The Role of KiSS1/GPR54 Receptor Signaling Pathway in Cancer Progression

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August 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of

The degree of Master of Science

Abstract

Metastasis accounts for 90% of cancer morbidity and mortality (Fares et al. 2020). Unfortunately, most of the existing anti-metastatic drugs exhibit very limited efficacy as well as show high toxicity levels in cancer patients. Thus, identifying key signaling pathways and molecules that relay and control metastasis remains a high priority to design novel and efficient targeted therapeutics against metastatic cancers and to improve overall survival rates in cancer patients. Kisspeptin (KiSS1) is a polypeptide that belongs to the neuropeptide family of RFamide peptides. KiSS1 regulates the human reproduction system through controlling sex steroid levels and the secretion of gonadotropin releasing hormone (GnRH). While KiSS1 and its receptor (KiSS1R, GPR54) can suppress metastasis in melanoma, prostate and pancreatic cancers, they appear to promote tumor metastasis in breast and liver cancers. Thus, the role of KiSS1 signaling in tumor progression and metastasis remains unclear and may be context dependent. The aim and long-term goal of this project is to study and decipher the role of KiSS1 signaling in distinct solid tumor types, including those of the breast, prostate, melanoma and pancreas and to further investigate the therapeutic potential of KiSS1 receptor agonist in these tumors. For this, we used a KiSS1/GPR54 receptor agonist (Y-156-2), recently designed by ShangPharma Innovation, Inc., to examine the effects of KiSS1 signaling on tumor progression using different models of breast, prostate, melanoma and pancreatic, cancers. Using specific cancer cell lines representative of the different solid tumor types, we assessed the effects of KiSS1 receptor agonist on cell viability, cell migration, cytoskeleton reorganization as well as epithelial to mesenchymal transition. Our results indicate that the KiSS1 receptor agonist prevents cell migration in prostate and melanoma cancer cells depending on the cell type while no effect was observed in pancreatic and breast cancer cell lines. With respect to epithelial to

mesenchymal transition (EMT), we found that the agonist increases the expression of the epithelial marker E-cadherin, assessed with immunoblotting and immunofluorescence, in prostate cancer and decreases snail expression in melanoma. Furthermore, we found that the KiSS1 agonist promotes the trans-localization of the mesenchymal marker Snail from the nucleus to the cytoplasm in melanoma, prostate and pancreatic cancer cell lines. Altogether, our results suggest that the KiSS1 agonist (Y-156-2) may represent a suitable candidate to prevent or delay tumor metastasis in melanoma, prostate and pancreatic cancers and lay the foundation for future *in vivo* studies to further investigate the ability of the agonist to reduce the metastatic burden in preclinical models of melanoma, prostate and pancreatic cancers.

Résumé

Près de 90% des cas de morbidité et de mortalité associés au cancer sont causés par la métastase (Fares et al., 2020). La majorité des thérapies contre les cancers métastatiques ont une efficacité très limitée et ont des effets toxiques chez les patients. Nous devons donc privilégier l'identification des voies de signalisation qui favorisent la métastase afin de pouvoir développer des nouvelles thérapies ciblées contre celles-ci et d'améliorer les taux de survie chez les patients. La protéine KiSSpeptin (KiSS1) appartient à la famille de neuropeptides RFamide. KiSS1 joue un rôle important dans le système reproducteur humain en régulant la production d'hormones et la sécrétion de la gonadotropin-releasing hormone (GnRH). Toutefois, alors que KiSS1 et son récepteur (KiSS1R, GPR54) semblent diminuer la métastase dans le mélanome, le cancer de la prostate et le cancer du pancréas, ils semblent promouvoir la métastase dans le cancer du sein et le cancer du foie. La contribution de la signalisation de KiSS1 dans les processus de formation de tumeurs et de métastases semblerait donc dépendre du contexte. Le but ultime de ce projet est d'élucider le rôle de la signalisation de KiSS1 dans des tumeurs solides, tels que les tumeurs mammaires, de la prostate et de la peau, et d'évaluer le potentiel thérapeutique d'un agoniste du récepteur KiSS1 dans ces contextes. Nous avons donc utilisé un agoniste du récepteur KiSS1/GPR54 (Y-156-2), dévéloppé par la compagnie ShangPharma Innovation Inc., pour examiner les effets de la signalisation KiSS1 sur la croissance tumorale dans des modèles de cancer du sein, du pancréas, de la prostate et de la peau. Nous avons évalué les effets de KiSS1 sur la viabilité cellulaire, le potentiel de migration des cellules, la réorganisation du cytosquelette et la transition épithéliale-mésenchymale. Dans des lignées cellulaires provenant de ces cancers. En effet, nous avons démontré que l'agoniste KiSS1 diminue la migration cellulaire dans des lignées cellulaires de mélanome, de cancer de la prostate. Cependant, l'effet contraire a été

observé dans des lignées cellulaires de cancer du sein et de cancer du pancréas. Nous avons démontré que l'agoniste augmente l'expression de E-cadhérine dans le cancer de la prostate et qu'il diminue l'expression de Snail dans le mélanome par analyse de protéines et par immunofluorescence. De plus, en traitant des cellules de mélanome, de cancer du pancréas et de cancer de la prostate avec l'agoniste KiSS1, nous avons remarqué le changement de localisation de Snail du noyau au cytoplasme des cellules. Nos résultats suggèrent que l'agoniste KiSS1 (Y-156-2) semble pouvoir prévenir ou diminuer la formation de métastases dans le mélanome, le cancer de la prostate et le cancer du pancréas. Cet agoniste pourrait donc être utilisé dans des études *in vivo* afin de pouvoir mieux évaluer son potentiel anti-métastatique dans des modèles précliniques de la peau, de la prostate et du pancréas.

Acknowledgements

The work in this dissertation would not have been possible without the guidance, the help, and the support of many individuals who by various means contributed and extended their valuable assistance in the completion of this thesis. It is to them I owe my profound gratitude.

First, to Dr. Jean-Jacques Lebrun, who undertook to be my supervisor and supported me from the beginning of my journey and believed in my ability to succeed. Also, for supporting me to go through the bureaucracy of the system, his kindness and love is something to remember.

To Dr. Sabah Hussein for his continuous guidance and support in every meaning as he has been a father, mentor, and an important part of my family in this country.

To Nicolas Knoppers-Turp for his support over my legal documents with IRCC, for all of his encouragement during the hard times I have passed through and being there for me.

To my supervisory committee members:

Dr. Giovanni DiBattista for his thoughtful comments and input toward my studies also for all interesting insights and discussions with me about the political future of this country.

Dr. Bertrand Jean-Claude for his unconditional support and love he provided from day one in this school.

To Dr. Robin Beech for his support and time to understand the administrative issues I have faced inside the university and helping me to overcome them.

To Dr. Inga Murawski for her generous support in reviewing and assisting me in the editing of my academic writing.

To all family members, and all those who supported and encouraged me during the completion of this research.

To my tremendously loving grandparents and parents, for their continual unconditional love and support and for their undivided devotion and commitment.

To all my friends, especially Mostafa Ghozlan, Alaa Moamer, and Najat Binothman, Anwar Shams, Ibrahim Hachim, who not only offered their generous assistance to help me to understand experimental techniques, but also encouragement and fun times throughout my work, also to those provided me with their caring and love on daily basis, Samar Elzein, Hajer Mohammad, Maryam Fazeli, Taghreed Ayash, Vita Sonjak, Nisreen Mohammad.

Dedication

I heartily would like to dedicate this thesis to:

My utterly loving grandmother, Kamala Mustafa and my amazingly caring grandfather, Mohamed Suleiman, who kept my spirits up when the muses failed me, for their continual unconditional love and support and for their undivided devotion and commitment throughout all my life.

My beloved parents and siblings, Mom Maryam Mohammad and Dad Abed AL Jaleel Abubaker,

My sisters, Hanaa, Somia, Iman, Aya, Ebtihal & My brothers, Abubaker and Mohammad

And to those friends:

Prof. Sabah Hussain, to whom my words will not be enough to express my gratitude for his unconditional encouragement and support to me every single day throughout my entire MSc journey, from beginning to end.

Mr. Nicolas Knoppers-Turp who has supported and encouraged me during my studies by providing unconditional legal assistance to complete my degree forever appreciative for his support.

Dr. Mostafa Ghozlan and Dr. Alaa Moamer for training me with full patience and providing me with everything I have needed unconditionally, their presence made this journey more joyful and tolerable.

Contributions of authors

I have conducted all the data in this thesis by myself, yet it would not be possible without the assistance of the following:

My supervisor who have contributed to the experimental design, helping, and supporting with the troubleshooting the challenges throughout the project.

Mostafa Ghozlan, Alaa Moamer, and Anwar Shamas have trained me in preforming cell culture and all the techniques I have used in this thesis.

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List of Abbreviations

Abbreviation	Meaning
DAPI	4,6-diamidino-2- phenylindole
DMEM	Dulbecco's modified Eagle medium
DMFS	Distant metastasis free survival
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E-CAD	Epithelial cadherin
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum;
FDA	Food and drug administration
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3- kinase
PTHRP	Parathyroid hormone related protein
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rb	Retinoblastoma protein
RGP	Radial growth phase
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species;
RPM	Revolutions per minute
RPMI	Roswell park memorial institute medium
R-SMAD	Receptor-regulated Smad
SAPK	Stress-activated protein kinase
SBE	Smad binding element
SC	Stem cell
SCC-SC	Squamous cell carcinoma stem cell
SCR	Scrambled
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SNON	Ski-related novel gene
SOCS	Suppressor of cytokine signaling
SSM	Superficial spreading melanoma
STAT	Signal transducer and activator of transcription
TBRI	Type I TGFβ receptor
TAK1	TGFβ-activated kinase 1
TGFB	Transforming growth factor β
BMPs	bone morphogenetic proteins

GDFs	Growth differentiation factors
GDNF	Glial cell line-derived neurotrophic factor
TGIF	TG-interacting factor
TF	Transcription factors
MAPK	Mitogen-activated protein kinase
TIC	Tumor-initiating cell
AKT/PKB	Protein kinase B
PTKs	Protein tyrosine kinases
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
PI3K	Phosphoinositide 3-kinases
c-Src	Proto-oncogene tyrosine-protein kinase
RhoA	Ras homolog family member A
TIEG1	TGFβ-inducible early-response gene
Rac1	Ras-related C3 botulinum toxin substrate 1
Cdc42	Cell division control protein 42 homolog
CDKs	cyclin-dependent kinases
$p15^{INK4B}$	Cyclin-dependent kinase inhibitor protein
$p27^{KIP}$	Cyclin-dependent kinase inhibitor 1B
K-Ras	GTPase transductor protein
TNBC	Triple negative breast cancer
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumor necrosis factor-α
TNM	Tumor -Node -Metastasis
TPA	Tissue -type plasminogen activator
TYRP1	Tyrosinase -related protein 1
TBRII	Type II TGFβ receptor
uPA	Urokinase-type plasminogen activator
UPAR	Urokinase plasminogen activator receptor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VGP	Vertical growth phase
α-MSH	α-melanocyte-stimulating hormone
βME	β-mercaptoethanol
CSCs	cancer stem cells
EMT	Epithelial–mesenchymal transition
SNAIL	Zinc finger protein SNAI1
ZEB	Zinc-finger E-box-binding
bHLH	Basic helix-loop-helix transcription factors
PAR	Partitioning defective
SCRIB	Scribble
MMPs	Matrix metalloproteinases
MMCT	Microcell-mediated transfer
PK	Pharmacokinetic
PD	pharmacodynamics

KPs	Kisspeptins
KiSS1R	KiSS1 receptor
GPR54	G-protein-coupled receptors 54
PAM	Peptidyl-glycine-alpha-amidating monooxygenase
GnRH	Gonadotropin-releasing hormone
MSGs	Metastasis suppression genes
ESR1	Estrogen receptor alpha
PGR	Progesterone receptor
ERBB2	Human epidermal growth factor receptor 2
GnRH-R	Gonadotropin-releasing hormone receptor
HPG	Hypothalamic-pituitary-gonadal
DMEM	Dulbecco's Modified Eagle's Medium
CNS	Central nervous system
F-12K	Ham's F-12K (Kaighn's) Medium

Chapter One: INTRODUCTION

1.1 Cancer

Cancer is considered the second leading cause of mortality and accounts for approximately 1 in every 6 deaths globally (Bray, Ferlay, et al. 2018). It accounts for 25% of annual death rates in developing countries (Ferlay, Colombet, et al. 2018). Cancer is defined as the ability of cells to divide uncontrollably through acquisition of new properties such as gene abnormalities (Sonnenschein, Soto et al. 2014). Thus, it can be considered as a genetic disorder within the cell genome resulting from the accumulation of mutations (Ferlay, Colombet, et al. 2018). The immune system is able to recognize and destroy the abnormal cells that may have malignant potential or neoplastic characteristics (Jain, Zhang, et al. 2017). However, when these cells escape immune surveillance, this will result in cancer development, which threatens the body with metastatic disease (Maule and Merletti 2012; Jain, Zhang, et al. 2017). The immune system produces molecules that both inhibit or promote tumor cells with metastatic potential. Therefore, identifying and studying the factors that regulate this immune response is essential for the treatment of cancer (Massagué 2012). There are multiple hallmarks of cancer observed among different types of cancer, including resistance to apoptosis, continuous growth and proliferation through activation of proliferative singling pathways, escaping the immune surveillance, increased angiogenesis, the ability to invade and metastasize to other organs, sustained metabolism, and the downregulation of the growth-supressing signaling pathways (Hanahan and Weinberg 2011). Each of these mechanisms contribute to the cells becoming malignant or cancerous. The following figure illustrates the incidence and mortality for cancer cases worldwide. (Bray, Ferlay, et al. 2018)(Figure 1-1).

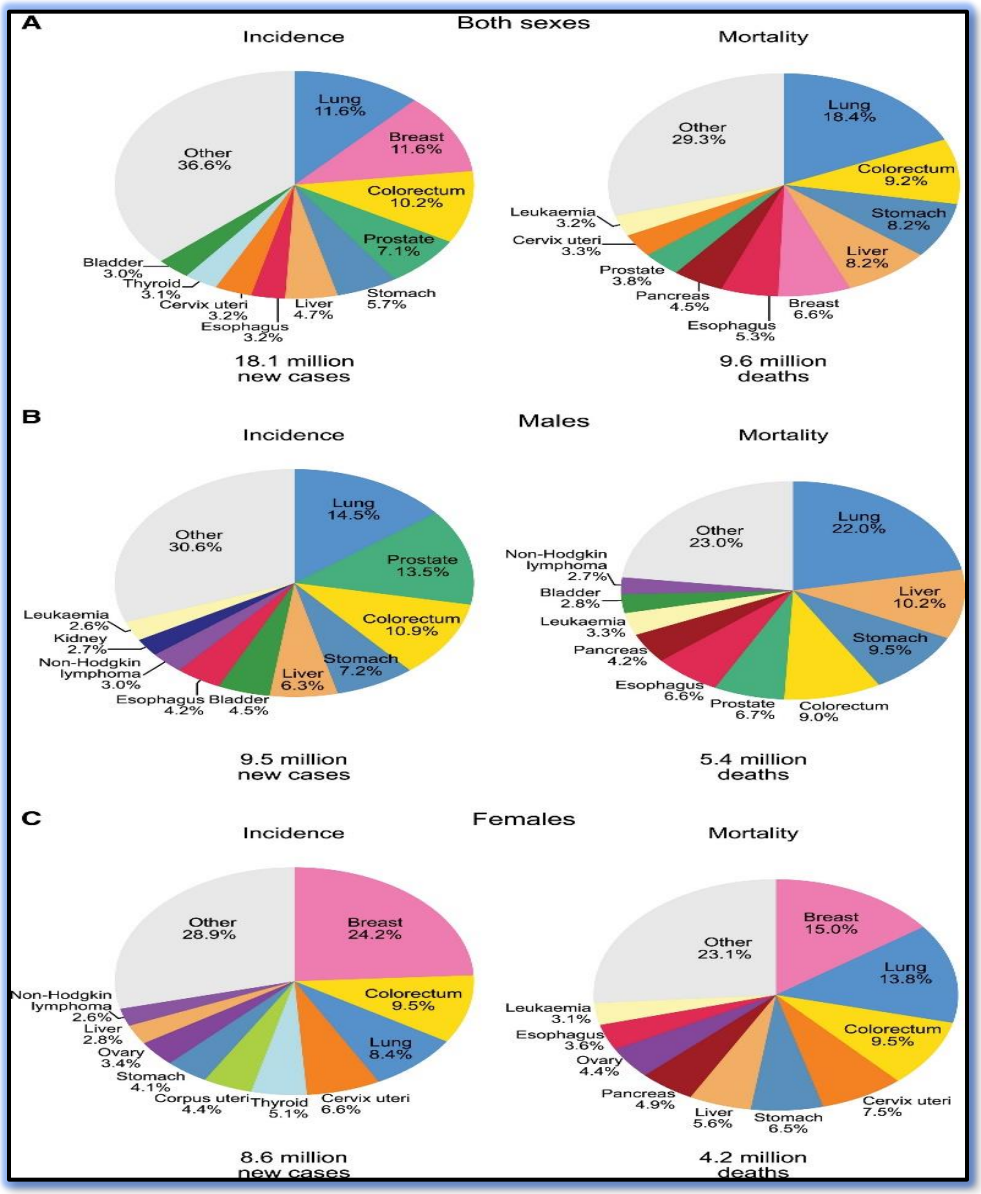


Figure 1-1: The distribution of incidence and mortality rates for the 10 most common cancers in 2018.

