VIP INDUCES SNAIL1, A MASTER EMT REGULATOR: UPREGULATION IN A375 CANCER CELLS

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Biochemistry

June 2021

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

VIP is neurotransmitter with pleiotropic functions in mammals. It is expressed by a large number of tissues, including the CNS, PNS, innate and adaptive immune systems. VIP has two endogenous G-protein coupled receptors, termed VPAC 1 and VPAC2. VIP signaling through VPAC1 receptor has been documented to transactivate EGFR in healthy and cancerous cells leading to the activation of multiple downstream signaling pathways. EGFR signaling is a potent inducer of the master regulator EMT, called Snail1, which is a zinc-finger, transcription factor that is associated with downregulating epithelial markers like E-cadherin, while upregulating mesenchymal markers necessary for invasion and metastasis. We hypothesize that VIP upregulates Snail1expression in cancer cells. Our results showed that VIP treatment of epithelial cells increased Snail1 expression transiently at 1h and 4h then returned to basal levels at 24h. This research has implications in development of targeted therapies for cancer.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Dorsam for all his guidance and support. I would also like to thank my committee members, the Chemistry and Biochemistry department chair, and the Graduate school for their assistance.

DEDICATION

I would like to dedicate this thesis to my family for their love and support, especially my beloved mother who was always there for me, through every step of the way. Mother you are a candle that lights my life, I thank you for everything you do, you are a true blessing, I love you.

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LIST OF ABBREVIATIONS

7TM .....7 Trans-Membrane

a.a.....Amino acid

Ab .....Anti-body

AC.....Adenylate Cyclase

β- Actin.....Beta Actin

BB .....Binding Buffer

BSA .....Bovine Serum Albumin

BCS.....Bovine Calf Serum

cAMP.....Cyclic Adenosine Monophosphate

C-ter .....C-Terminus

CNS .....Central Nervous Systems

CD4.....Cluster of Differentiation 4

DNA.....Deoxy ribonucleic Acid

EDTA .....Ethylene diamine tetra acetic acid

EGFR .....Epidermal Growth Factor Receptor

EGF.....Epidermal Growth Factor

EMT.....Epithelial- Mesenchymal Transition

g .....Grams

GPCR.....G-Protein Coupled Receptor

h .....Hours

HDAC.....Histone Deacetylase

Her2 .....Human Epidermal Growth Factor Receptor 2

IF.....Immunofluorescence

Kd .....Dissociation Constant

L .....Liters

LMB.....Leptomycin B

µM .....Micro molar

mM.....Milli molar

mL.....Milli Liters

min .....Minutes

MeOH .....Methanol

mRNA.....Messenger ribonucleic Acid

MEMα .....Minimum Essential Media α

MMP.....Matrix Metalloprotease

N-ter.....N-Terminus

nM.....Nano Molar

NLS.....Nuclear Localization Signal

NES.....Nuclear Export Sequence

ON.....Over Night

PACAP .....Pituitary Adenylate-Cyclase Activating Peptide

PFA.....Paraformaldehyde

PKA .....Protein Kinase A

PBS .....Phosphate Buffered Saline

PNS.....Peripheral Nervous Systems

Pak1 .....p21 Activated Kinase 1

p/s.....Penicillin/Streptomycin

REG .....Regulatory Domain

RPM.....Revolutions per Minute

RT .....Room Temperature

SCP .....Small C-terminal domain phosphatase

Tx-100 .....Triton X-100

T reg.....T regulatory cell

Th2.....T Helper 2

TGFβ.....Tumor Growth Factor β

VEGF.....Vascular Endothelial Growth Factor

VPAC .....Vasoactive intestinal peptide / Pituitary Adenylate Cyclase receptor

VIP.....Vasoactive Intestinal Peptide

ZF.....Zinc Finger

INTRODUCTION

VIP

Vasoactive Intestinal Peptide (VIP) is a secreted 28 amino acid (a.a.) neuropeptide predominately expressed in the central and peripheral nervous systems (CNS and PNS) of mammals that belongs to the secretin superfamily (1). VIP was first discovered in 1969 when it was purified from swine intestines and described to have vasodilator properties, which prompted its rather historical name (28). A decade later, VIP was shown to be highly expressed within the Central nervous system and Peripheral nervous system and therefore accepted as a neuropeptide/ neurotransmitter. In the 1980's and 1990's additional discoveries in the field demonstrated molecular actions within the immune systems showing potent anti-inflammatory effects and an inducer of anti-inflammatory, T regulatory cells (Treg). Therefore, over the past 50 years, the pleiotropic molecular functions of VIP have been linked to vasoactive properties of blood vessels, secretagogue action within the pulmonary and gastrointestinal systems, neurotransmitters in the nervous system, and immunoregulator in both the innate and adaptive immune systems. VIP a master regulator of the central circadian rhythm that defines mammalian feeding behaviors based on solar time (8).

VIP is synthesized by endocrine and immune cells (8), and is delivered by VIPergic nerve fibers that innervate many tissues and organs, including the mucosa-associated lymphoid organs of the lungs and gut (2). Within the gastrointestinal tract (GIT), VIP is involved in smooth muscle relaxation, water and ion homeostasis (6), gastric acid secretions, and peristalsis by inhibiting smooth muscle contraction and mucus secretion from goblet cells. VIP also maintains normal barrier function by promoting differentiation, proliferation and cell adhesion of epithelial

cells in the intestines. VIP deficiency is associated with changes in microbiota structure, biodiversity and decreased body weight (4).

VIP acts as an immunomodulatory agent to restore homeostasis of the immune system. It plays a pivotal role in immune disorders such as Rheumatoid Arthritis, Irritable Bowel Syndrome and Multiple Sclerosis. Evidence for such restorative immunological actions for VIP is based on how it blocks inflammation by down-regulating co-stimulatory molecules on dendritic cells and generating regulatory T cells (4). VIP secretion by activated CD4 T_{h2} cells is a secondary autocrine/paracrine source that can modulate immune function as a T_{h2} cytokine, and as a result it can potentially treat certain autoimmune diseases, clinically. For example, it is protective against collagen induced arthritis and inflammatory bowel disease by acting as an immunosuppressive agent possibly through the generation of antigen-specific Tregs (3). PACAP (Pituitary adenylate-Cyclase Activating Peptide) shares 68% homology with VIP, so VIP is thought to have evolved by exon duplication from PACAP (2). PACAP has two forms, PACAP27 and PACAP38. Both PACAP and VIP belong to the secretin super family (5).

