, the protein levels of E-cadherin remained unchanged. Cadherin switching is a common occurrence in carcinogenic cells undergoing EMT but has yet to be fully understood in context of HCC. Analysis of tissue samples from patients diagnosed with HCC found that there was no significant indication of cadherin switching, with only 5 of the 63 patient samples in the cohort displaying strong N-Cadherin and reduced E-Cadherin IHC staining in HCC tumor cell membrane[69]. This prompted the need to analyze transcription factors that regulate EMT, such as Snail, Slug, and Twist in order to determine which protein(s) were responsible for this change in EMT phenotype. Snail and Twist proteins were reduced in Ad.SND1sh-infected cells, while Slug was

universally down regulated in all HCC cell lines with SND1 knockdown. Based on this data, we can conclude that adenovirus-mediated knockdown of SND1 reduces Slug expression in HCC cells, leading to a decrease in N-Cadherin, which causes cells to lose mesenchymal features resulting in reduced migratory and invasive capacity. Also, certain HCC phenotypes may also display reductions in Snail and Twist protein levels. We believe this may be the result of genetic heterogeneity in HCC cell phenotypes.

Recent studies have been published linking EMT to the generation of stem cell like properties in cancer cells[70]. A similar study found that when IHC was performed on 27 HCC patient samples, EMT markers E-Cadherin and N-Cadherin were down regulated 63% and upregulated in 81%, respectively, of patient samples. Also, of note was that 78% of those patient samples were found to have significant upregulation of TIC marker CD133[71]. To assess if SND1 knockdown affects cancer stemness in HCC cells, lysates from control and adenovirus-infected cells were probed for TIC marker CD133 and HCC marker α-fetoprotein (AFP). A reduction in both CD133 and AFP was observed in Ad.SND1sh-infected cells from all three HCC cell lines. This finding is significant because Slug overexpression has been shown to enhance CD133 expression in the HCC cell line HepG2[72]. In addition, $CD44^+$ $CD133^+$ HCC cells have been found to correlate with enhanced AFP expression and poor differentiation[73]. Based on the established literature and data obtained from this study, it can be inferred that SND1 knockdown reduces Slug expression, which in turn reduces CD133 and AFP protein expression in human HCC cells.

Further investigation is needed to better understand how adenovirus-mediated knockdown of SND1 leads to decreased Slug protein levels in HCC cell lines. Since no

significant changes were detected in E-Cadherin protein levels of Ad.SND1sh-infected HCC cells, it would be recommended to probe for other EMT other mesenchymal markers, such as Vimentin, Fibronectin, and Laminin-5. Probing for these proteins may prove to further distinguish how reduced SND1 expression affects HCC cells, particularly in the case of Hep3B cells where Ad.SND1sh-infection significantly reduced migration but had no significant effect on N-Cadherin protein levels. Rhodamine phalloidin staining of Ad.SND1sh HCC cells may display a difference in actin organization, which is expected to be altered with reduced migratory and invasive capacity. With AFP and CD133 reduction being confirmed with SND1 knockdown, tumor sphere formation capacity and drug resistance need to be assessed to successfully validate diminished TIC phenotype in human HCC cells. Once the phenotypes and molecular pathways associated with adenovirus-mediated knockdown of SND1 have been fully investigated *in vitro*, this gene therapy system should be assessed using nude mice to determine its efficacy *in vivo*.

In conclusion, we have shown that an adenovirus vector system is efficient in delivering targeted gene therapy to HCC cells in vitro, with adenovirus-mediated knockdown of SND1 resulting in reversal of EMT and reduction in liver cancer stem cell markers AFP and CD133 via reduction of Slug protein expression.

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