Pie charts demonstrating the distribution of incidence and mortality rates for (A) both sexes, (B) males, and (C) females. The area of the pie chart segments is proportional to their respective number of cases (incidence) or deaths (mortality). Non-melanoma skin cancers are included in the "other" category. Adapted from (Bray, Ferlay, et al. 2018).

1.2 Hallmarks of cancer

Cells which exhibit hallmarks of cancer acquire evolutionary advantageous characteristics that promote the transformation of phenotypically normal cells into malignant ones; they also promote the progression of malignant cells while sacrificing and exploiting the host tissue (Hanahan and Weinberg 2000)(Figure 1-2).

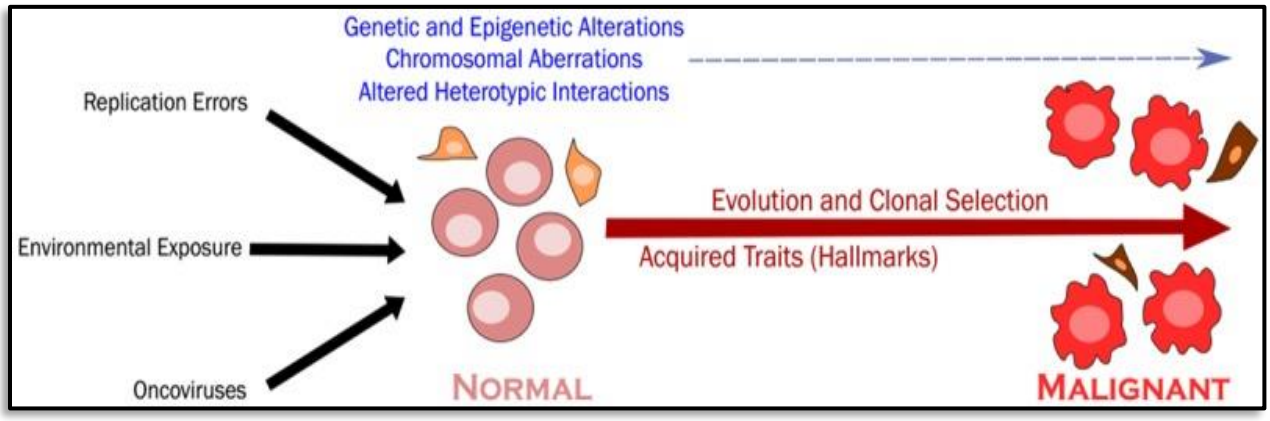


Figure 1-2: Transformation process from normal cells to malignant cells.

Adapted from Hanahan *et al.* 2011 (Hanahan and Weinberg 2011).

To date, major hallmarks of cancer have been identified including (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) the ability to evade apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, (6) tissue invasion and metastasis (Hanahan and Weinberg 2011), (7) deregulated cellular energetics, (8) avoiding immune destruction, (9) genome instability (Vogelstein, Papadopoulos, et al. 2013), (10) mutations (Martincorena, Roshan, et al. 2015) (Hanahan and Weinberg 2011) (Figure 1-3).

Self-sufficiency in growth signaling has been one of the most well-studied hallmarks of cancer. These studies have highlighted that this process is carried out by cytokines which are crucial components of cellular biological structure and they play a vital role in cancer biology. Amongst these cytokines is the transforming growth factor beta (TGF-β), which is expressed in most cell types, and plays multiple roles during normal development through its signaling pathways (Kubiczkova, Sedlarikova, et al. 2012). The TGF-β signaling pathway has been linked to hyperproliferative diseases, cancer development, inflammatory and autoimmune diseases, immunosuppression, and tumour metastasis (Chen and Wahl 1999; Massague, Blain, et al. 2000; Wakefield and Roberts 2002).

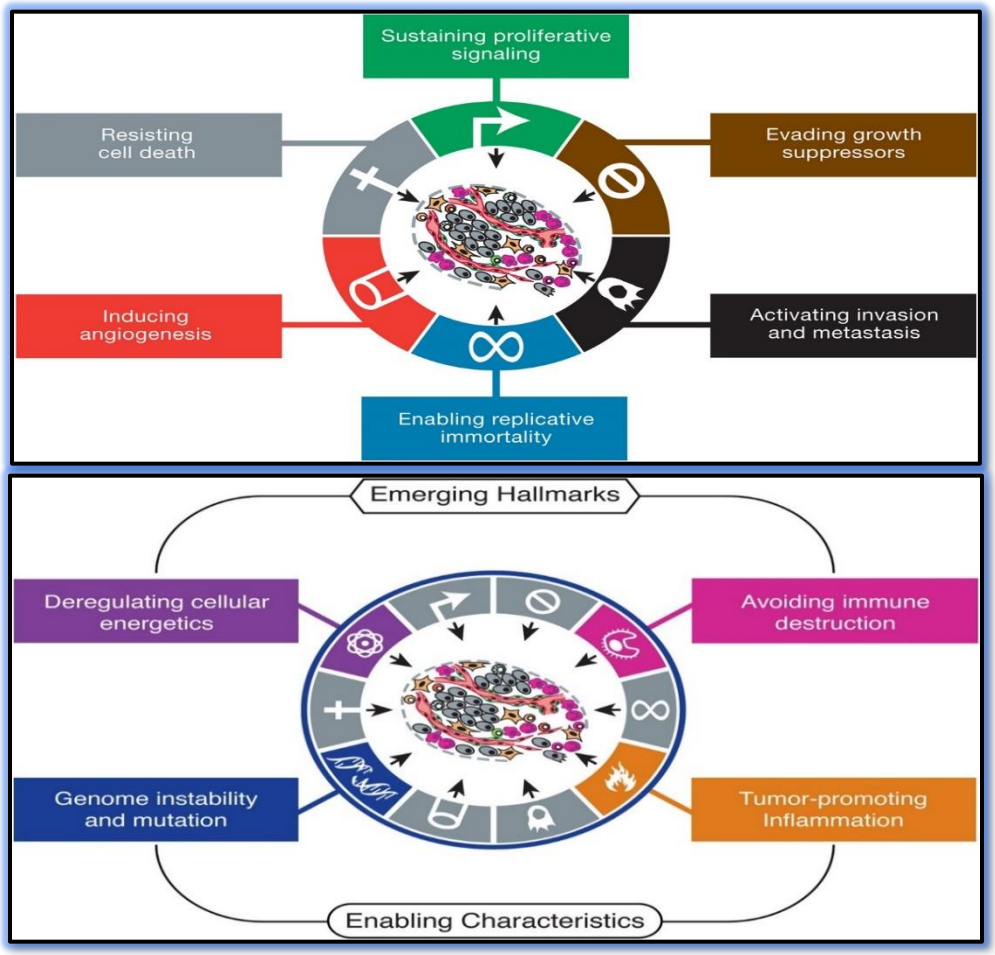


Figure 1-3: The hallmarks of cancer.

Upper Panel: An illustration demonstrating the six hallmarks of cancer first proposed by Hanahan and Weinberg 2000. Lower Panel: An illustration demonstrating the two emerging hallmarks of cancer [deregulation of cellular energetics and avoidance of immune destruction] as proposed by Hanahan and Weinberg (2011).

1.3 The Transforming growth factor-β (TGF-β)

1.3.1 TGF-β family

TGF-β is the prototype of the TGF-β family of growth and differentiation factors. The TGF-β family consists of a group of proteins, including the activin/inhibin family, bone morphogenetic proteins (BMPs), growth differentiation factors (GDFs), the TGF-β subfamily, and the glial cell line-derived neurotrophic factor (GDNF) family (Kubiczkova, Sedlarikova, et al. 2012; Massagué 2012). There are three known isoforms of TGF-β, namely, TGF-β1, TGF-β2 and TGF-β3, which are expressed in mammalian tissues. All these isoforms have conserved regions and function through the same receptor signaling pathways.

1.3.2 TGF-β signaling

TGF-β signals through canonical and non-canonical signalling pathways (Figure 4). The canonical signaling pathway involves the activation of the SMAD proteins through phosphorylation of two serine/threonine kinase receptors (TGFβR1 and TGFβRII) and the recruitment of receptor-regulated SMADs (R-SMADs), SMAD2 and SMAD3. Once SMAD2/3 are recruited to the receptor complex, they undergo phosphorylation and are then released to the cytoplasm where they will heteromerize with the common partner (Co-SMAD), SMAD4. The heterotrimer complex is subsequently translocated into the nucleus where it binds to specific transcription factors (TF) and induces the transcription of TGF-β-dependent target genes. In contrast, non-canonical signaling is independent of SMAD activation and involves the activation of different signaling pathways after ligand binding such as Notch signaling, MAP kinases, AKT/PKB pathway, GTP-binding proteins pathway, PTK pathway, NF-κB, and Wnt/β-catenin pathway (Roberts, Anzano, et al. 1985; Kubiczkova, Sedlarikova, et al. 2012; Massagué 2012).

1.3.3 TGF-β functions

The TGF-β superfamily regulates various biological processes such as growth, development, tissue homeostasis, and immune system regulation. The distinct roles of the TGFβ-subfamily members are highly dependent on the cell type, growth conditions, and the presence of other growth factors. For instance, TGF-β inhibits epithelial, endothelial, neural as well as hematopoietic and immune cells but stimulates various mesenchymal cells (Massague, Blain, et al. 2000)(Figure 1-4). Furthermore, TGF-β supresses physiological development of the central nervous system (CNS) while in prostate cancer, TGF-β1 overexpression correlates with tumor progression, cell migration, and angiogenesis (Kubiczkova, Sedlarikova, et al. 2012; Massagué 2012).

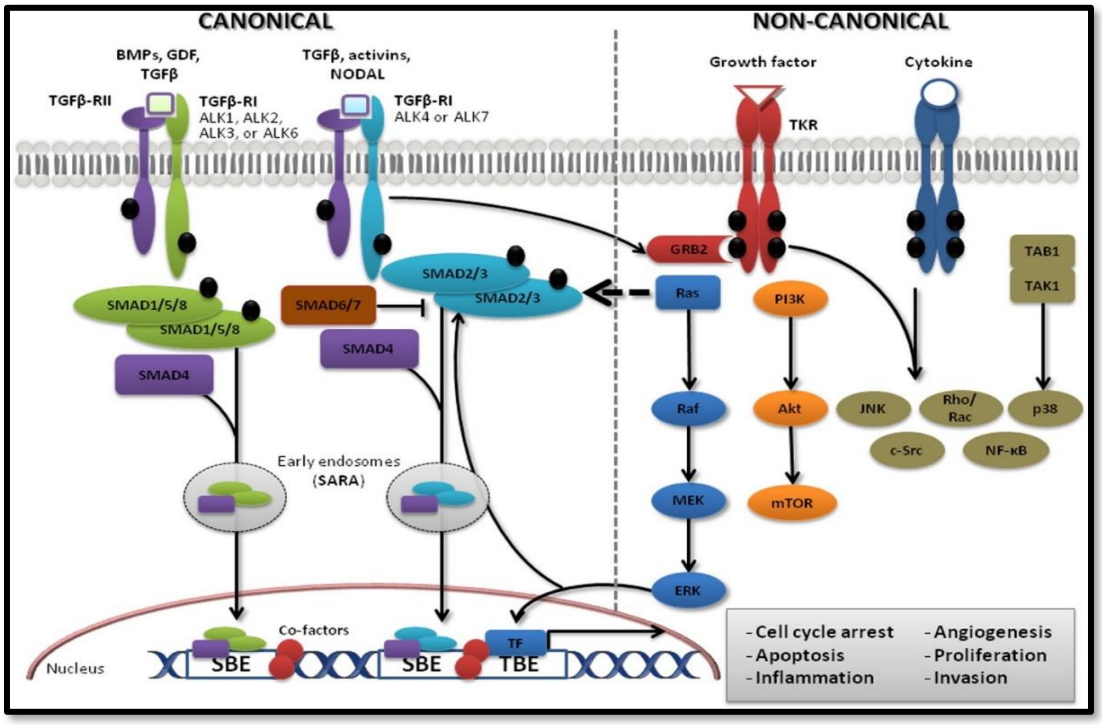


Figure 1-4: An overview of the TGF-β family signaling pathways.

The TGFβ superfamily pathway activities are mediated by both canonical (SMAD-dependent) and non-canonical (SMAD-independent) pathways. The non-canonical TGFβ signaling pathway involves mediators such as PI3K/AKT, MAPKs, c-Src, NF-κB, or small GTPases such as RhoA, Rac1 & Cdc42. Adapted from Neuzillet, Tijeras-Raballand, et al. 2015.

1.3.4 The role of TGF-β in cancer

In cancers, TGF-β has dual paradoxical roles where it elicits tumor-suppressive effects in normal cells and early carcinomas, but also exhibits tumour-promoting effects in advanced cancers (Moustakas, Pardali, et al. 2002; Galliher, Neil, et al. 2006) depending on the cell context, tumor stage, and other factors. TGF-β induces cell cycle arrest at the G1 phase though inhibition of c-myc and up-regulation of cyclin-dependent kinase inhibitors (CDKIs) $p15^{INK4B}$ and $p27^{KIP}$(Ikushima and Miyazono 2010). TGF-β acts as a tumor suppressor in normal epithelium via inhibiting cell proliferation and inducing apoptosis. By contrast, during tumor progression, the TGF-β suppressive effect is lost and replaced by tumor promoting effects in later stages of cancer. Indeed, in advanced tumors, particularly those of the breast, TGF-β will promote cell migration and invasion, induce epithelial-to-mesenchymal transition, promote the vascularisation and inhibit the host immunosurveillance, thereby promoting the metastatic spread of the primary tumor (Moustakas, Pardali, et al. 2002; Ikushima and Miyazono 2010). Moreover, TGF-β regulates the expression and the activity of chemokines and chemokine receptors that play various roles in inflammatory cell recruitment (Coussens and Werb 2002). In addition, TGF-β induces epithelial-to-mesenchymal transition (Moustakas, Pardali, et al. 2002; Tian, Neil, et al. 2011).

1.3.4.1 The role of TGF-β in pancreatic cancer

TGF-β1 was shown to play a role in pancreatic cancer. Indeed, *Smad4* deletion was observed in late neoplastic progression of histologically recognizable carcinoma (Bardeesy, Cheng, et al. 2006; Ahmed, Bradshaw, et al. 2017). Other studies reported that the deletion of *Smad4* or *TGFβRII* in pancreatic epithelium was not sufficient to initiate pancreatic cancer development or to induce invasive carcinoma (Shen, Tao, et al. 2017). However, when K-Ras was activated in pancreatic cells, loss of *Smad4* or *TGFβRII* or *Smad4* haplo-insufficiency led to progression to high-grade tumors (Zhao, Liang, et al. 2016). These results suggest, that Smad4 mediates the tumor inhibitory action of TGF-β signaling, particularly in the progressive stage of tumorigenesis (Ahmed, Bradshaw, et al. 2017).

1.3.4.2 TGF-β & breast cancer

In normal mammalian breast development, TGF-β is involved in establishing proper mammary gland structures and apoptosis induction. In breast cancer, TGF-β promotes tumor progression and metastasis in basal-like breast cancer. KiSS1, a tumor suppressor gene, was identified as a downstream target of the canonical TGF-β/Smad2 pathway in triple negative breast cancer cells, whereby KiSS1 expression was shown to be required for TGF-β-induced cancer cell invasion (Tian, Al-Odaini, et al. 2018). Studies from our lab have highlighted the clinical utility of KiSS1 as a potential therapeutic target (Tian, Al-Odaini, et al. 2018)(Figure 1-5).