VIP amino acid sequence:

H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-O-M-A-V-K-K-Y-L-N-S-I-L-N

PACAP27 amino acid sequence:

H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-O-M-A-V-K-K-Y-L-A-A-V-L

PACAP38 amino acid sequence:

H-S-D-G-I-F-T-D-S-Y-S-R-Y-R-K-O-M-A-V-K-K-Y-L-A-A-V-L-G-K-R-Y-K-O-R-V-K-N-K

VIP Receptors

VIP is known to bind at least two receptors, VPAC1 and VPAC2, VPAC stand for Vasoactive Intestinal Peptide/Pituitary Adenylate Cyclase Activating Polypeptide Receptor. Both

receptors can bind to VIP and PACAP at physiological concentrations, whereas PAC1 (Pituitary Adenylate Cyclase Activating Polypeptide Receptor) binds to PACAP with higher affinity (12).

VIP receptors are seven transmembrane, Group 2-B, G-protein coupled receptors (GPCRs) that belong to the secretin/glucagon family (9, 10). The VPAC1 receptor is an archetype of class B GPCR (11). All GPCRs share a common structure of 7 transmembrane domains (7TM), 3 intracellular and 3 extracellular loops, an amino terminal extracellular domain and a carboxyl terminal intracellular domain (12). In this group, the N-terminus is structured (> 120 residues), which represents the major binding site for its natural ligand, so VIP's N-terminus interacts with VPAC1 extracellular side (11). GPCRs have a common signaling mechanism where they interact with G-proteins to produce second messengers in the cell like cAMP, IP, DAG and Ca+2 (12). Class II GPCRs couple to Gαs - adenylate cyclase-cAMP predominantly (12) but differential coupling between Gαs, Gαi and Gαq can result from VIP/ VPAC1 signaling depending on cell type and context (9).

VPAC1 is widely expressed in mammals. It binds VIP with high affinity (≈1nM K_d) (9). In immune cells, VIP modulates numerous cellular activities like chemotaxis, proliferation, apoptosis and differentiation (9), only if these cells express VPAC1 and are in close proximity to VIPergic nerves. Of these immune cells, B cells don't have enough VPAC1 receptors to be detected, whereas T cells have high levels of it. Upon activation, CD4 + T cells show 10-fold less VPAC1 than naïve ones (10). Many inflammatory disorders and diseases have abnormally high VPAC1 levels, examples are solid tumors (lung, brain, breast and prostate), irritable bowel disease and rheumatoid arthritis (9).

VIP and its Receptors in Cancer

In the annual cancer facts and figures for 2021, the most common types of cancers are breast (30%), colon (16%) prostate (26%) and lung &bronchus (25%). These cancers predominantly express functional VPAC1 receptors which are, and in very rare cases express VPAC2 (e.g., certain human and rat leukemic cancers) (14), so these receptors can be targets for imaging and targeted delivery. By using radiolabeled VIP analogues to localize tumors in humans and animals, Nano-particles coupled to VIP to deliver cytotoxic agents to high-VPAC1-expressing tumors (13). VIP acts differently depending on tumor type, VIP modulates growth, differentiation and migration of many human tumors (breast, prostate and lung) promoting carcinogenesis, or inhibits growth of others (e.g. renal) (13, 14). VPAC1 is overexpressed in many cancers like breast, prostate, colon, liver, thyroid and pancreatic (13). In different breast cancer cell lines, VIP activates VPAC1 thereby turning on AC, which leads to higher VEGF expression and secretion, inducing growth that can be inhibited by VPAC1 antagonists (13). In fact, VIP receptors antagonists were found to inhibit proliferation of many solid tumors (51 of 56 human lung cancers) (14).

VIP stimulates transactivation of Her2/Neu and EGFR in breast cancer. Transactivation happens when EGFR is activated by another receptor, typically a GPCR. Upon ligand binding to a GPCR, the signal produced will induce activity of a transmembrane metalloproteinase, activated metalloproteinase will cleave (ecto-domain shedding) a pro- EGF producing a mature EGF as a substrate for EGFR, which binds to EGFR activating it and inducing an intracellular signal (29).

In colon cancer, studies revealed that highest overexpression of VPAC1 correlated with increased translocation of activated EGFR to the cytoplasm. In prostate cancer, VIP addition

causes an increase in cAMP and transactivation of Her2/Neu through PKA-ERK which leads to increase in c-Fos, VEGF and Cox2. This induces a malignant transformation, proliferation and increased cell motility (13).

EMT

EMT (Epithelial-Mesenchymal Transition) is a genetic developmental program that is evolutionarily conserved (18). It is a dynamic process to acquire cell motility with simultaneous loss of adhesive ability for body organization during embryonic development and wound healing (15, 16). EMT is a progressive and reversible process in which epithelial cells undergo a change in phenotype, during this transition cell-cell junction structures are disassembled, causing a change from a cobble stone to an elongated spindle like appearance of the cells (20) characterized by decreased epithelial markers like E-Cadherin and increased mesenchymal markers like fibronectin (16). E-Cadherin is an adherens junction protein, or gate keeper that suppresses EMT events, dissemination and motility of the cell that maintains the normal morphology of epithelial cells (15, 18). During EMT, rearrangement of cytoskeletal proteins is a key step. Features of EMT include more cell spread morphology, downregulation of E-Cadherin, upregulation of Snail1 (transcription factor), vimentin and fibronectin (structural proteins) (15, 17).

EMT is regulated by many Transcription factors such as Snail, Slug and Twist (16). Snail1 is required for EMT initiation, while Twist acts to maintain late EMT. Cells receive signals from the microenvironment to initiate and regulate EMT including TGFβ, hypoxia and EGF (19). Absence of snail1 severely impacts mesoderm formation during embryogenesis, thus making Snail1 a key marker of EMT.

EMT has characteristics that are beneficial for tumorigenesis, as malignant cells become invasive and mobile, resistant to pro-apoptotic signals, and phenotypically transform their metabolism to that of stem cells (20). EMT can be induced by one of many transcriptional repressors, yet multiple ones are expressed simultaneously in cancer cells (19). TGFβ and VEGF are not necessarily transcription repressors, but are EMT inducers and can upregulate Snail in a variety of cancers (16). EMT is a key player in prostate cancer progression (18). Metastasized cancer tumors rather than primary ones are responsible for most cancer deaths (17). According to the American cancer society, 81% of cancers are invasive.

Snail induces EMT, accelerates cancer metastasis by utilizing the enhanced invasive ability, induction of multiple immunosuppression and immuno-resistant mechanisms, like immunosuppressive cytokines, regulatory T cells, impaired dendritic cells and cytotoxic T lymphocyte resistance. Snail blockade inhibits both cancer invasion and the multi immunosuppressions at the same time yielding efficient inhibition of cancer metastasis, this targeting can be useful in immuno-therapy (16).

Snail1

Snail was first discovered in drosophila (fruit fly) where embryos that didn't express snail failed to develop mesoderm layer and died early during embryogenesis (23). In mammals, Snail1 expression is necessary for early phases of embryonic development. Snail1 knock out mice embryos are embryonically lethal as they don't finish gastrulation (21, 15, 19, and 25). Snail can induce cellular invasion during embryonic development, and in epithelial cells this invasion starts with increasing motility, production of extracellular matrix degrading enzymes and cell-cell connections/junctions (22). Snail1 is a DNA binding protein acting as a transcriptional repressor as it blocks E-Cadherin expression by binding to its promoter (21) causing the cell to

detach from neighbors and migrate (25). It represses tight junction proteins, matrix metalloproteases, and mesenchymal marker expression such as vimentin and fibronectin (indirectly), as well as other transcription factors expression (21, 24). Snail1 is a key transcription repressor of E-Cadherin (15) and is one of the ultimate inducers of EMT (19). Snail is a critical regulator of multiple signaling pathways that lead to EMT and cell migration (15). For these reasons, Cancer cells have usurped this transcription factor for a survival advantage. Snail1 is a key factor in metastasis of melanoma, bladder, and breast, colorectal and pancreatic carcinomas. In these tumors, both tumorigenic and non-tumorigenic epithelial cancer cells (19) downregulate E-Cadherin, which positively correlates with increased expression of snail. Snail1 plays a role in in EMT by suppressing E-Cadherin which in turn is a suppressor of invasion during cancer metastasis (27). Snail overexpression induces breast cancer recurrence and apoptosis resistance (15, 19).

The snail super family is divided into two subfamilies; Snail and Scratch. Snail family contains three members; snail1, snail2 (slug) and snail3 (24). These are zinc finger (ZF) transcription factors (27). A zinc finger is a specialized peptide domain that is stabilized by a zinc ion. Cysteine and histidine residues bind to zinc ions to bind DNA with a specific affinity depending on the amino acid sequence of the finger (31).

There are two regions in this protein; N-terminal domain (a.a. 1-151) and a C-terminal domain (a.a. 152-264). N-ter has the SNAG domain which is important for repression (24) and recruiting HDACs (21), and NES (nuclear export sequence) region which is important for subcellular localization and the destruction box domain. C-terminus has 4 zinc fingers, that recognize and bind to a specific DNA sequence and serves a nuclear localization signal (NLS) (24, 25, and 27).

Snail1 localization can be cytoplasmic or nuclear or both, depending on the cell type. Activity of Snail1 is controlled by phosphorylation. In the nucleus when snail is phosphorylated it is less active as a repressor or activator of transcription (25). De-phosphorylated Snail1 is more stable (15) and is only present in the nucleus to enhance its activity and induce EMT (27). The N-terminus region can be phosphorylated to control its repressive activity, or to signal for nuclear export, or ubiquitinylation and degradation (21). Phosphorylation of the destruction box domain creates a recognition site that induces degradation by Ubiquitin-Proteasome system. Therefore, control of the nucleo-cytoplasmic localization of Snail is crucial for its activity (25) and there are multiple kinases that are responsible for snail1 phosphorylation like pak1 (30) and CRM1 (15).

In 2010, Valdehita *et al.* showed that VIP through VPAC1 trans-activated EGFR and elicited a downstream signal in breast cancer cells, while Yang *et al.* 2005, suggested that EGFR signaling activated Pak1 which in turn can phosphorylate snail1 at S246 to facilitate its movement back to the nucleus for enhanced activity. Dorsam *et al.* 2010 did a microarray study in which CD4 T cells total mRNA was collected after VIP treatment, results showed an increase in snail1, Pak1 and EGFR levels. Taking all this together, we hypothesize that VIP signaling induces snail1 upregulation, to test this hypothesis we added VIP to A375 cells for 24h and performed IF. Our results showed an increase in snail1 after 1h, 4h. Future directions will include measuring snail1 mRNA and protein levels by qPCR and western blotting, respectively, upon VIP addition after 1h and 4h, using siRNA for Pak1/ VPAC1.