1.3.4.3 The role of TGF-β in angiogenesis

The formation of blood vessels by tumor cells is a vital process for tumor growth, invasion, and metastasis. TGF-β plays a crucial role in promoting angiogenesis in the tumor microenvironment by inducing the expression of pro-angiogenic factors (Liu, Chen, et al. 2018). The TGF-β signaling pathway also plays a significant role in the maintenance of cancer stem cells (CSCs), thus highlighting the role of TGF-β in the recurrence of the disease following anticancer therapy (Bellomo, Caja, et al. 2016).

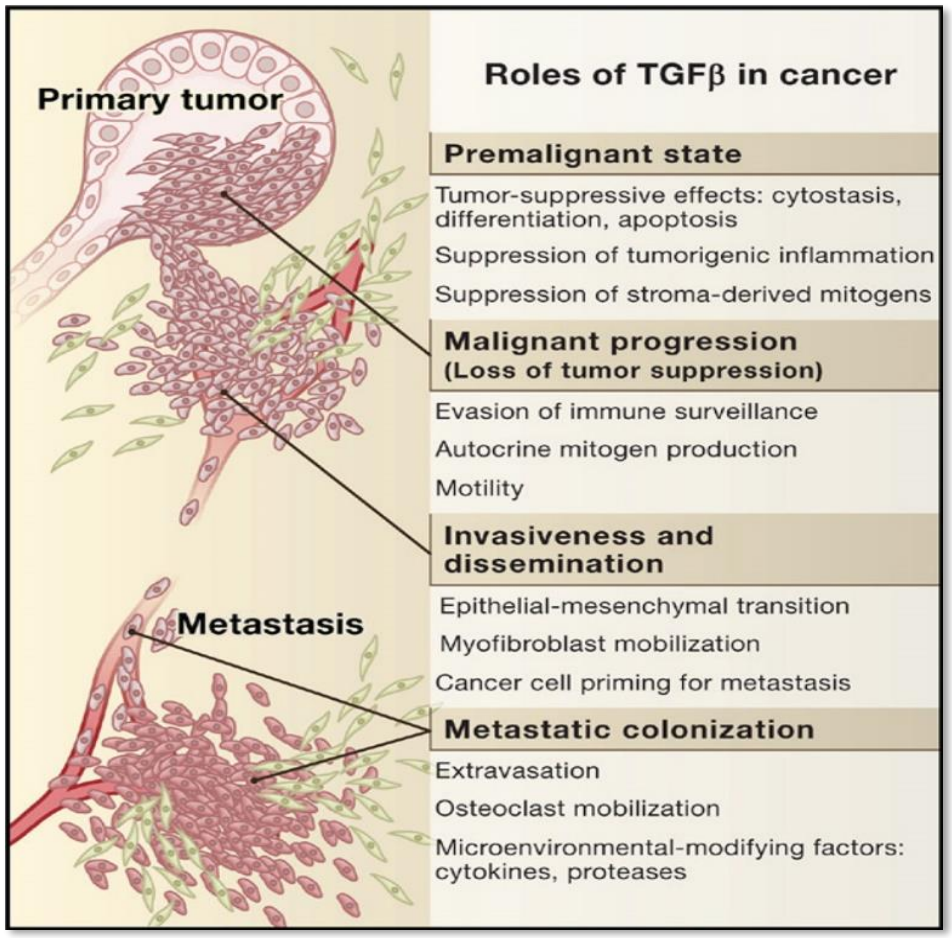


Figure 1-5: Roles of TGF-β in cancer.

In normal and pre-malignant cells TGF-β acts as a tumor suppressor and promotes homeostasis by inducing cytostasis, differentiation, and apoptosis. It also acts to suppress

inflammation and modulates stroma-derived mitogens. Upon tumor progression, TGF-β can no longer suppress tumor initiation. Accordingly, tumor cells use TGF-β to evade immune system surveillance, and autocrine mitogen production, thereby transforming into an invasive phenotype. Reproduced from (Shi and Massague 2003).

1.4 Cancer metastasis

Cancer metastasis is defined as the movement of cancer cells from the primary tumor to surrounding tissues and to distant organs and is considered the primary cause of cancer morbidity and mortality (Fidler 2003; Seyfried and Huysentruyt 2013). Metastasis is a complex process as primary tumors migrate to secondary organs (Weiss 1990) and is considered as an ongoing challenge in the clinical management of cancer (Luzzi, MacDonald, et al. 1998; Chambers, Naumov, et al. 2001). Tumor metastasis occurs through lymphatic spread, the haematogenous route, or by disseminating into body cavities (Parker and Sukumar 2003; Valastyan and Weinberg 2011). Coordination between the activation of metastasis-promoting genetic programs and the inhibition of metastasis-suppressing programs in tumors is required to complete this process (Figure 1-6).

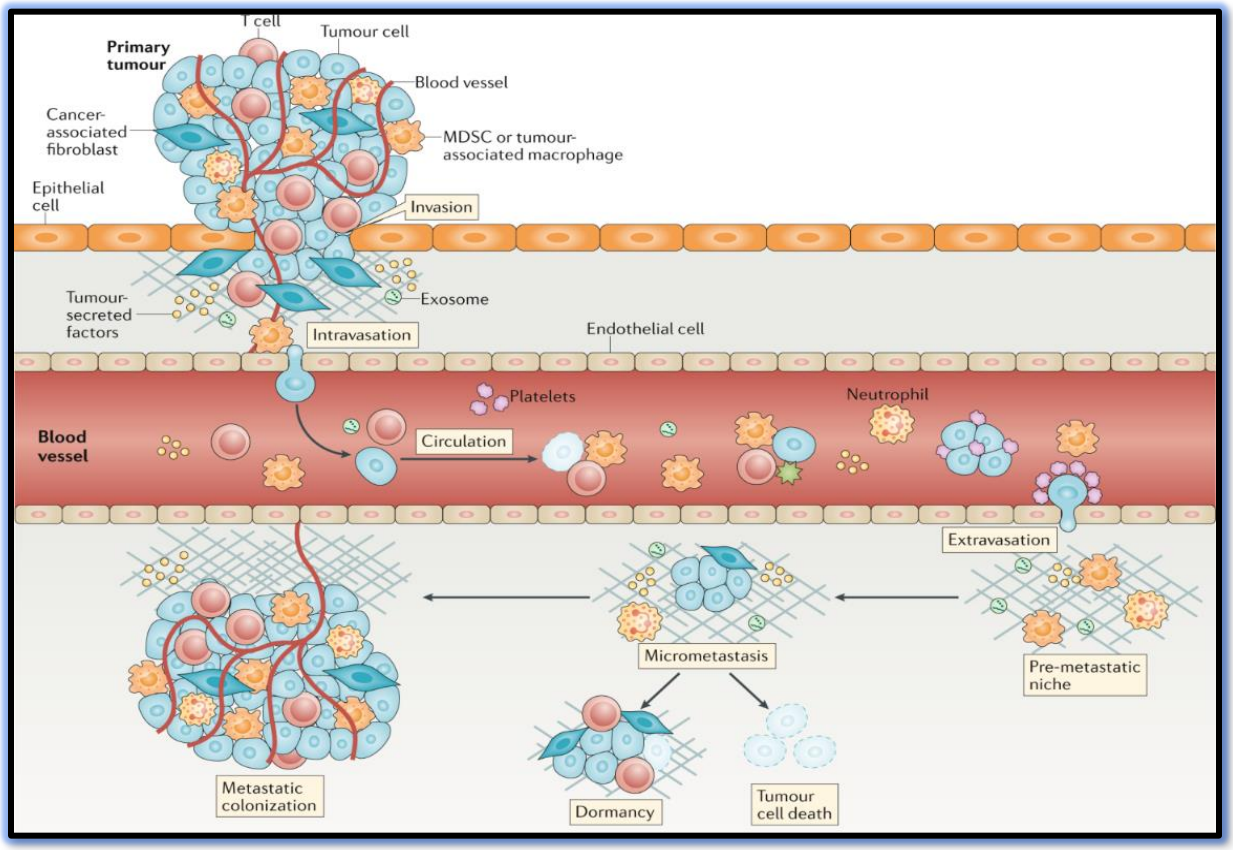


Figure 1-6: Principal steps in metastasis.

Transformation of normal epithelial cells leads to carcinoma *in situ*, which, upon the loss of adherens junctions, evolves toward the invasive carcinoma stage. Following basement membrane degradation, tumor cells invade the surrounding stroma, then migrate into blood or lymph vessels, and disseminate into distant organs. Reproduced from Anderson, Balasas, et al. 2019.

1.5 Epithelial-Mesenchymal Transition (EMT)

Epithelial–mesenchymal transition (EMT) is a reversible trans-differentiation process of cells that change from an immotile epithelial phenotype to a motile mesenchymal phenotype. The process of EMT occurs during cancer development and progression. EMT also plays a major role in cancer cell invasion and migration. This process is regulated by several transcription factors such as SNAIL (van Meeteren and ten Dijke 2012; Timmerman, Grego-Bessa, et al. 2004), zinc-finger E-box-binding (ZEB) (Gregory, Bert et al. 2008), and basic helix-loop-helix transcription factors (Peinado, Olmeda et al. 2007; De Craene and Berx 2013) In addition, EMT requires the presence of several growth factors and the activation of several signalling pathways, in addition to other factors such as hypoxia (Imai, Horiuchi et al. 2003, Sahlgren, Gustafsson et al. 2008) and mechanical stress (Farge 2003).

Under normal circumstances, EMT is observed during embryogenesis, and in adult tissues (Lim and Thiery 2012). For the EMT process to begin, the epithelial cell–cell contacts (Huang, Guilford et al. 2012) have to be disassembled and epithelial cells have to lose their tight junctions, adherens junctions, desmosomes, gap junctions as well as have to lose cell polarity via the disruption of the crumbs, partitioning defective (PAR) and scribble (SCRIB) polarity complexes (Moreno-Bueno, Portillo, et al. 2008). Cells then start repressing the expression of epithelial genes through activation of mesenchymal gene expression. This results in the reorganization of the epithelial actin architecture and the acquisition of a highly mobile and invasive phenotype characterized by the formation of lamellipodia, filopodia and invadopodia, and upregulation of matrix metalloproteinases (MMPs) which degrades extracellular matrix (ECM) proteins (Yilmaz and Christofori 2009; Bergert, Chandradoss, et al. 2012; Meirson and Gil-Henn 2018) (Figure 1-7).

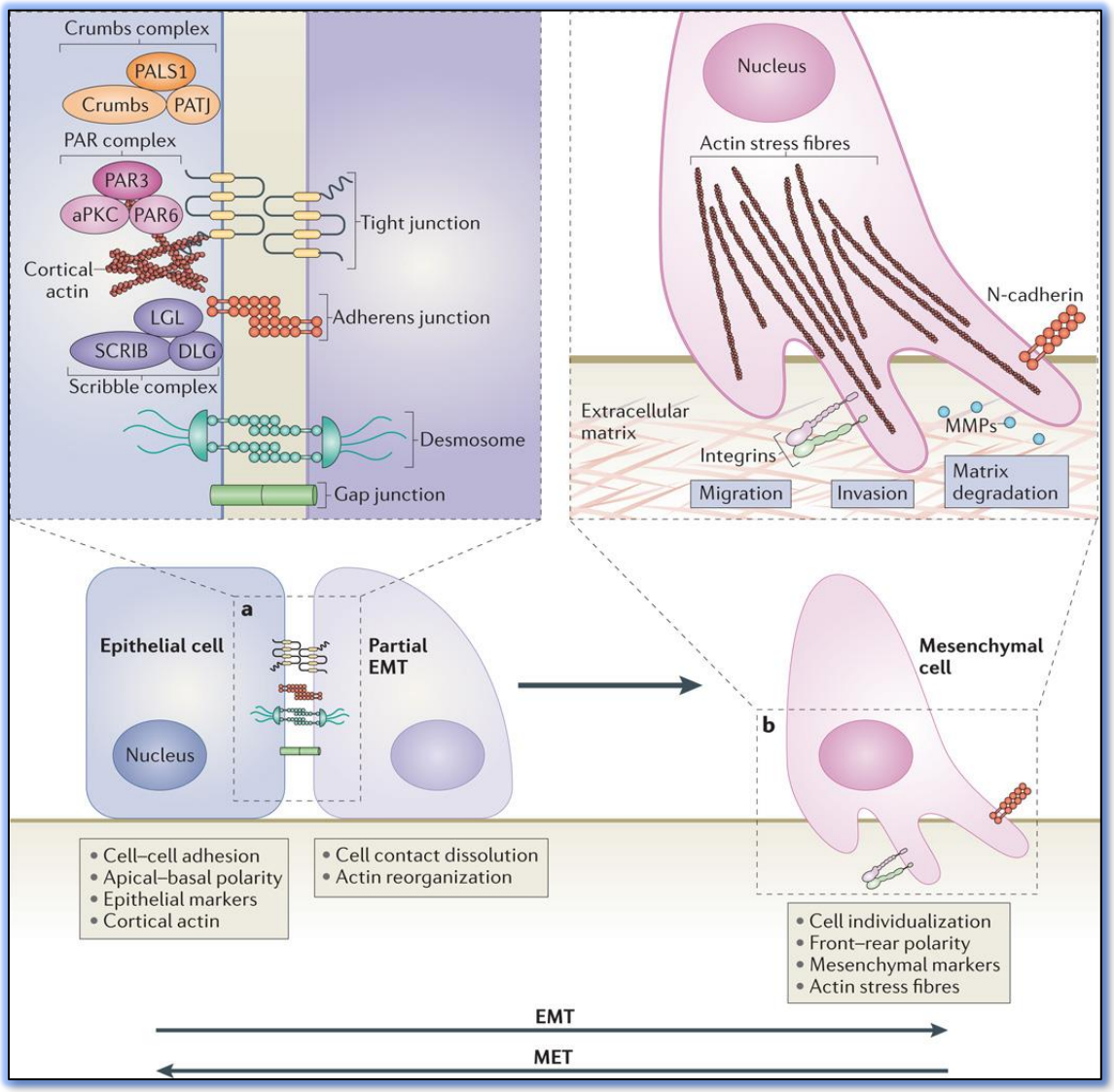


Figure 1-7: An overview of EMT and MET processes.

Reproduced from (Lamouille, Xu, et al. 2014)

1.6 Metastasis suppressive genes (MSG)

Metastasis suppressive genes (MSG) are those involved in the inhibition of metastasis. Various therapies have been designed to mimic MSG in treating cancer patients. To date, twenty three MSGs have been identified and appear to have inhibitory effects on metastasis and tumor formation (Yoshida, Sokoloff et al. 2000; Kauffman, Robinson, et al. 2003). The discovery of MSGs was accomplished by using microcell-mediated transfer (MMCT) to introduce copies of normal chromosomes into cancer cells which have significant chromosomal mutations. For instance, when chromosomes 2, 7, 8, 10, 11, 12, 13, 16, 17, and 20 were introduced into different cancer cells, the metastatic potentials of these cells were significantly inhibited suggesting that normal chromosomes are coding for genes that suppress metastasis (Lee, Miele, et al. 1996; Yoshida, Sokoloff, et al. 2000).

1.7 The Development of anti-metastatic agents

To develop anti-tumor or anti-metastatic therapeutic agents, many factors should be considered: (1) the identification of potential therapeutic targets, (2) the use of highly relevant experimental and pre-clinical models that best reflect the actual tumor or metastatic environment, and (3) the role of immune cells infiltrating the tumor site and their role in promoting metastasis. Preclinical models must closely resemble the actual *in vivo* microenvironment, but this is not always possible. As such, the more closely one is able to mimic the cancer microenvironment, the better chance we have at understanding the underlying metastatic pathways and the better chance we have at identifying potential novel therapeutic targets. Additional considerations in preclinical models include pharmacokinetic (PK) profiles and pharmacodynamic (PD) markers that provide an understanding of anti-metastatic drug effects, which can subsequently be used in the clinic.

Determining the safety, the PK profile and PD characteristics should be the main goals of pre-clinical studies to ensure that the drug has all the desired biological, pharmacological, and therapeutic effects. In order to determine a biological proof of concept in cancer patients, examining PD effects on the dose of anti-metastatic agents before surgery and validating surrogate end points of clinical efficacy are considered and could be used to speed up the development timeline(Anderson, Balasas et al. 2019)(Figure 1-8).