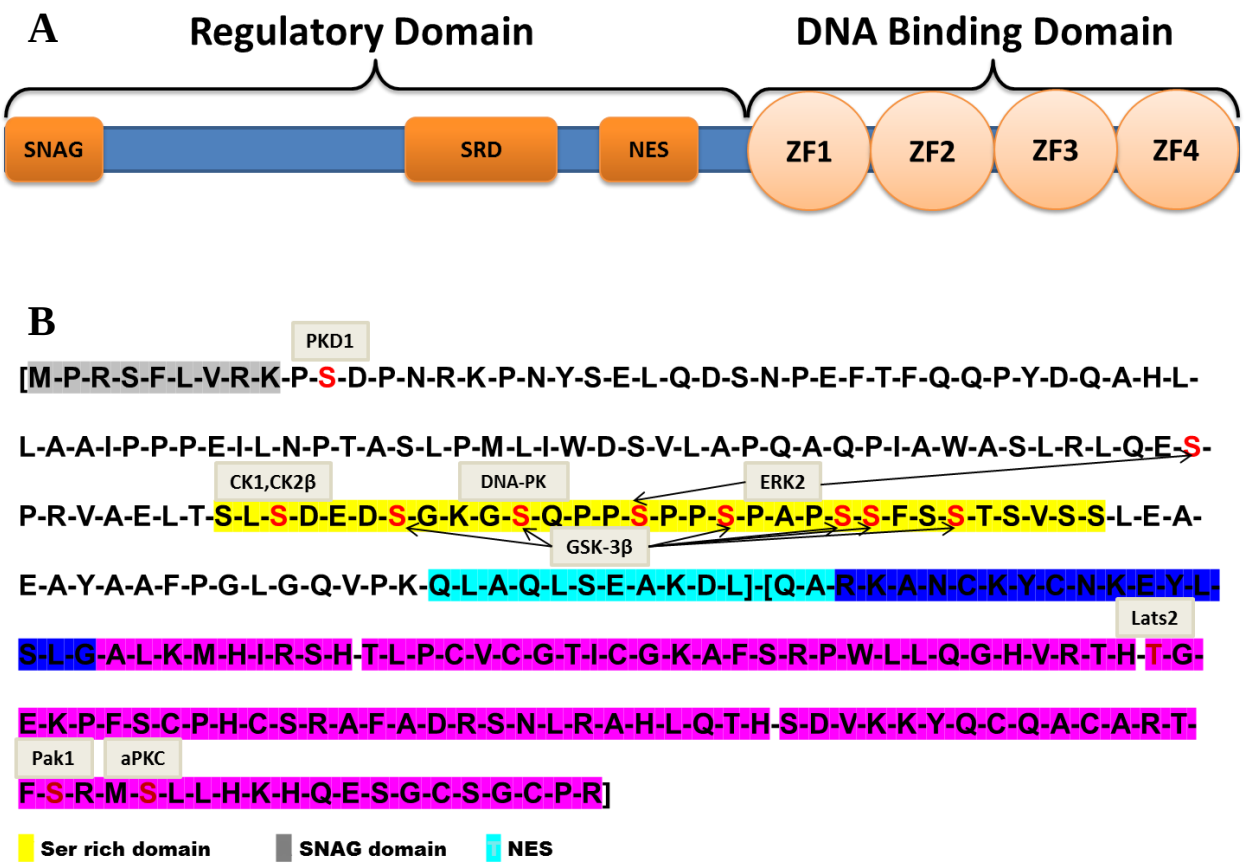

Figure 1. Snail1 protein structure and amino acid sequence. A) Full protein structure with important domains marked. B) Amino acid sequence of snail1, red ser and Thr are phosphorylated by the indicated kinases. Grey is SNAG domain, yellow is SRD, cyan is NES, purple is zinc finger, blue is a mix of NES and zinc finger. Snail1, NM_005985.

MATERIALS AND METHODS

Table 1. Reagents Used in Research.

Reagent	Source
A375, MCF7 and T47D Cell lines	Kind gift from Dr. Archana Dhasarathay at UND
MEM α medium	Thermo Scientific, Logan, Utah
Trypsin EDTA, 1X	Corning, Manassas, VA
PBS, 1X	Thermo Scientific, Logan, Utah
Penicillin/streptomycin	Cellgro, Manassas, VA
BCS (Bovine Calf Serum)	Corning, Manassas, VA
Alexa Fluor® 647 Goat Anti-Rabbit IgG (H+L)	Invitrogen, Grand Island, NY
Chamber culture slides	BD Biosciences, Bedford, MA
UltraCruz mounting medium	Santa Cruz Biotechnology, Dallas, TX
Rabbit anti-mouse /human SNAI1 (H-130)	Santa Cruz Biotechnology, Dallas, TX
Rabbit anti-mouse /human Actin (C-11)	Santa Cruz Biotechnology, Dallas, TX
30% BSA	Rockland Immunochemicals, Gilbertsville, PA
Leptomycin B	Cell Signaling, Danvers, MA
16% w/v MeOH free formaldehyde solution	Thermoscientific, Rockland, IL

Tissue Culture

A375, MCF7 and T47D cells were used in this research. Cell lines were grown in DMEM medium supplemented with 10% BCS and 1% P/S. In a T-25 flask, when cells reached a confluency of about 90% they were split by aspirating off medium and washed with 1X PBS, followed by incubation with 3ml of 1X Trypsin-EDTA and incubated at 37 °C incubator for 5 min. Two (2) mL of supplemented medium were added to deactivate the Trypsin. Cells were transferred to a new 15 mL centrifuge tube, washed twice with 5 mL 1X PBS. Cells were centrifuged at 1500 RPM (500 g) for 5 min at RT. Supernatant was aspirated and cells were re-suspended in 1 mL of 1X PBS, counted using a hemocytometer, and seeded at $5X10^5$ into a new T-25 culture flask containing 5 mL of supplemented medium and incubated at 37 °C incubator with 5% CO_2. Old medium was replaced every 2-3 days with fresh medium.

Immunofluorescence

Cells ($1X10^4$ cells/ml) were seeded in a four chamber-glass slide (Chamber culture slides) grown to approximately 85% confluency overnight (≈24 h) in 5% CO_2 incubator at 37 ℃. Cells were washed with 1X PBS, fixed with 4% MeOH free PFA for 15 min at room temperature (24 °C) and permeabilized with 1% Triton X-100 for 7 min at RT. Fixed cells were washed with 1X PBS, incubated with Binding Buffer (BB; 1% BSA) for 20 min at 4 ^oC. Diluted 1^o Ab (anti-Snail1, Rabbit anti-mouse /human SNAI1 (H-130)) was added and cells were covered and incubated over night at 4 ^oC and washed with 1ml of 1X PBS for 10 min between and repeated twice. Cells were incubated with 2^o Ab diluted in BB-and incubated for 1h in the dark at RT, washed again with 1X PBS as above, removed plastic chambers and added one drop-of Ultra Cruz mounting medium, covered with glass slip, sealed and examined with a Zeiss Axio Imager M2 microscope.

RESULTS

Successful Optimization of Immunofluorescence (IF) For SNAIL1 Protein

In order to successfully test our hypothesis, we established an IF protocol. To this end, we optimized the IF protocol to identify the best fixative, primary and secondary antibody concentrations for SNAIL1 protein detection.