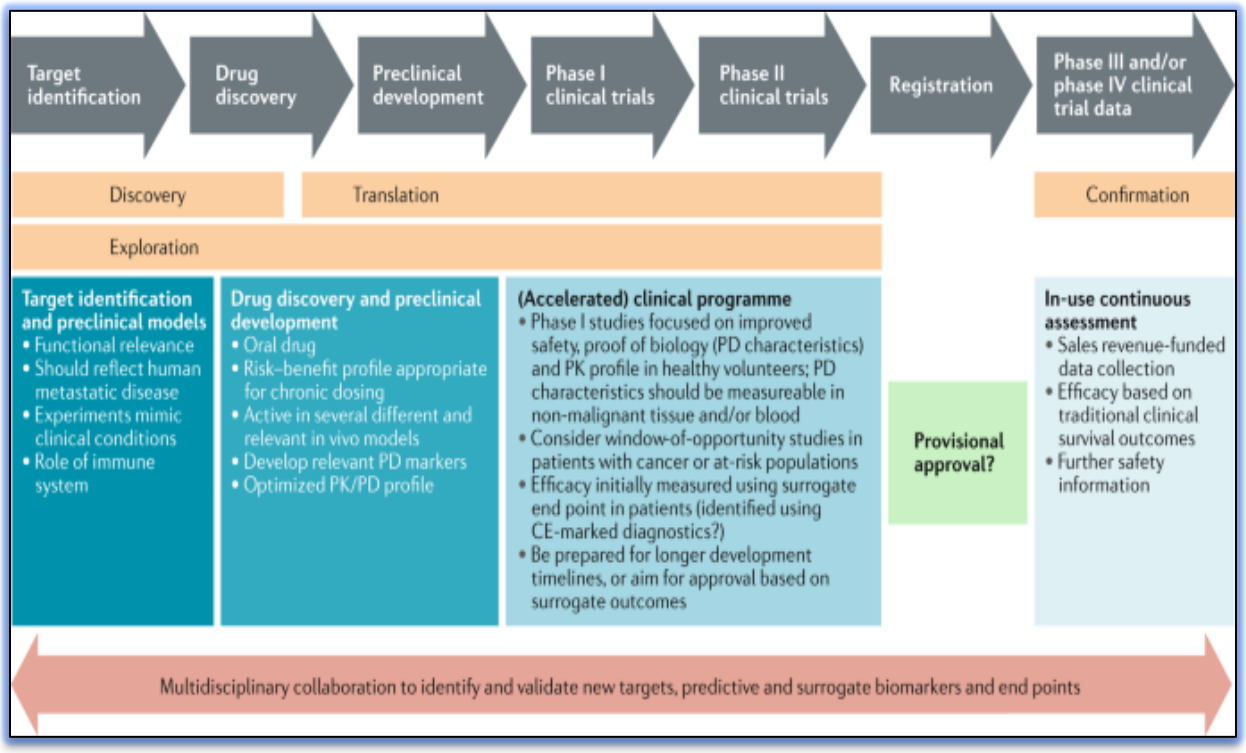


Figure 1-8: An overview of the different steps of drug development.

Reproduced from Anderson, Balasas et al. 2019.

1.8 Kisspeptin (KiSS1)

1.8.1 Discovery

Kiss1 was identified in 1996 by Lee *et al*. during their investigation of genes that suppress metastasis of cutaneous melanoma. They introduced chromosome 6 to a highly metastatic melanoma cell line using a subtractive hybridization method (Lee, Miele, et al. 1996). These authors identified seven cDNA clones that were highly expressed in non-metastatic cells compared to metastatic cells (Lee and Welch 1997). One of the seven identified clones was *KiSS1* cDNA. *KiSS1* got its name from the location of its discovery in Hershey, Pennsylvania, USA, which is also the home of the famous Hershey's Kiss chocolate. The structure and functional roles of KiSS1 were extensively investigated in different types of cancer (Lee, Miele, et al. 1996; Trevisan, Montagna, et al. 2018).

1.8.2 Characterization of KiSS1 protein

1.8.3 KiSS1 sequence analysis

The *KiSS1* gene encodes pre-pro-Kisspeptin protein (145 amino acids) which is then processed through proteolytic cleavage in the serum into smaller but biologically active peptides called Kisspeptins (KPs). These peptides include a common form of 54 amino acids, as well as other smaller forms of 14, 13, and 10 amino acids (kp-54, kp-14, kp-13 and kp-10). The post-translational proteolysis occurs at two dibasic residues in pre-pro-kisspeptin at positions 66-67 and 123-124 (Trevisan, Montagna, et al. 2018) (Figure 1-9).

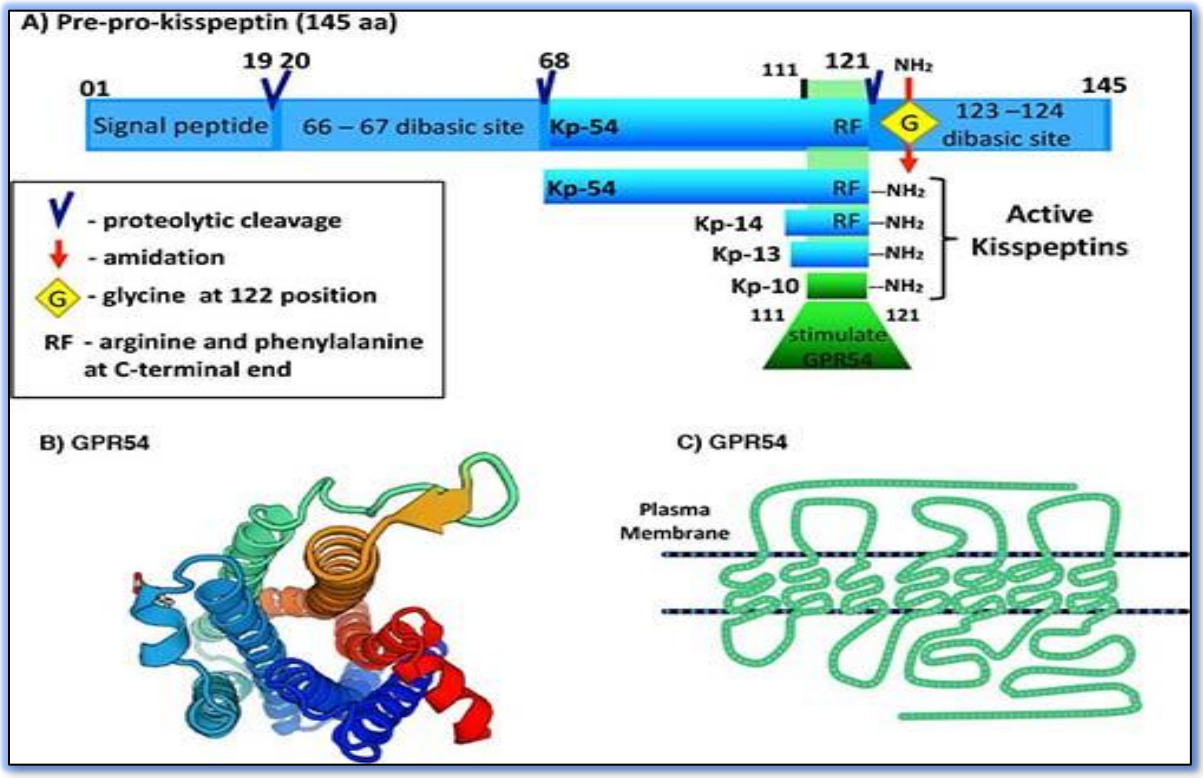


Figure 1-9: A diagram showing the structure of Kisspeptin (KiSS1) protein and KiSS1 receptor (GPR54) in humans.

(A) Pre-pro-kisspeptin and path of proteolytic cleavage; (B) GPR54 structure from SWISS-MODEL Q969F8 (KISSR_HUMAN); (C) GPR54 structure in plasma membrane with seven transmembrane helices, an extracellular N-terminal domain and ends with C-terminal cytoplasmic domain. Reproduced from (Trevisan, Montagna, et al. 2018).

KPs possess a highly conserved 10 amino acid RF-amide C terminus core sequence. The last two amino acids are arginine and phenylalanine that receive an amine group transferred from glycine at position 122 to residue 121, which is the C-terminal end of the mature peptide (Figure 1-10). kp-10 has the shortest sequence to fully stimulate the GPR54 and consequently increases phosphatidylinositol turnover. The KISS1 receptor , KISS1R (GPR54), is a 398-amino acid protein of the G_q class of G proteins coupled to phospholipase C (Lee, Nguyen, et al. 1999; Kirby,

Maguire, et al. 2010). GPR54 possesses an extracellular N-terminal domain which is followed by seven transmembrane helices and ends with a C-terminal cytoplasmic domain of about 70 residues (Kotani, Detheux, et al. 2001). This intracytoplasmic C-terminal region has the ability to bind to the catalytic and regulatory subunits of phosphatase 2A, which allows it to form complexes with protein partners involved in receptor signaling (Lee, Nguyen et al. 1999; Kotani, Detheux, et al. 2001). Thus, GPR54 signaling would increase intracellular Ca^{2+} levels, activate calcium-dependent signaling pathways and activate mitogen-activated protein kinase (MAPK) p38, extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) in GnRH neurons (Kotani, Detheux, et al. 2001).

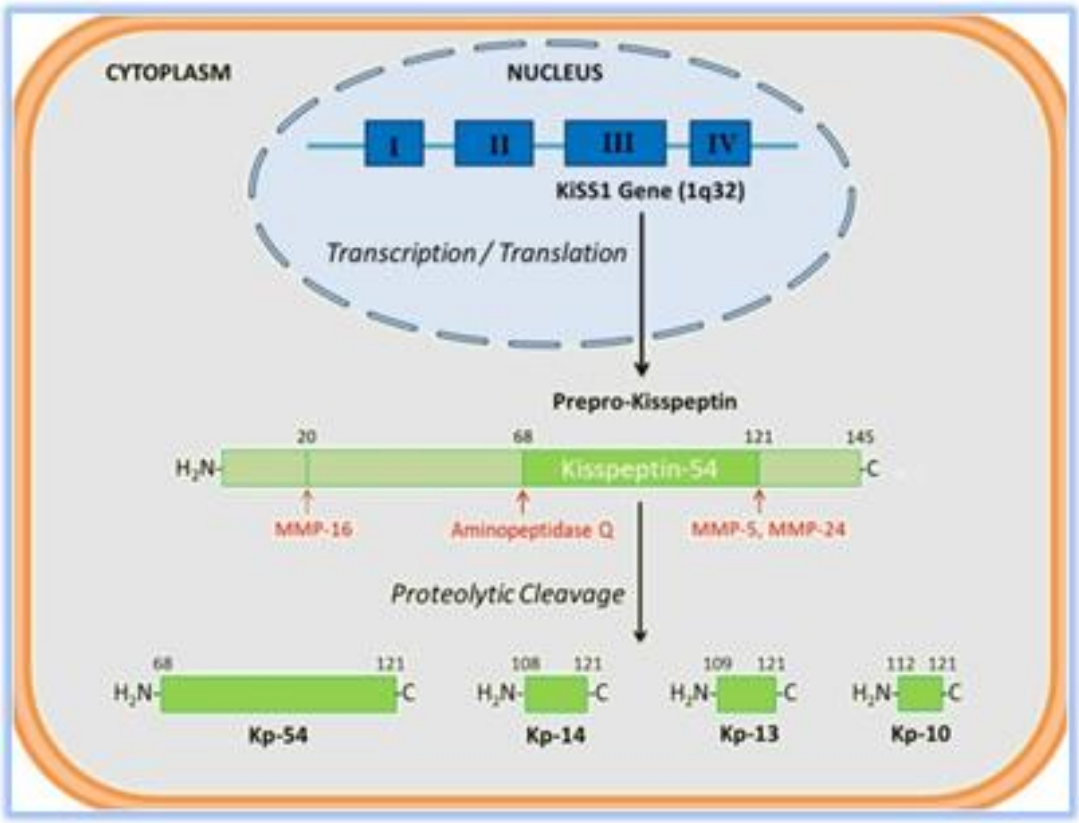


Figure 1-10: Kisspeptin processing.

KiSS1 protein is cleaved by furins or prohormone convertases based upon the amino acid sequence of the pre-pro-kisspeptin molecule, leading to the formation of several different sized kisspeptins all possessing a similar C terminus. Adopted from (Muir, Chamberlain, et al. 2001).

1.8.4 KiSS1 processing and secretion

Little is known about processing and secretion of KiSS1 because of the lack of specific KiSS1 antibodies and the relatively short half-life (30 seconds) of the KiSS1 prepropeptide. It is predicted that the processing could be carried out via three steps: endoproteolytic processing at the dibasic cleavage sites, followed by the elimination of the basic residues at the c-terminus by the carboxypeptidase enzyme, and finally the amidation of the glycine by peptidyl-glycine-alpha-amidating monooxygenase (PAM). It is suggested that the previous steps occur later in the secretory pathway as shown in (Figure 1-10) (Kotani, Detheux, et al. 2001).

1.8.5 KiSS1 derivatives are GPR54 ligands

G-protein-coupled receptors (GPCRs) are a large family of membrane bound proteins, which share a common structure. All GPCRs consist of seven transmembrane alpha helix structures. GPCRs function as receptors for a wide range of small peptides or polypeptides and lipids (Ohtaki, Shintani, et al. 2001). The KiSS1 receptor (KiSS1R) is a member of the GPCR family due to its structural homology. It was labeled as an orphan G-coupled receptor because at the time of its discovery, no ligands were identified (Muir, Chamberlain, et al. 2001). The *KiSS1R* gene is located on chromosome 19p13.4, and it consists of five exons and 4 introns that encode for 398 amino acids (75 k Da). Various studies investigated the stimulation of GPR54 using ~1500 different ligands, while measuring the response using calcium mobilization. These studies revealed

that KiSS1 derivatives function as ligands for GPR54. All the KiSS1 products bind and activate GPR54 but kisspeptin 10 has the highest affinity to the receptor (Muir, Chamberlain, et al. 2001; Ohtaki, Shintani, et al. 2001).

1.8.6 Tissues expressing KiSS1 and KiSS1R

KiSS1 was discovered initially during studies aimed at identifying tumor suppressors in melanoma and was found to specifically suppress metastatic characteristics. Subsequent studies revealed that KiSS1 can also function as a vital regulator of sexual maturation in humans. Moreover, KiSS1 was identified in the pancreas, the kidney and the placenta, among other tissues (Kotani, Detheux, et al. 2001; Muir, Chamberlain, et al. 2001).

1.8.7 Regulation in the developmental stage

In newborn rats, KiSS1 was detected in the hypothalamus of both sexes. The expression level is low at birth and begins to increase 25 days postpartum. At later stages of development, KiSS1 levels are maintained until it reaches its highest level at the pre-puberty stage, followed by a transient increase at the time of puberty. KiSS1 expression is higher in ovaries of mature rats compared to immature rats (Trevisan, Montagna, et al. 2018).

1.8.8 Feedback regulation by gonadal steroids

The delicate process of sexual maturity and puberty is highly controlled by the Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus and is regulated by feedback loop mechanisms of the gonadal steroids which negatively regulate the hypothalamic hormones. Interestingly, KiSS1 is considered an upstream regulator of GnRH. Loss of Kisspeptin

signaling leads to hypogonadotrophic hypogonadism in humans and mammals. Kisspeptin interacts with other neuropeptides to regulate GnRH pulse generation. Also, it is believed that Kisspeptin signaling is regulated by nutritional status and stress. (Navarro, Castellano, et al. 2004; Navarro, Castellano, et al. 2005; Bhattacharya and Babwah 2015).

1.8.9 KiSS1 in cancer biology

Cancer development and progression require a complex array of genes and pathways which render cancer cells unresponsive to suppressive signals, resulting in the evasion of immune surveillance, eventually leading to invasion and distant metastasis. Considering the high mortality of metastatic tumours, it is critically important to identify specific genetic mutations at the molecular level that will lead to metastasis thereby enabling the development of successful therapies. Metastasis suppressor genes inhibit metastasis in malignant tumor cells and are downregulated or mutated in various cancer cells. KiSS1 is one of the MSGs identified to date, which led to investigating its suppressive role in different types of cancer. Several studies have reported that KiSS1, KPs, and KiSS1R regulate the development and progression of several cancers, where they act as suppressors of tumorigenesis and metastasis in melanoma, pancreatic and prostate cancers, while they act as tumor promoters in breast and liver cancers (Stathaki, Stamatiou et al. 2019; Mfakri, Pissimissis, et al. 2008).

1.8.9.1 The role of KiSS1 in promoting metastasis

Triple negative breast cancers (TNBC) consist of a very heterogeneous group of tumors defined as a basal-like subtype lacking estrogen receptor alpha (ESR1), progesterone receptor (PGR), and human epidermal growth factor receptor 2 (ERBB2). TNBC patient prognosis is

extremely poor as most of the patients show high grade tumors that are mostly metastatic at the time of diagnosis. KiSS1 and KiSS1R promote metastasis in breast cancer (Martin, Watkins, et al. 2005; Papaoiconomou, Lymperi, et al. 2014; Guzman, Brackstone, et al. 2019). The first report of Lee and Welch showed KiSS1 to be a tumor suppressor in the breast cancer MDA-MB-435 cell line (Lee and Welch 1997). Later studies on this particular cell line confirmed that this cell line is actually a melanoma that metastasized to the breast that was unintentionally reported to be a TNBC cell line (Rae, Creighton, et al. 2007). Nonetheless, the later use of other well-established breast cancer cell lines revealed that KiSS1 indeed promotes metastasis instead of suppressing it, thus adding KiSS1 to a huge family of pathways that play a dual role in cancer.

Liver cancer is a leading cause of cancer death in the world with a 75% increase in incidence between 1990 and 2015. *KiSS1* and *KiSS1R* mRNA are relatively elevated in surgically resected hepatocellular carcinoma samples in comparison with non-cancerous liver (Ikeguchi, Hirooka, et al. 2003).

1.8.9.2 The role of KiSS1 in suppressing metastasis

1.8.9.2.1 Melanoma

Melanoma was the first malignant disease where KiSS1 and KiSS1R were reported to suppress metastasis both *in vitro* and *in vivo*, where the re-expression of KiSS1 in the metastatic melanoma cell line diminished its metastatic characteristics (Welch, Chen, et al. 1994; Lee, Miele, et al. 1996). Another study found that KiSS1 expression is diminished in melanomas deeper than 4mm compared with its expression level in nevocellular nevi (moles) and primary melanomas (Shirasaki, Takata, et al. 2001).

1.8.9.2.2 Pancreatic cancer

Most patients with pancreatic cancer have locally advanced tumors and/or metastases. KiSS1 and KiSS1R are expressed in the pancreatic islets, in the endocrine alpha and beta cells; and they regulate glucose and insulin secretion. Elevated levels of KiSS1and KiSS1R levels were detected in the early stages of disease and are progressively downregulated with the advancement of the cancer (Nagai, Doi, et al. 2009; Stathaki, Stamatiou, et al. 2019).

1.8.9.2.3 Prostate cancer

Prostate cancer is a common disease in men of 40 years of age and older and is considered the second leading cause of cancer mortality. The first line of treatment for prostate cancer is prolonged administration of gonadotropin-releasing hormone receptor (GnRH-R) agonists that induce androgen deprivation. Administration of potent and long-acting kisspeptin agonists was reported to decrease serum testosterone levels via suppression of the hypothalamic-pituitary-gonadal (HPG) axis, suggesting that treating prostate cancer patients with kisspeptin agonists is likely to improve prostate tumor outcomes (Wang, Jones, et al. 2012). Further studies concluded that KiSS1 expression correlates negatively with clinical staging and an *KiSS1* mRNA expression is decreased in a highly metastatic cancer cell line. Thus, kisspeptin conducts its role on prostate cancer cells both indirectly via the HPG axis and directly thereby highlighting its clinical value in treating prostate cancer (Nash and Welch 2006; Beck and Welch 2010; Cho, Li, et al. 2012). Table 1 summarizes the roles of KiSS1 in various cancer types.

1.8.9.2.4 Summary of the role of KiSS1 different cancer types

Table 1: Summary of KiSS1 roles in multiple types of cancer.

Reproduced from (Stathaki, Stamatiou et al. 2019)

	KiSS1 mRNA	Kiss1R mRNA	Effect	Prognosis	References
Melanoma	↑		-Tumor suppressor -Induces dormancy state in melanoma cells	Better	(Martins et al., 2008; Lee et al., 1996c; Cvetković et al., 2013; 93)
	↓		Metastases occurrence	Bad	
Breast Cancer	↑	↑	-Tumor suppressor -Inhibits EMT -Induces -metastasis -Invasion -drug resistance in triple negative tumors	Contraversial	(Cvetković et al., 2013; Cho et al., 2009b; Mooez et al., 2011; Martin et al., 2005; Ji et al., 2013; Ulasov et al., 2012; Teng et al., 2011; Zajac et al., 2011; Xie et al., 2012; Cho et al., 2011)
	↓	↓		Bad	
Pancreatic Cancer	↑		Metastasis inhibition	Better	(Yan et al., 2001b; Nash et al., 2007; Song and Zhao, 2015; Wang et al., 2016; McNally et al., 2010b; Nagai et al., 2009; Liang and Yang, 2007)
	↓	↑	Metastases occurrence	Bad	
	↓	↑		Poor	
	↓		Metastasis occurrence	Poor	
Prostate Cancer	↑		In benign tumors Inhibition of Angiogenesis in xenografts	Good	(Cho et al., 2009a; Wang et al., 2012; Curtis et al., 2010)
	↓	↓	In advanced tumors	Poor	
	↓		More aggressive tumors	Poor	

1.9 Hypothesis and Rationale

According to the literature, KiSS1 has dual roles in various types of cancer; and its function differs according to the type and the stage of the disease as well as the microenvironment. Some studies showed that KiSS1 plays a suppressive role in melanoma, prostate and pancreatic cancers, while other reports showed that KiSS1 is a tumor promoter in liver and breast cancers. Furthermore, our group investigated the role of KiSS1 in triple negative breast cancer, and concluded that KiSS1 promotes TGF-β -mediated metastasis through its canonical SMAD-dependent pathway (Tian, Al-Odaini et al. 2018) Altogether, these studies indicate that KiSS1 plays a vital role in cancer metastasis which requires further investigation.

KiSS1 is overexpressed in primary tumors and non-metastatic cancers and is downregulated upon the progression of the tumors, suggesting it has a potential suppressive role in cancers. Given the tentative tumor suppressive role played by KiSS1, we hypothesized that a KiSS1 receptor agonist would exert negative effects on cell mobility and invasiveness of metastatic melanoma, pancreatic, and prostate cancer cells.

1.10 Aims

The main aim of this study is to elucidate the functional roles of the KiSS1 receptor signaling pathway in tumorigenesis and cancer progression of several types of solid tumors. This aim will be attained through:

1. Identification of the effect of KiSS1 receptor agonist on cancer cell viability and motility.
2. Investigation of the impact of a KiSS1 receptor agonist on epithelial mesenchymal transition.
3. Identification of the role of a KiSS1 receptor agonist in cytoskeleton reorganization of cancer cells.

Chapter Two: METHODS

2.1 Reagents & Chemicals

2.1.1 KiSS1 receptor agonist

A KiSS1R agonist **(**Y-156-2) and a negative control were provided by Dr Robert Drakas and ShangPharma Innovation, Inc. Non-disclosure statement: The specific sequence or structure of the KiSS1R agonist (Y-156-2: MW= 1959.32) and negative control peptide (Y-20: MW = 1358.50) cannot be disclosed as proprietary to ShangPharma Innovation. However, in vitro and ADME data, including protocols for activation and migration assays were provided by ShangPharma Innovation and are included as Supplementary Materials. The negative control is a scrambled peptide. 10 nM is the dose that was used of Y-20 in all the assays.

2.2 Cell lines and cell culture

Eight different cancer cell lines were used. Prostate cancer cell lines: PC3 (grade IV, adenocarcinoma, epithelial) and LNCaP (carcinoma, epithelial). Breast cancer cell lines: MDA-MB-231(adenocarcinoma, epithelial) and T47D (ductal carcinoma, epithelial). Pancreatic cancer cell lines: HPAF2 (adenocarcinoma, epithelial) and BxPC3 (adenocarcinoma, epithelial. Melanoma cell lines: WM1232 (metastatic human melanoma) and A375M (malignant melanoma). Most of the cells were obtained from ATCC.

RPMI-1640 Medium (RPMI) supplemented with fetal bovine serum to a final concentration of 10% was used as complete medium to culture the WM1232, LNCaP, BxPC3, and T47D cell lines. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS) to a final concentration of 10% was used to culture the A375m and MDA-MB-231 cell lines. Ham's F-12K (Kaighn's) Medium F-12K Medium supplemented with 10% FBS was used to culture PC3 cells. All media were supplemented with 1% penicillin/streptomycin as antimicrobial

/antimycotic. All cells were grown at 37 ºC in humidified atmosphere incubators containing 5% CO_2. Cells were passaged using Trypsin EDTA 0.25%.

2.3 Scratch wound healing migration assay

Cells were grown to full confluence in 6-well plates in a suitable complete medium after which cells were starved overnight and a linear scratch was made through the monolayer using a sterile pipette tip (200µl). Cells were photographed using an inverted microscope at zero hours. Cells were then treated with the KiSS1R agonist Y-156-2 or the appropriate negative control. Cells were photographed again 24 hours later. The difference in the wound width between the two time points was calculated to reflect the effect of Y-156-2 in inducing wound closure.

2.4 Cell viability assay

The MTT assay is a colorimetric assay used to measure cellular viability and cytotoxicity of chemicals, where 3-(4,5, dimethylethiazol-22-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to measure the activity of mitochondrial succinate-dehydrogenase enzyme which is only found in living cells. Blue formazan product is produced due to the cleavage of a tetrazolium ring in the active mitochondria, where the amount of formazan produced is directly proportional to the number of active/live cells. Cells were cultured in their suitable complete media as explained above, then trypsinized (0.25% trypisin, cellgro) and normalized with low-serum medium (Medium with 2% FBS). Cells were counted and volume adjusted to have 5000 cells/100 µl and 2500 cells/100 µl. Cells were seeded in 96-well plates into two different groups of either 5000 cells or 2500 cells/100 µl and each group was treated or not with Y-156-2 plus a negative control for 24 hours at 37 ºC in the cell culture incubator. Twenty-four hours later, 25 µl of MTT solution (5 mg/ml) was added to the medium in each well and cells were further incubated at 37 ºC for two

hours. The reaction was then terminated by adding 100 µl DMSO to lyse the cells and release the blue crystals. To adjust the pH, Sorenson glycine buffer was added. The amount of colour was quantified using the Epoch™ Microplate Spectrophotometer (BIOTEK Instruments Inc.) at wavelength of 570 nm with a reference wavelength of 690 nm.

2.5 Western blot analysis

2.5.1 Sample preparation and protein quantification

Each cell line was cultured in a suitable complete medium as described above. Cells were treated with Y-165-2 and negative control for 72 hours. A non-treated control was conducted in parallel as well. Seventy-two hours later, old media were discarded, and cells were washed with PBS, lysed for 5 minutes over ice with 1% triton X-100 in RIPPA buffer supplemented with 100µM PMSF, 10µg/ml aprotinin, and 10µg/ml pepstatin. Cell lysates were centrifuged at 14,000 rpm for 15 minutes at 4°C. Protein content was measured using the BCA protein assay kit (Thermo Scientific). Cell lysates were mixed with 6x SDS loading buffer and boiled to 98 ºC for 5 minutes.

2.5.2 SDS-PAGE and transfer

Sample lysate containing 50 µg total protein were equally loaded and separated on a SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) electrophoresis apparatus (Bio-Rad). Then, proteins were transferred in a semi-dry apparatus (Bio-Rad) containing transfer buffer to a nitrocellulose membrane at a constant voltage of 15 V for 45 minutes.

2.5.3 Immunoblotting

After protein transfer, membranes were blocked using 5% non-fat milk-TBST buffer for 1 hour at room temperature and were then washed with TBST 3 times, each for 10 minutes.

Membranes were incubated overnight with primary antibodies diluted in gelatin solution at 4°C with gentle rocking. The next day, membranes were washed with TBST 3 times, each for 10 minutes. Afterwards, the secondary antibodies were added in blocking buffer 5% non-fat milk-TBST for 1 hour at room temperature. Membranes were washed with TBST 3 times, each for 10 minutes, and then membranes were treated with ECL chemiluminescent reagents (Bio-Rad) for 5 minutes in the dark. The proteins then were visualized using the ChemiDoc Imaging System (Bio-Rad). Densitometric analysis of protein levels was done using Image Lab Software (Bio-Rad).

2.6 Immunofluorescence

Cells were grown on coverslips to reach 80% confluence. The fixation process was performed on coverslips coated with cells in 4% Paraformaldehyde for 15 minutes at room temperature, followed by a permeabilization process with 0.1% Triton X-100 (Fisher). Cells were subsequently incubated with the primary antibody overnight at 4 °C followed by an incubation of the secondary antibody and DAPI (4,6-diamidino-2- phenylindole) for 1 hour at room temperature. Mounting media (Lerner # 13800) was used to mount the coverslips on glass slides and slides were stored at 4 °C. Confocal microscopy was performed using a Zeiss LSM 780 confocal microscope equipped with the oil immersion objective Plan-Apochromat 63x/1.4 Oil M27 (FWD=0.19 mm). The intensity of fluorescence was measured using Zen software, same number of cells were measured in each condition.

2.7 Statistical analysis

were performed using paired t test analysis accordingly. Results were shown as means ± SEM and $P < 0.05$ was considered as cut-off for significant association.

Chapter Three: RESULTS

3.1 Breast cancer

3.1.1 KiSS1/GPR54 receptor agonist has no effect on migration of triple negative breast cancer cells.

The role of KiSS1\GPR54 signaling in triple negative breast cancer is pro-metastatic, as shown by our previous work. This prompted us to further investigate its receptor agonist effect in promoting cell migration in breast cancer (Fratangelo, Carriero, et al. 2018; Tian, Al-Odaini, et al. 2018). To determine the effect of the KiSS1/GPR54 receptor agonist on breast cancer cell migration, a wound-healing assay was performed using the MDA-MB-231 cell line. The KiSS1/GPR54 receptor agonist had no effect on the cell migration of MDA-MB-231 cells. (Figure 3-1).

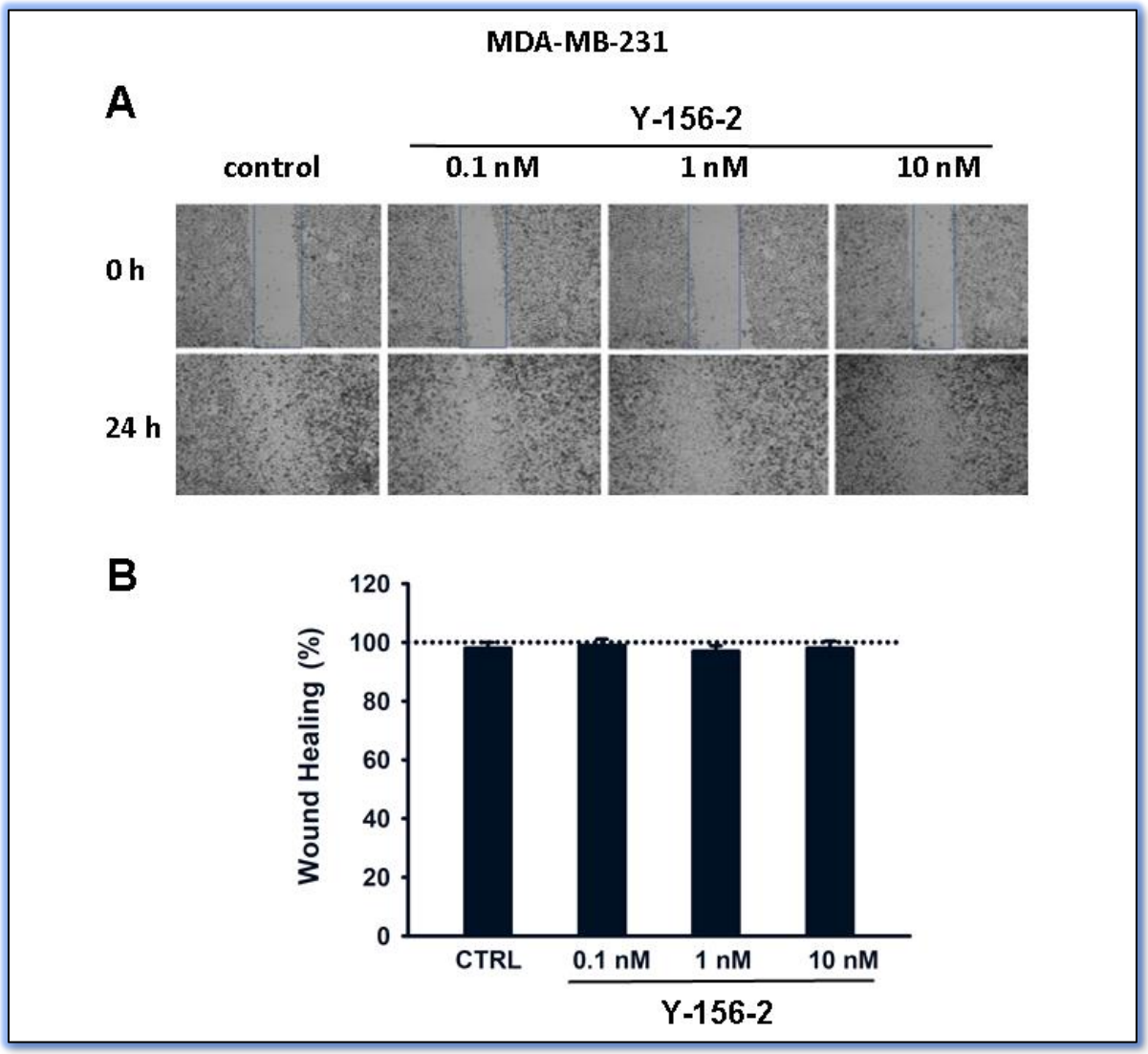


Figure 3-1 KiSS1/GPR54 receptor agonist (Y-156-2) has no effect on cell migration of triple negative breast cancer cells.