The cross-linking paraformaldehyde (PFA) fixative resulted in lower background signal compared to the precipitate-forming methanol (MeOH) fixative. Fixatives work by "fixing" the cellular compartmentalization in order to visualize subcellular location of proteins of interest. The first fixation strategy involved 4% PFA that retains cellular integrity by cross-linking molecules via a methylene bridge (33). A second major fixing strategy utilizes ice cold 95% MeOH that precipitates proteins and cellular membranes, rather than cross-linking, in an attempt to provide a snap shot of subcellular molecules (32). A375 cells were grown to 90% confluency on glass-chamber slides and fixed with either MeOH or PFA prior to secondary Ab incubation diluted to 1/500. Figure 1 clearly shows unacceptably high background signal using the organic MeOH fixative compared to the aldehyde PFA fixative (compare top middle image to bottom middle image in figure 2) when visualized by fluorescent microscopy. A possible explanation for these results could be that MeOH causes greater binding surfaces for the secondary antibody thereby resulting in non-specific binding between precipitated molecular structures and the secondary antibody.

Successful Checkerboard Strategy for Identifying the Optimal Anti-SNAIL1 Primary Antibody (Ab) and Secondary Ab Concentrations

In order to measure Snail protein with the highest signal to noise ratio, we next set out to determine the optimal dilutions needed for both primary anti-Snail antibody and ALEXA-

conjugated secondary antibody. To this end, checkerboard analyses of primary and secondary Ab dilutions were evaluated using a cell line known to express Snail1 (26). Primary Ab dilutions smaller than 1/500 gave little to no detectable signal, while secondary Ab dilutions greater than 1/2000 gave high background signal (Figure 2). These studies suggested to us that the optimal primary antibody dilutions would be 1/100-200 for the primary antibody and 1/2000 for the secondary antibody, respectively. These antibody dilutions were used for all subsequent studies.

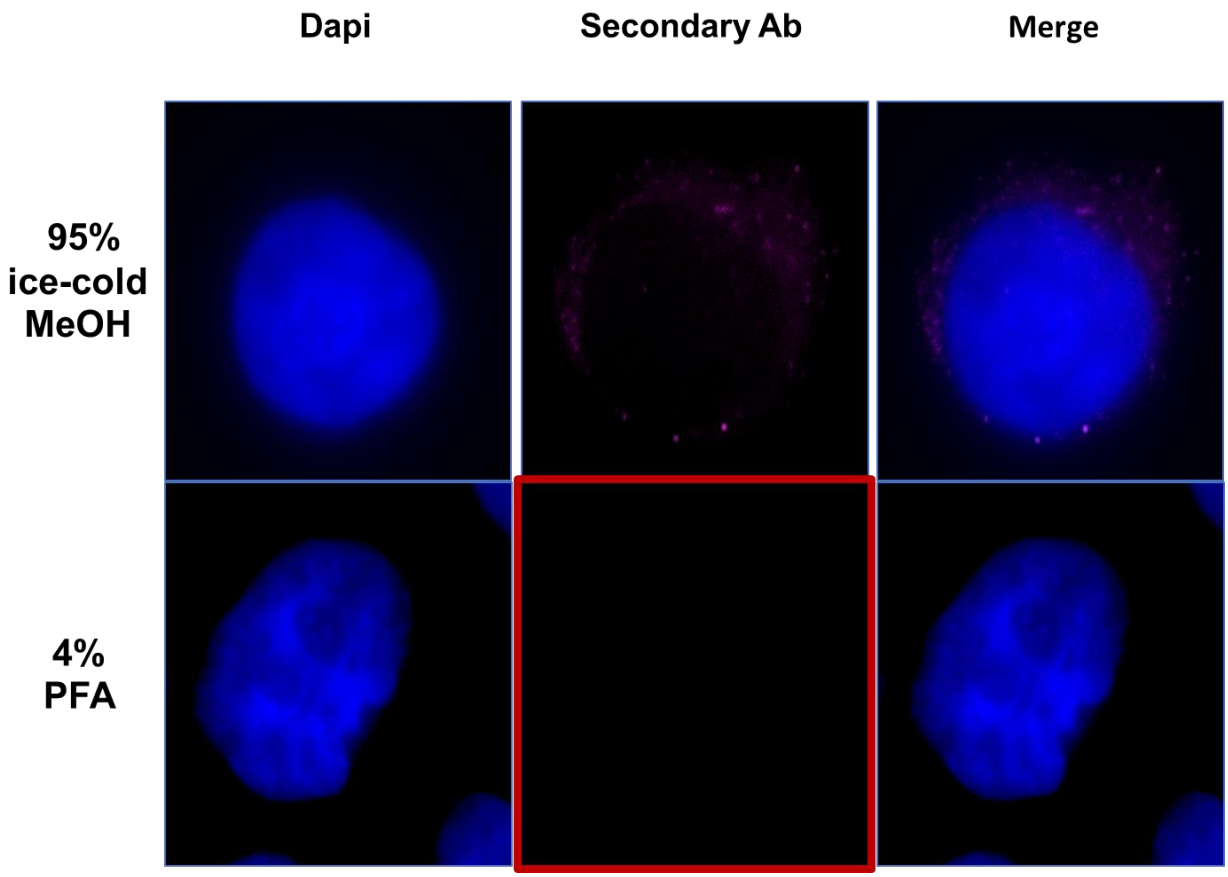

Figure 2. Optimization of fixation. A375 cells were seeded (1 X 10^4 cells) onto glass slides and fixed with: ice-cold, 95% methanol (*top panel*), or 4% paraformaldehyde (PFA, *bottom panel*). Cells were subsequently stained with an Alexa-conjugated secondary Ab (1/500) and counter-stained with Dapi. Photographs were taken with a Zeiss Axio Imager M2 microscope (100X).