A: MDA-MB-231 cells were subjected to a scratch wound healing assay (cell migration) and were treated with negative control (10 nM) or three different concentrations of Y-156-2. Wound healing was measured 24 hours after wounding and was expressed as percentage of maximum (complete healing).

B: Means ± SEM of wound healing (expressed as percent of maximum). (N=3).

3.1.2 KiSS1/GRP54 receptor agonist has no effect on cell viability of triple negative breast cancer cells.

We examined the effect of the KiSS1/GPR54 receptor agonist on the viability of MDA-MB-231 cells. The KiSS1/GPR54 receptor agonist had no effect on the viability of these cells (Figure 3-2).

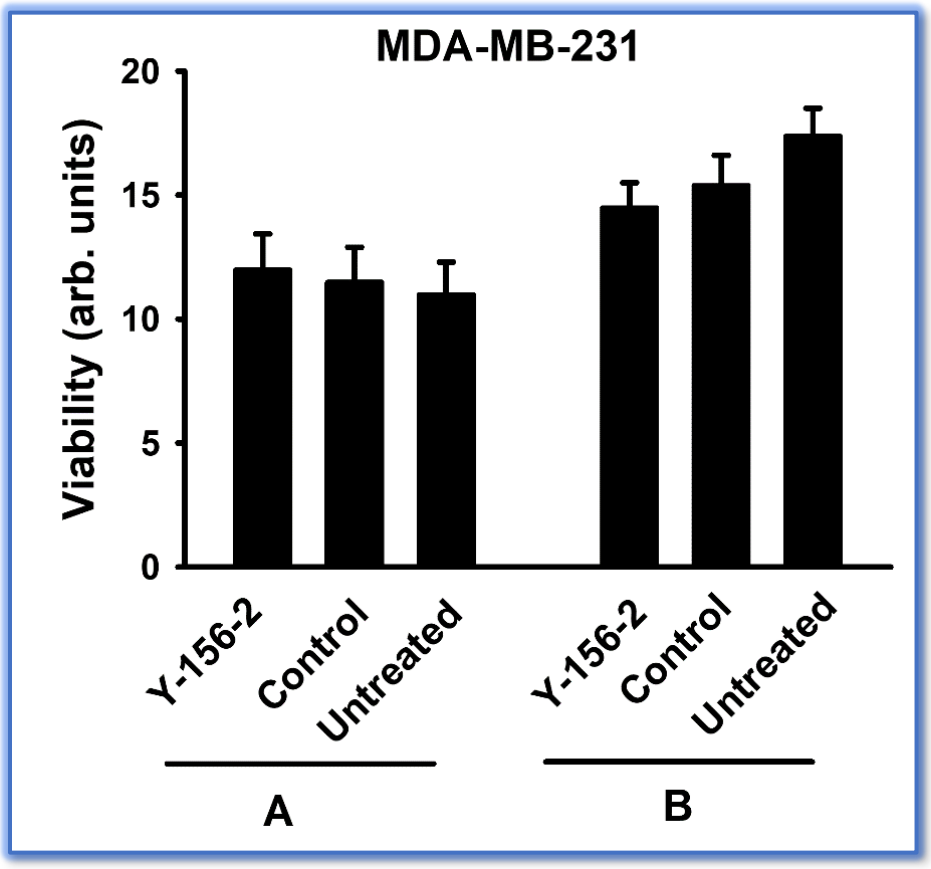


Figure 3-2 KiSS1/GPR54 receptor agonist does not affect triple negative breast cancer cell viability.

MDA-MB-231 cells were stimulated for 24 hours with Y-156-2, control peptide (10 nM) or left untreated. In condition A, 2500 cells were used, while 5000 cells were used in condition B. Data are means ± SEM. Arb. Unit refers to arbitrary units. (N=3).

3.1.3 KiSS1/GPR54 receptor agonist upregulates epithelial-mesenchymal transition markers in triple negative breast cancer.

To further investigate the role of the KiSS1/GPR54 receptor agonist in triple negative breast cancer, we examined whether it affects EMT processes in these cells by measuring the expression of several EMT markers in the MDA-MB-231 cell line. The KiSS1/GPR54 receptor triggered a significant increase in the intensities of Snail and Twist transcription factors compared to control peptide, suggesting that the KiSS1/GPR54 receptor agonist promotes EMT programming in triple negative breast cancer cells (Figure 3-3).

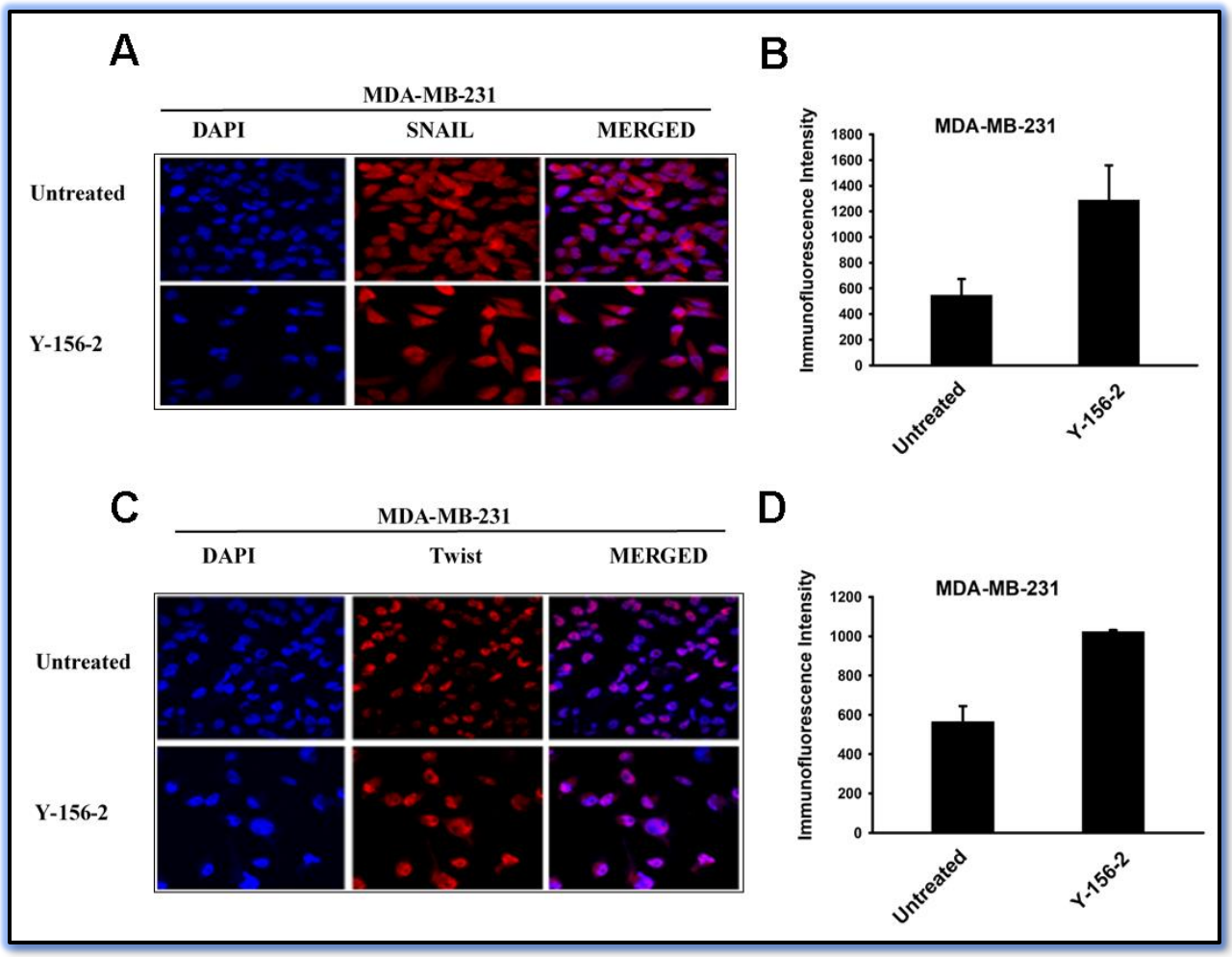


Figure 3-3 KiSS1/GPR54 receptor agonist upregulates the expression of Snail and Twist proteins in MDA-MB-231 cells.

A) Representative confocal immunofluorescence images of Snail1 (red) and nuclei (DAPI, blue) in untreated MDA-MB-231 cells and those treated with Y-156-2 (10 nM).
B) Mean ±SEM of Snail immunofluorescence intensities (n=3).
C) Representative confocal immunofluorescence images of Twist (red) and nuclei (DAPI, blue) in untreated MDA-MB-231and those treated with Y-156-2 (10 nM).
D) Means ±SEM of Twist immunofluorescence intensities (n=3).

To further investigate the role of the KiSS1/GPR54 receptor agonist in regulating EMT in breast cancer cells, we assessed Snail, E-Cadherin, and Vimentin protein levels in two cell lines. One represents the least aggressive luminal A breast cancer (T47D), the second an aggressive triple negative breast cancer (MDA-MB-231). E-Cadherin and Vimentin protein levels in both cell lines were not altered by Y-156-2 treatment. By comparison, Snail1 protein expression in MDA-MB-231 cells treated with Y-156-2 increased compared to those left untreated or treated with control peptide. (Figure 3-4).

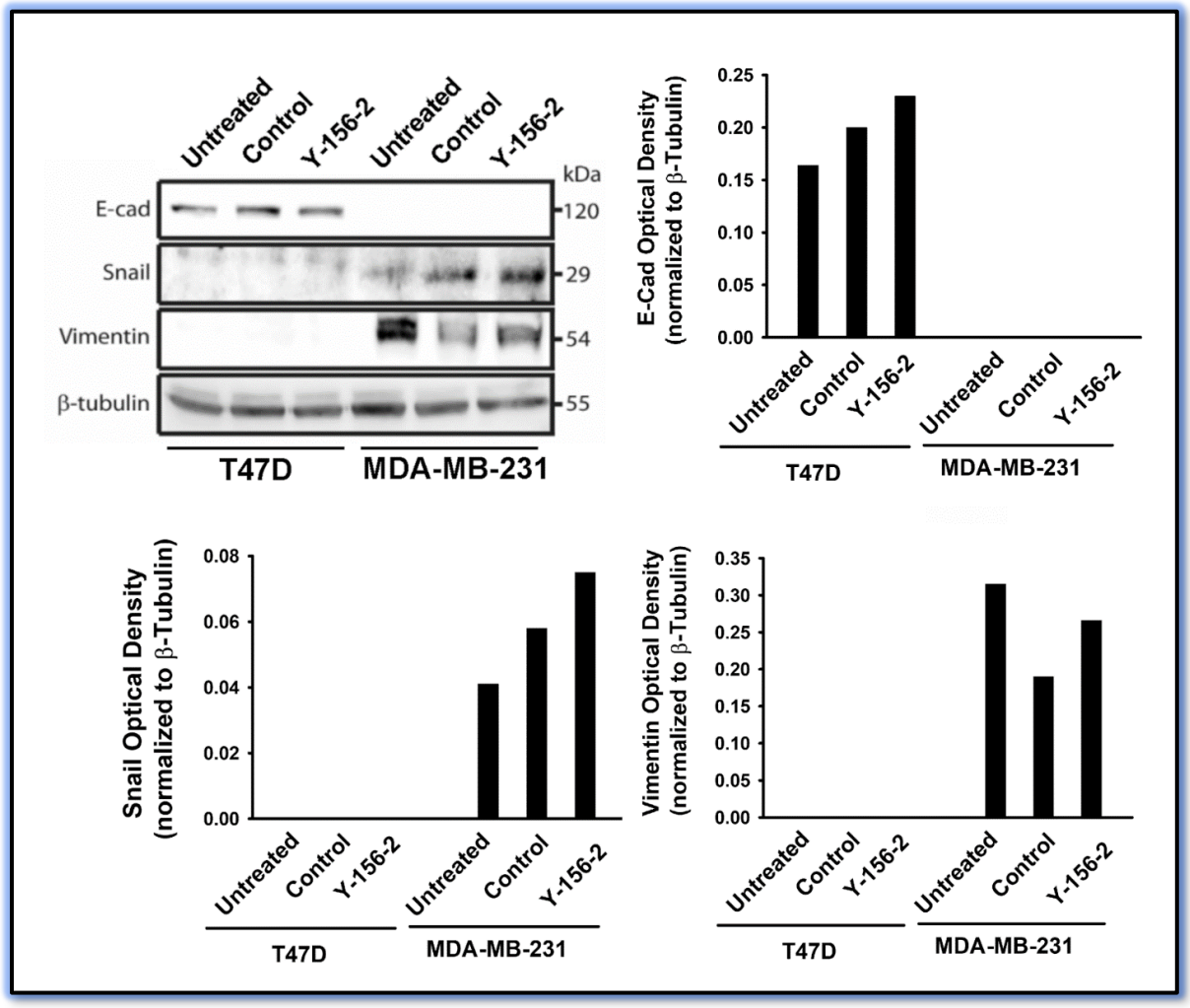


Figure 3-4 KiSS1/GPR54 receptor agonist trends to induce Snail1 expression in MDA-MB-231 breast cancer cells.

T47D and MDA-MB-231 cells were untreated or treated for 72 hours with control peptide (10 nM) or Y-156-2(10 nM). Cells were then lysed, and immunoblotting for E-Cadherin, Snail, Vimentin, and β-tubulin proteins was performed using specific primary antibodies. (N=1).

3.2 Pancreatic cancer

3.2.1 KiSS1/GPR54 receptor agonist does not stimulate pancreatic cancer cell migration

we investigated the effect of the KiSS1/GPR54 receptor agonist on pancreatic cancer migration and metastasis. Wound-healing assays were performed using HPAF2 and BXPC3 pancreatic cancer cell lines. Figure 3-5 illustrates that the KiSS1/GPR54 receptor agonist had no effect on HPAF2 and BXPC3 pancreatic cancer cell migration.

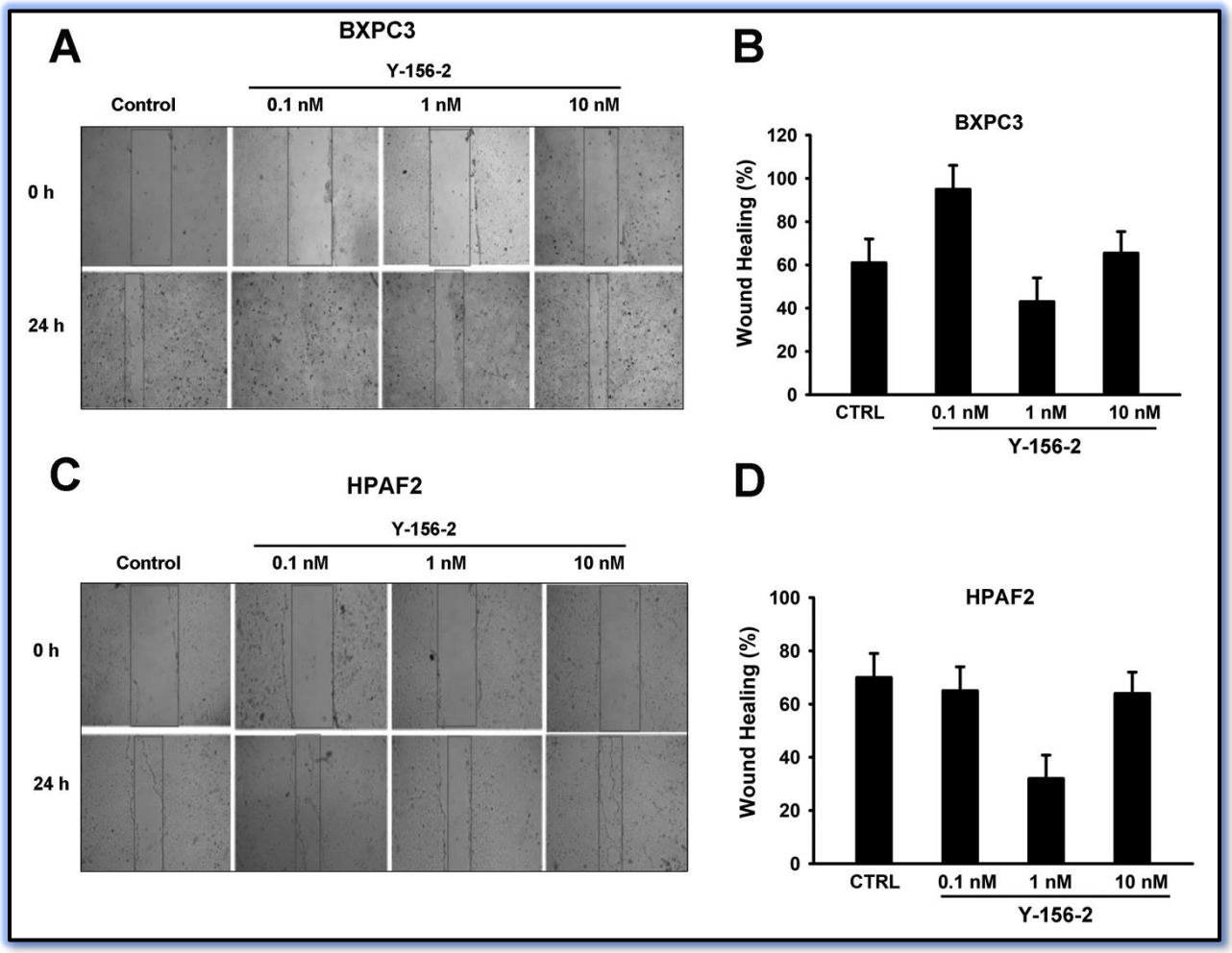


Figure 3-5 KiSS1/GPR54 receptor agonist has no effect on pancreatic cancer cell migration.

A & C) BXPC3 and HPAF2 pancreatic cancer cells were subjected to scratch wound healing assays (cell migration) and were treated with negative control peptide (10 nM) or three different concentrations of Y-156-2. The degree of wound healing was analyzed 24 hours after wounding and was expressed as percentage of maximum (complete healing).

B&D) Means ± SEM of wound healing (expressed as percent of maximum healing). (N=3).

3.2.2 KiSS1/GPR54 receptor agonist has no effect on pancreatic cancer cell viability

MTT assays using HPAF2 and BXPC3 pancreatic cancer cell lines revealed that Y-156-2 treatment had no effects on the viability of these cells (Figure 3-6).

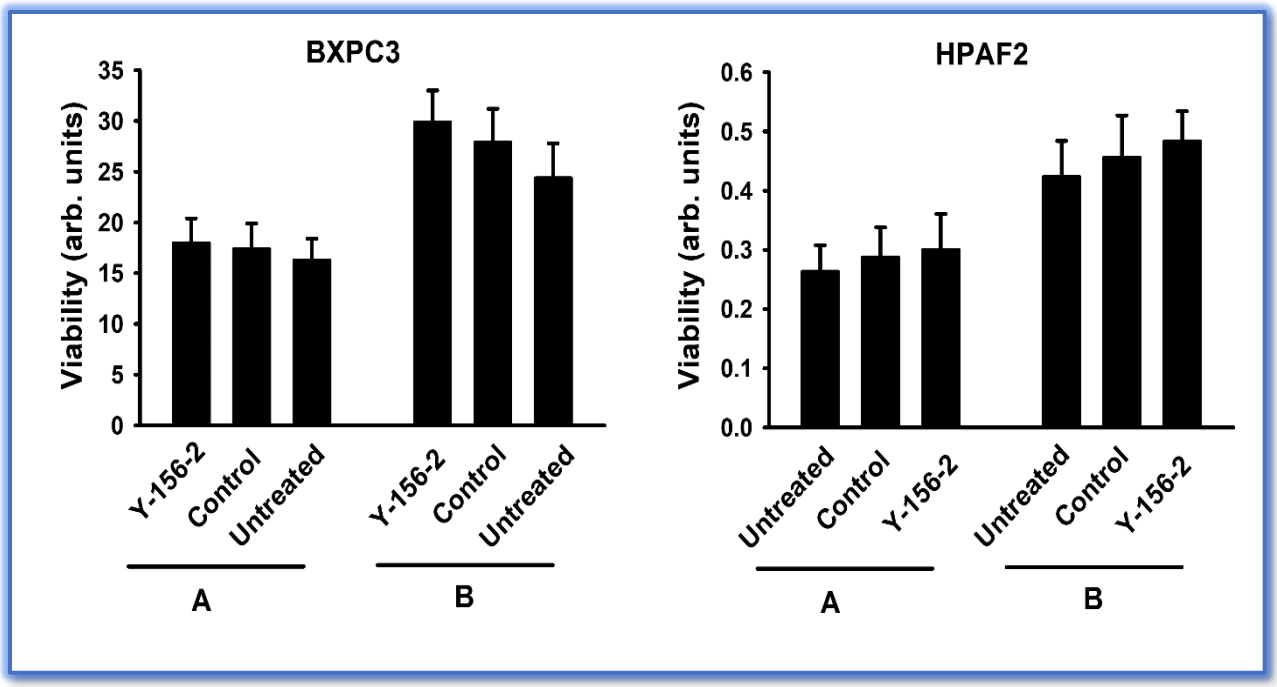
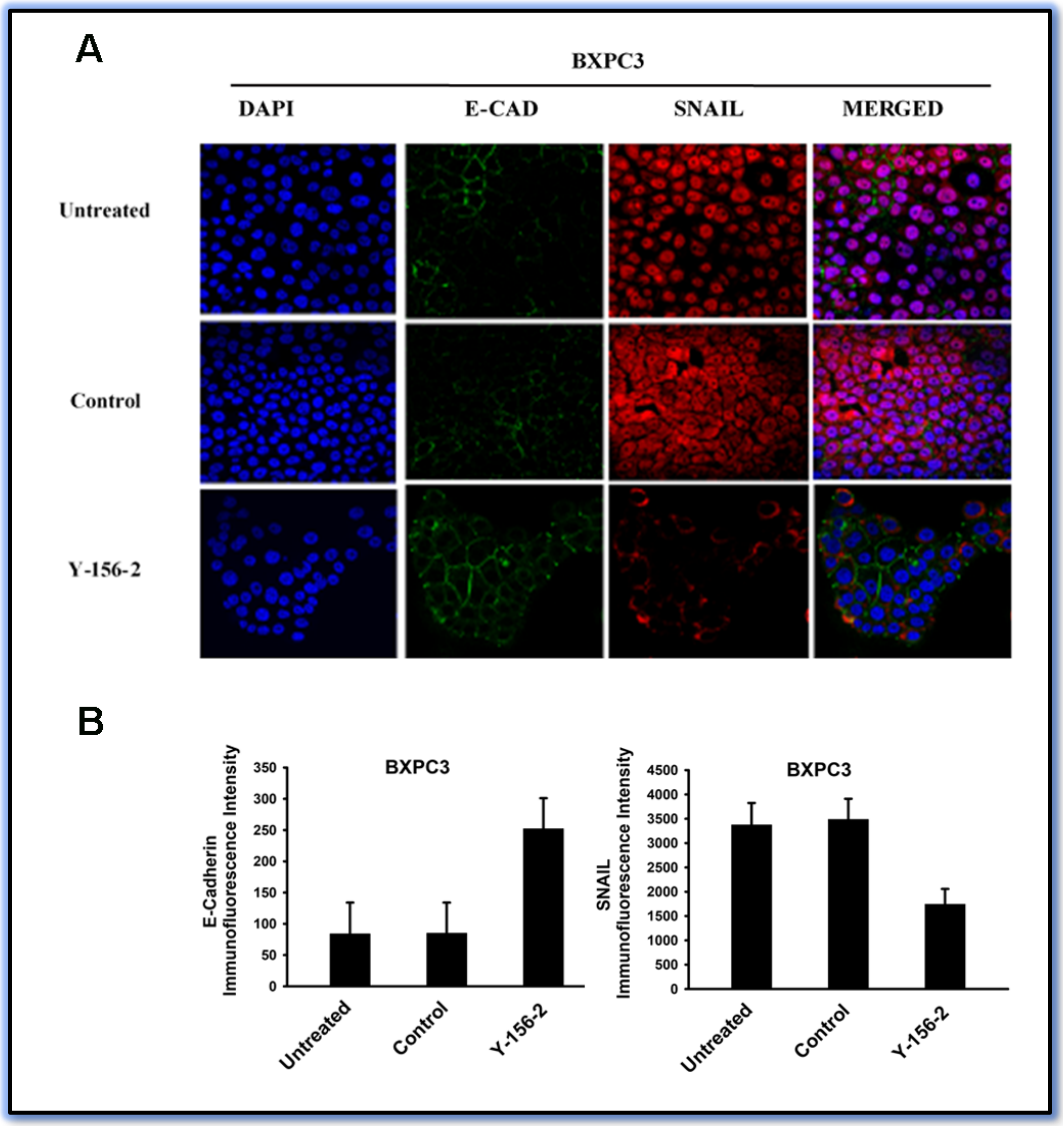


Figure 3-6 KiSS1/GPR54 receptor agonist has no effect on pancreatic cancer cell viability.

BXPC3 (left panel) and HPAF2 (right panel) cells were stimulated for 24 hours with Y-156-2, control peptide (Y-20) or left untreated. In condition A, 2500 cells were used, while 5000 cells were used in condition B. Data are means ± SEM. Arb. Units refers to arbitrary units. (N=3).

3.2.3 KiSS1/GPR54 receptor agonist induces epithelial-mesenchymal transitions markers in pancreatic cancer.

While E-cadherin protein fluorescence was readily detected in pancreatic cancer cells treated with the KiSS1 receptor agonist, Snail protein fluorescence was undetected compared to negative control and untreated conditions (Figure 3-7). This result suggests that the KiSS1/GRP54 receptor agonist has the potential to reprogram and to suppress the EMT process in pancreatic cancer cells.



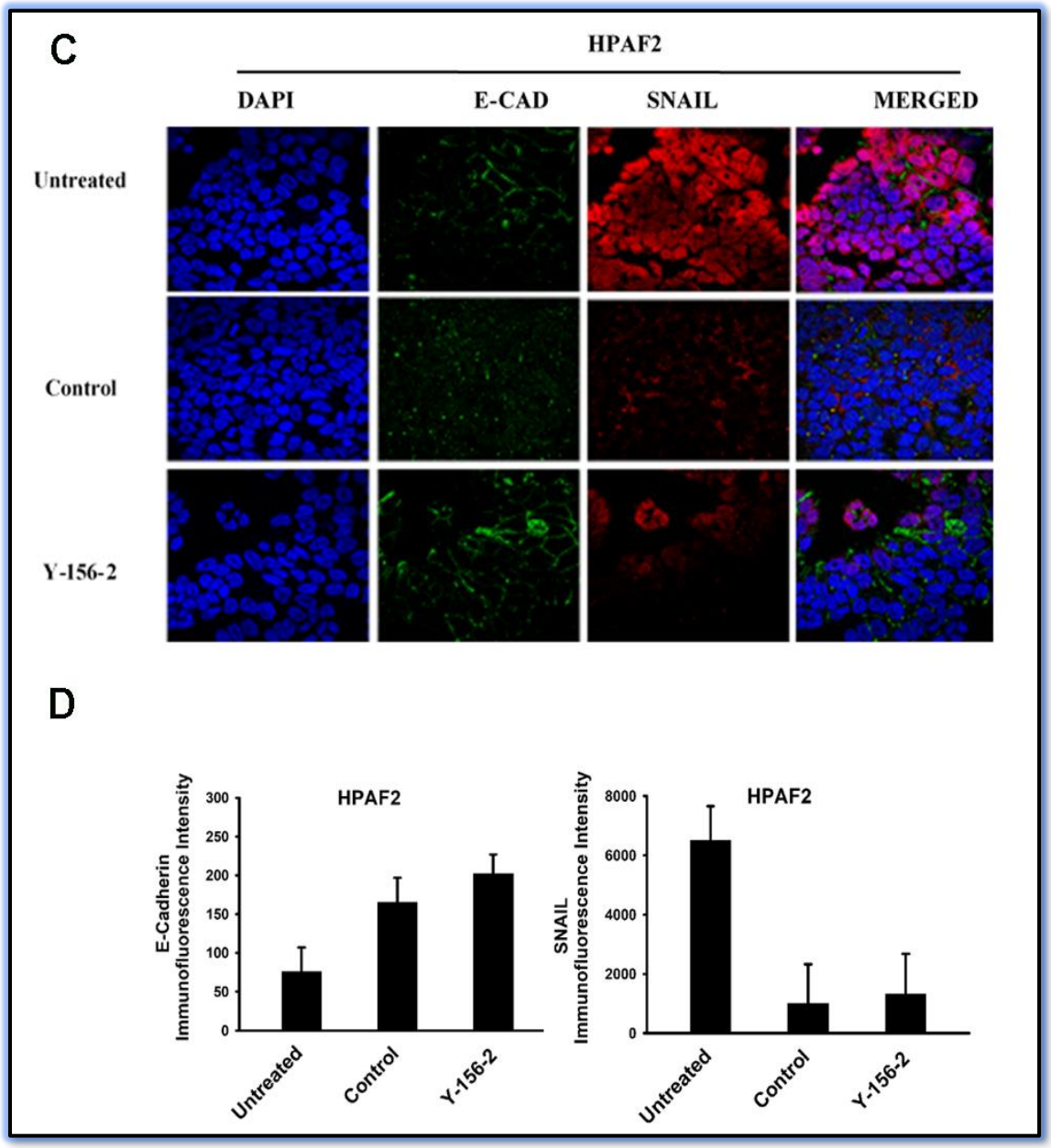


Figure 3-7 KiSS1/GPR54 receptor agonist modulates the expression of Snail and E-Cadherin in pancreatic cancer cells.

A & C) Representative confocal immunofluorescence images of membranous E-Cadherin (green), Snail (red), and nuclei (DAPI, blue) in untreated BXPC3 and HPAF2 cells and those stimulated for 72 hours with control peptide (10 nM) or Y-156-2 (10 nM). B & D) Means ±SEM of membranous E-Cadherin and Snail immunofluorescence intensities (n=3).

3.2.4 KiSS1/GPR54 receptor agonist trends to upregulate E-cadherin expression in pancreatic cancer.

To further confirm the role of the KiSS1/GPR54 receptor agonist in regulating EMT processes in pancreatic cancer, western blot analysis of E-Cadherin expression was performed using HPAF2 and BXPC3 pancreatic cancer cell lines. E-Cadherin expression in BXPC3 cells increased in response to KiSS1 receptor agonist treatment suggesting that these receptors regulate the epithelial phenotype in this type of pancreatic cancers. In HPAF2 cells, both control peptide and Y-156-2 decreased E-Cadherin levels compared to untreated cells (Figure 3-8).