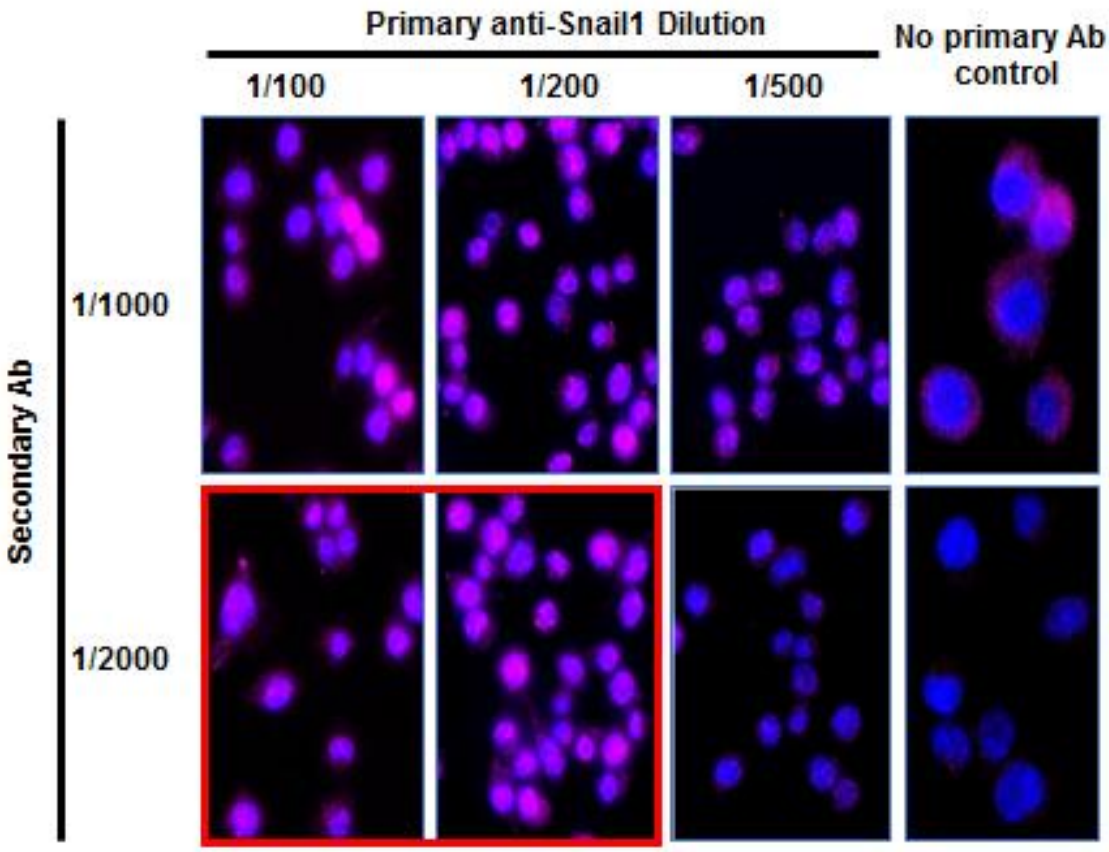

Figure 3. Primary and secondary Ab concentration optimization. A375 cells were seeded (1 X 10^4 cells) onto glass slides and fixed with 4% PFA. Cells were stained with a rabbit anti-mouse/human snail1 Ab at the indicated concentrations (top row), followed by staining with an Alexa conjugated goat anti rabbit secondary Ab at the indicated concentrations (left column). Cells were counter stained with Dapi. Optimal conditions for all future experiments were primary Ab 1/100-1/200 and secondary Ab 1/2000 (red rectangle). Cells were counter stained with Dapi and photographs were taken with a Zeiss Axio Imager M2 microscope (x20).

Validation of the Anti-Snail1 Polyclonal Antibody

Thus far, optimization of the IF procedure has identified a suitable fixative and working antibody dilutions. To finalize this optimization, we attempted to authenticate the specificity for the SNAIL1 antibody and to rule out off-target protein detection. We first tried two irrelative antibodies specific to actin and E-cadherin that would reveal different subcellular localization patterns compared to Snail1. As expected, the actin antibody (1/100) gave a ubiquitous, hazy subcellular localization throughout the cell, whereas E-cadherin (1/100) gave a fibrous, cellular-

junction subcellular localization – MCF-7 cells were used instead of A375 cells as there is higher E-Cadherin expression in these cells (25).

The second method was incubating cells with a nuclear-transporter inhibitor that would prevent proteins exported from the nucleus (e.g. Snail1 protein), and thus cause accumulation in the nucleus (Figure 4).

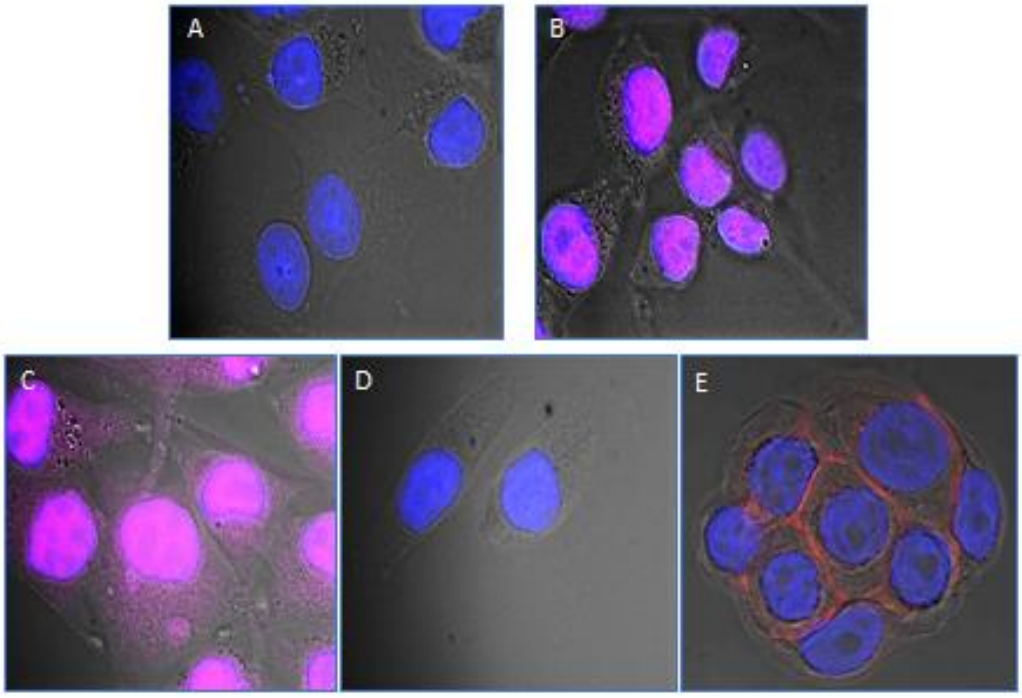

Figure 4. Validation of anti-snail1 antibody. **A-D.** A375 cells or **E.** MCF-7 cells were seeded (1 X 10^4 cells) onto glass slides and fixed with 4% PFA. **A-E** Cells were incubated with **A.** no primary Ab, **B.** Snail1 primary Ab (1/100), **C.** Actin primary Ab (1/100) or **D and E.** E-Cadherin primary Ab (1/100), followed by staining with an Alexa-conjugated goat-anti-rabbit secondary Ab (1/2000). Cells were counter stained with Dapi and photographs were taken with a Zeiss Axio Observer Z1 LSM 700 confocal laser scanning microscope (x100).

We stained A375 cells with different primary antibodies, including anti-β- Actin and anti-E-Cadherin. A second cell line, called MCF-7 cells, was used that are known to express Snail1, low E-Cadherin. Cells stained with anti-β-Actin antibody showed intense signal both in the cytoplasm and nucleus, but there was no detectable signal with anti-E-Cadherin as these cells are high in snail1 which in turn lowers E-Cadherin expression, as expected.

LMB was used as a second method for validating snail1 signal during the IF staining of A375 cells. LMB (0 -50 nM) caused a concentration-dependent retention of signal within the nucleus based on DAPI counterstaining (Figure 5).

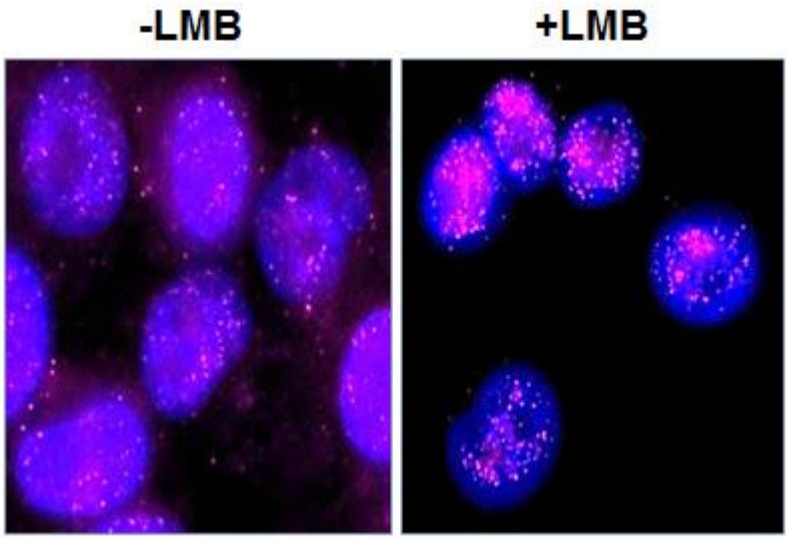

Figure 5. Nuclear export inhibition elevates Snail1 nuclear staining. A375 cells were seeded ($1 \text{ X } 10^4$ cells) onto glass slides and incubated in the absence (-LMB) and presence of 50 nM LMB (+LMB) for 24 hr. Cells were fixed with 4% PFA and stained with an anti-SNAI1 primary Ab (1/100) followed by an Alexa-conjugated goat-anti-rabbit secondary Ab (1/2000). Cells were counter-stained with DAPI and photographs taken by a Zeiss Axio Imager M2 microscope (x100).

In summary, these data support the notion that IF signal from indirect staining of various epithelial cell lines with anti-Snail1 polyclonal antibody is detecting authentic Snail1 protein. This conclusion was supported by the subcellular localization pattern seen in the epithelial cells (cytoplasmic and nuclear) that is distinct from other proteins (e.g. actin), or not detectable altogether (E-cadherin). Moreover, LMB treatment of epithelial cells successfully altered the subcellular localization pattern of Snail1 from a cytoplasmic/nuclear distribution to almost exclusively nuclear. These observations suggest that the anit-Snail1 antibody recognizes snail1 protein in a specific manner.

VIP Induces Snail1 Protein Upregulation in A375 Cells

To test our hypothesis, VIP was added to the cells at different time points to find the peak time of the cells' response to VIP at a concentration of 1×10^{-7} M compared to vehicle control (filtered bio-grade water). The time points that were included were 1h, 4h and 24h, followed by IF measurements. Exogenous addition of the VIP ligand cause a time-dependent increase in signal beginning at 1h, peaking at 4h and returning to basal levels at 24h (Figure 6). The increase in Snail1 signal was noticeable in the cytoplasm and was more intense in the nucleus. To conclude, VIP addition resulted in an increase in Snail1 protein at 1h and 4h, supporting our hypothesis.

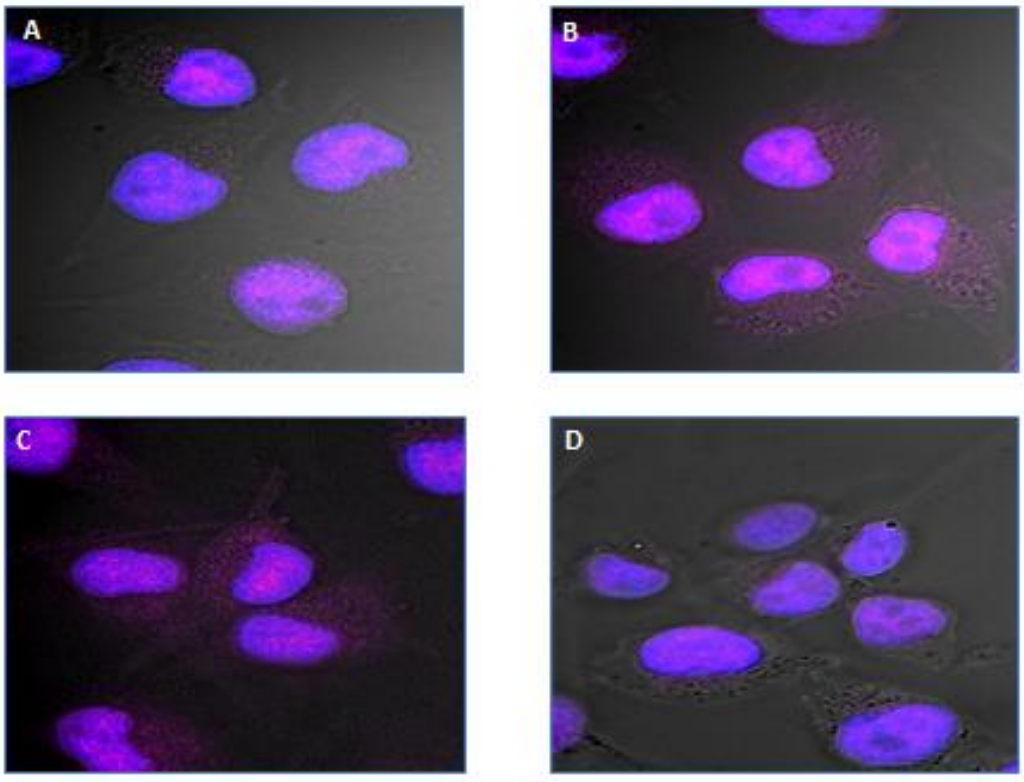

Figure 6. VIP transiently induces Snai1 increase in A375 cells. A375 cells were seeded (1 X 10^4 cells) onto glass slides and incubated with vasoactive intestinal peptide (VIP) at 1×10^{-7} M for **A.** 0h, **B.** 1h, **C.** 4h, and **D.** 24h. Cells were fixed with 4% PFA and stained with an anti-SNAI1 primary Ab (1/200) followed by an Alexa-conjugated goat-anti-rabbit secondary Ab (1/2000). Cells were counter-stained with DAPI and photographs taken by a Zeiss Axio Observer Z1 LSM 700 (x100).

DISCUSSION

In this study, we show that VIP affects the ultimate inducer of EMT for the first time, and because metastasized tumors are responsible for the majority of cancer fatalities, it is a very important target in cancer research.

Previously published by the Dorsam laboratory, microarray data revealed differentially expressed genes using in naïve CD4 T cells induced by exogenous addition of the VIP ligand (1×10^{-7} M) compared to vehicle control. This report identified a large number of genes that were upregulated, some of which are involved chemotaxis, including *snai1*, egfr and mmp and pak1 among many others. The increase in snail1 we see in figure 5 could be in part *de novo* snail1, following the central dogma, snai1 gene is transcribed to mRNA in the nucleus and transported to the cytoplasm for translation by the ribosomes to a fully functional, mature snail1 protein, this whole process depends on the extracellular (VIP from microenvironment) stimuli whether it's transient or not. To answer this question, cellular protein synthesis machinery can be blocked, and then detection of snail1 protein can be done after 1 h of VIP addition e.g. flow cytometry. Another question would be, if VIP is signaling through VPAC1. VPAC1 can be blocked prior to VIP addition, followed by snail1 detection. If snail1 was not upregulated, this would indicate that VPAC1 was the receptor VIP was signaling through otherwise it can be another VIP receptor, the same can be done for the other VIP receptors.

Another possibility for snail1 increase, is extending the half-life of preexisting snail1 (normal is about 25 min), being phosphorylated by pak1, for example, which induces snail1 to accumulate in the nucleus (27, 21) to repress more or to sustain induction of its target genes, thus it won't get marked for degradation by the proteasome after 25 min. This method is faster than

making new snail1 since it doesn't have to go through many steps of transcription and translation.

In 2013, Zhang XH, *et al* showed that EGFR signaling induces snail1 upregulation. Another study provided evidence for GPCRs transactivating tyrosine kinases (EGFR) in breast cancer cells (1).

We hypothesized that VIP could be affecting snail1 since cancer cells are known to hijack normal cellular mechanisms like EMT and immunosuppression.

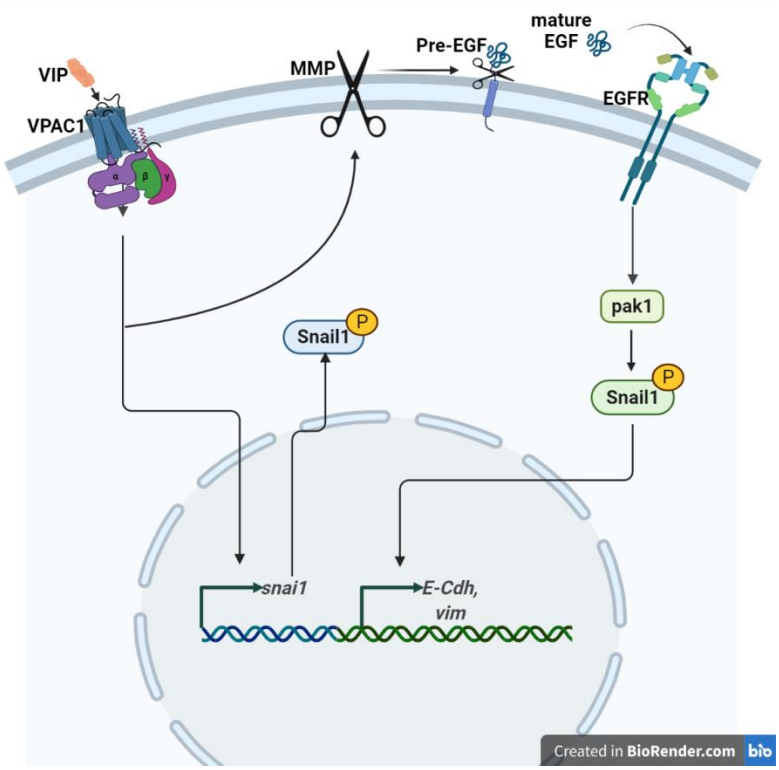

Figure 7. Schematic representation of our hypothetical model. VIP signals through VPAC1 eliciting a down-stream signaling cascade, ultimately causing an increase in snail1 gene expression, another product of this signaling is EGFR transactivation (activated MMP cleaves pre-EGF, which in turn can bind to EGFR and cause its activation). Activated EGFR would induce pak1 phosphorylation of cytoplasmic snail1, this would allow snail1 to be transported back to the nucleus, to bind promoter regions in the DNA of epithelial and mesenchymal genes. This schematic is created with BioRender.com.

To this end, we used IF as a method to detect changes to snail1 protein when VIP was added at different time-points. IF is a common technique that uses the specificity of Abs to certain antigens (in this case, protein sequences) that are labelled with a fluorescent dye, to provide information about the sub-cellular localization and protein expression levels under a fluorescence microscope.

In the nucleus, snail1 is normally not found in nuclear speckles (regions in the nucleus that snail can bind DNA) that are sites of active RNA splicing (25). In figure 5, these speckles are observed at all time points except 4 h suggesting that at that time snail1 is binding to DNA to induce repression/expression of its target genes, one of which is its own promoter, down-regulating its expression in a negative feed-back loop mechanism. This has been found in epithelial and mesenchymal cells that possibly prevents unwanted effects like growth inhibition (21).

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