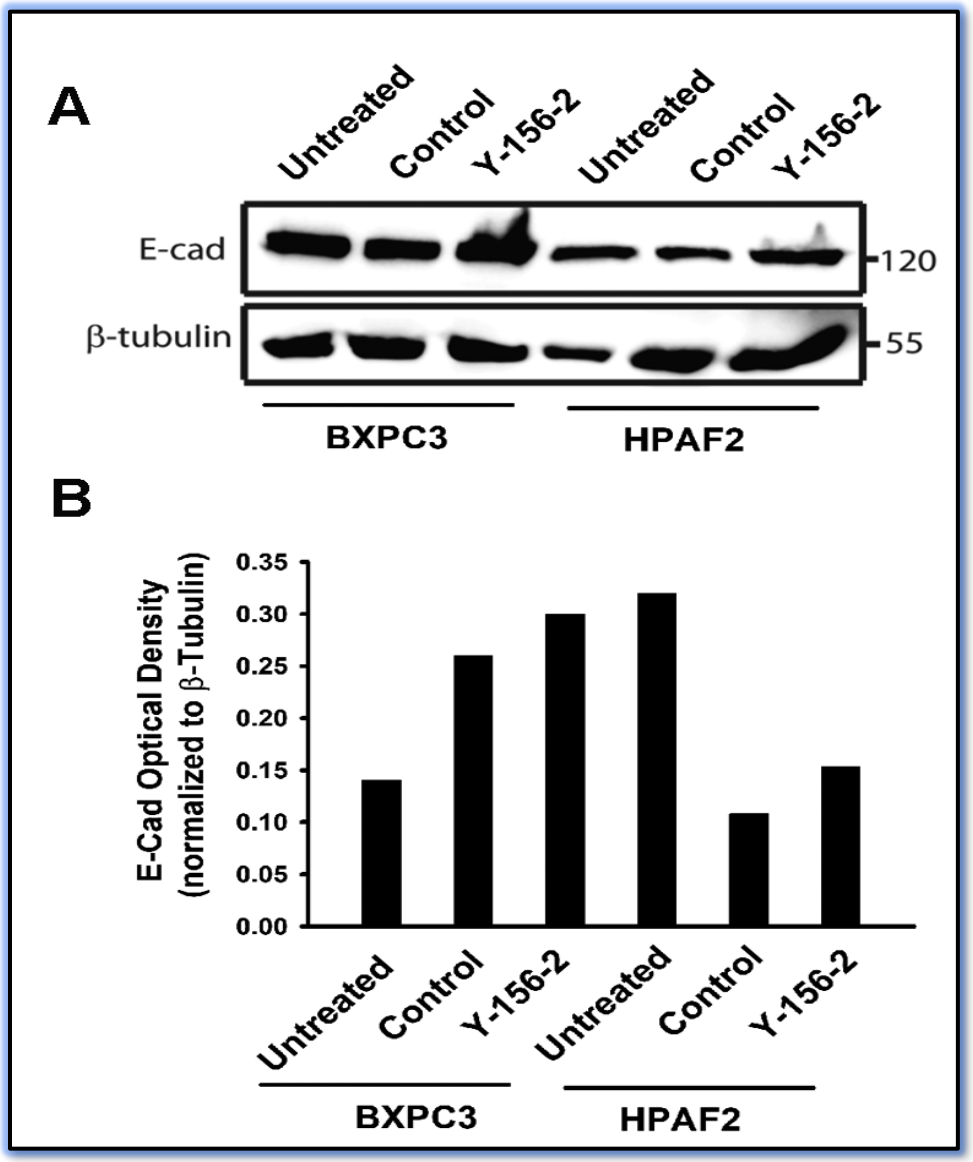


Figure 3-8 KiSS1/GPR54 receptor agonist trends to induce E-CAD expression in pancreatic cancer.

HPAFS and BXPC3 pancreatic cancer cells were left untreated or stimulated with control peptide (10 nM) or Y-156-2 peptide (10 nM) for 72 hours. Cells were then lysed, and immunoblotting was performed for E-Cadherin and β-Tubulin proteins using specific antibodies. Panel A shows E-Cadherin and β-Tubulin immunoblots while panel B shows the optical densities of E-Cadherin normalized for β-Tubulin. N=1.

3.3 Melanoma

3.3.1 KiSS1/GPR54 receptor agonist reduces migration in melanoma depending on the cell type.

Wound healing assays using the WM1232 and A375M cell lines were performed to investigate the effect of the KiSS1/GPR54 receptor agonist on melanoma cell migration. The Y-156-2 peptide inhibited wound healing of A375M cells but had no effect on wound healing of WM1232 cells. These results suggest that KiSS1/GPR54 receptor agonist reduces cell migration of A375M melanoma cancer cells but has no effect on migration of WM1232 cells (Figure 3-9).

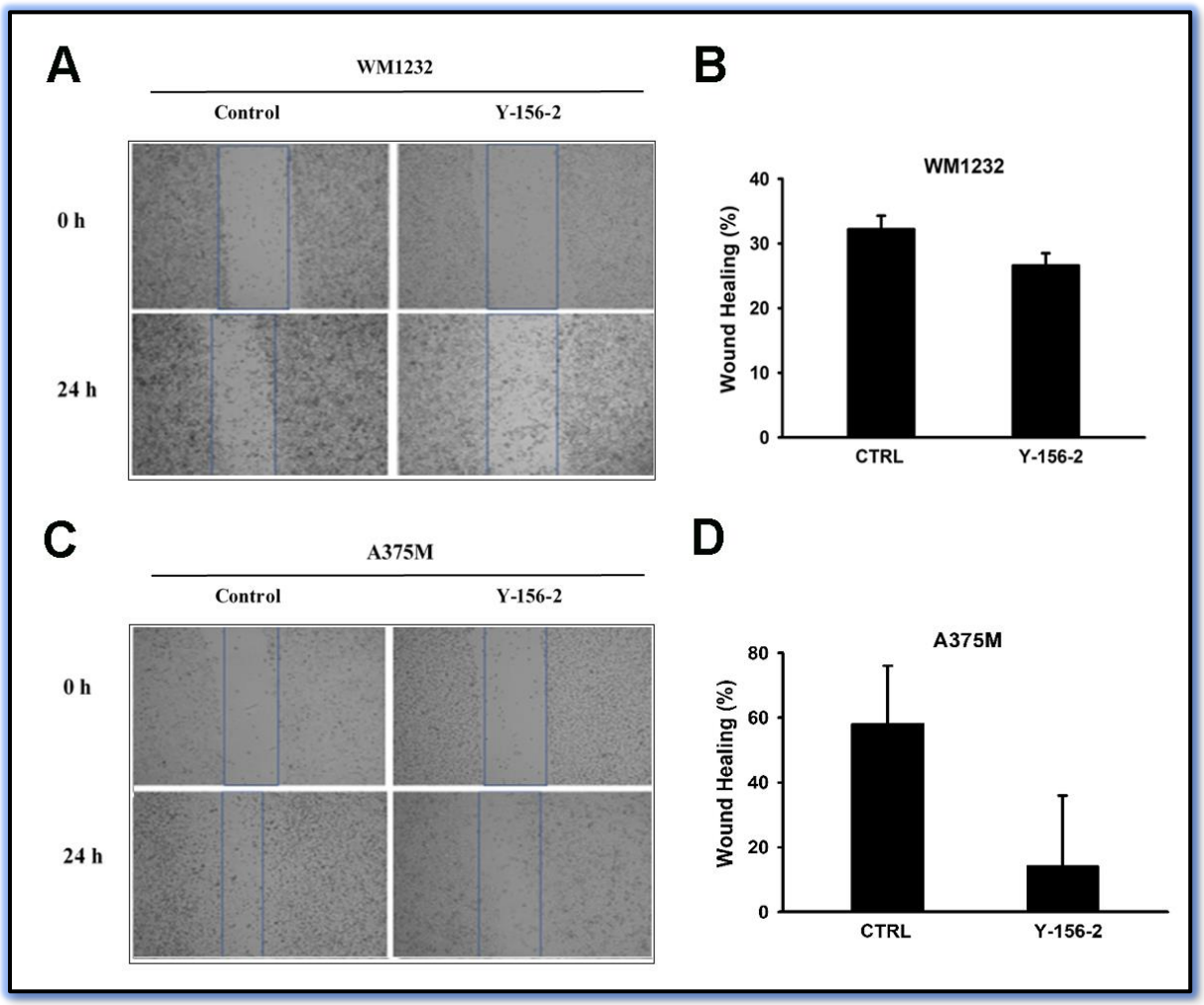


Figure 3-9 KiSS1/GPR54 receptor agonist regulates wound healing of melanoma depending on the cell type.

A & C) WM1232 and A375M melanoma cancer cells were subjected to scratch wound healing assays (cell migration) and were treated with control peptide (10 nM) or 10 nM of Y-156-2 peptide. Wound healing was analyzed 24 hours after wounding.

B&D) Means ± SD of wound healing (expressed as percent of maximum). (N=3).

3.3.2 KiSS1/GPR54 receptor agonist has no effect on melanoma cell viability

To determine the effect of the KiSS1/GPR54 receptor agonist on melanoma cell viability, A375M and WM1232 cells were stimulated with KiSS1/GPR54 receptor agonist, negative control peptide or untreated for 24 hours. The KiSS1/GPR54 receptor agonist had no effect on viability of WM-1232 and A375M melanoma cancer cells (Figure 3-10).

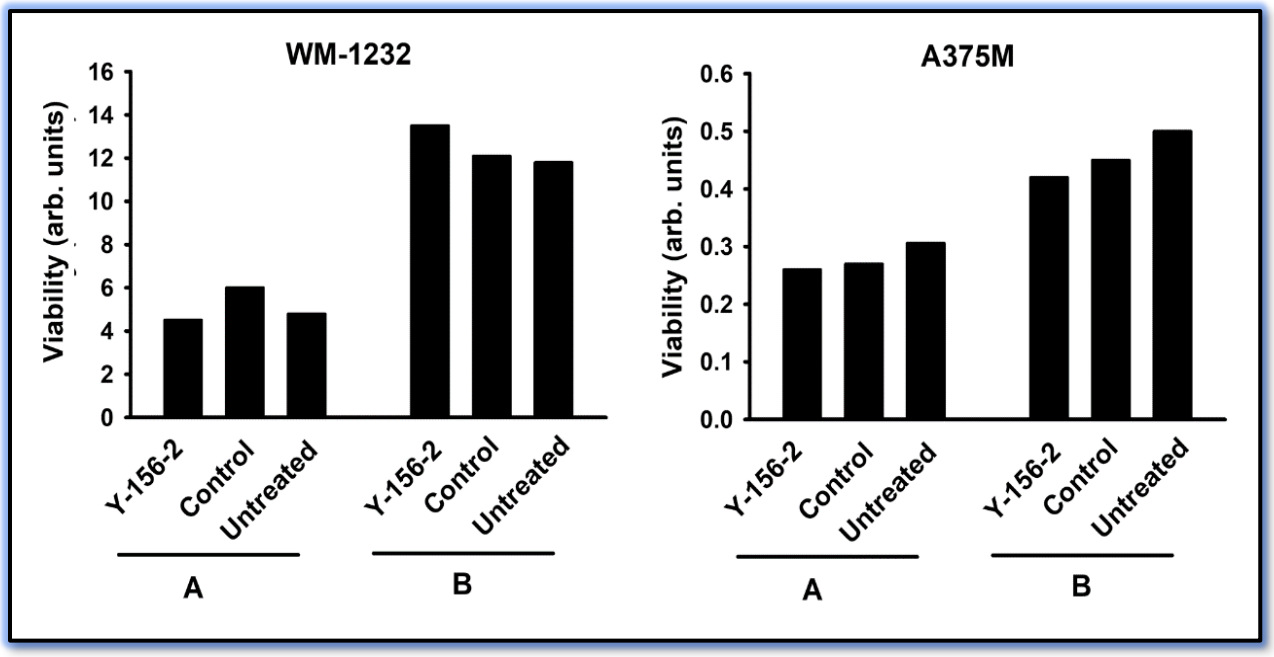
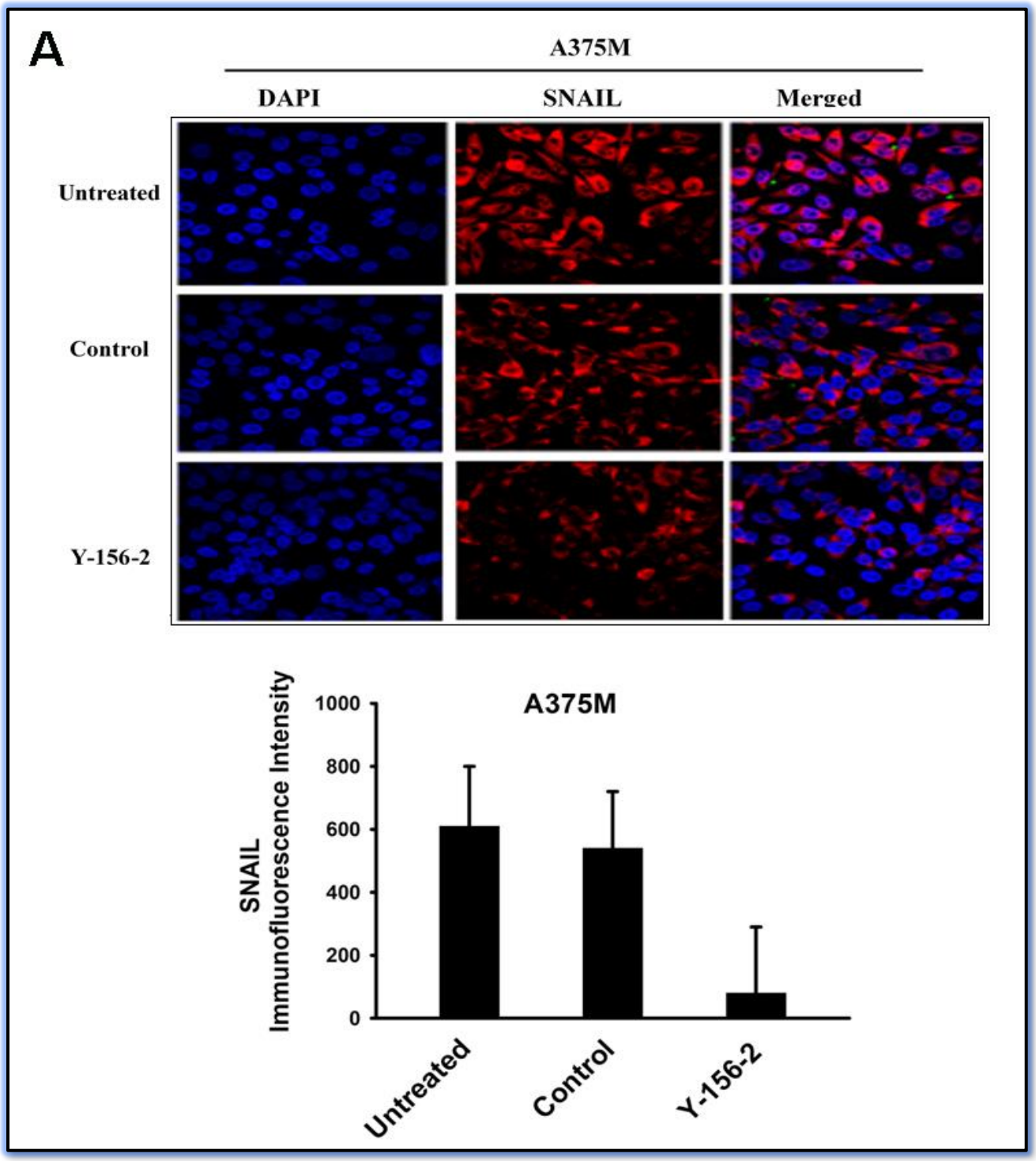


Figure 3-10 KiSS1/GPR54 receptor agonist has no effect on cell viability of melanoma cells.

WM1232 (left panel) and A375M (right panel) melanoma cancer cells were stimulated for 24 hours with Y-156-2 (10 nM), control peptide (10 nM) or left untreated. In condition A, 2500 cells were used, while 5000 cells were used in condition B. Data are means ± SEM. Arb. Units refers to arbitrary units. (N=1)

3.3.3 KiSS1/GPR54 receptor agonist downregulates epithelial-mesenchymal transition markers in melanoma.

To further decipher the role of KiSS1/GPR54 receptor agonist in melanoma, we studied its role in regulating EMT markers. KiSS1/ GPR54 receptor agonist treatment decreased Snail and Twist expression in A375M melanoma cells. Similarly, KiSS1/GPR54 receptor agonist treatment decreased Snail expression in WM1232 melanoma cells. These data suggest that KiSS1/ GPR54 receptor agonist may regulate EMT processes in melanoma.(Figure 3-11).


A
A375M
DAPI
SNAIL
Merged
Untreated
Control
Y-156-2
A375M
SNAIL Immunofluorescence Intensity
1000
800
600
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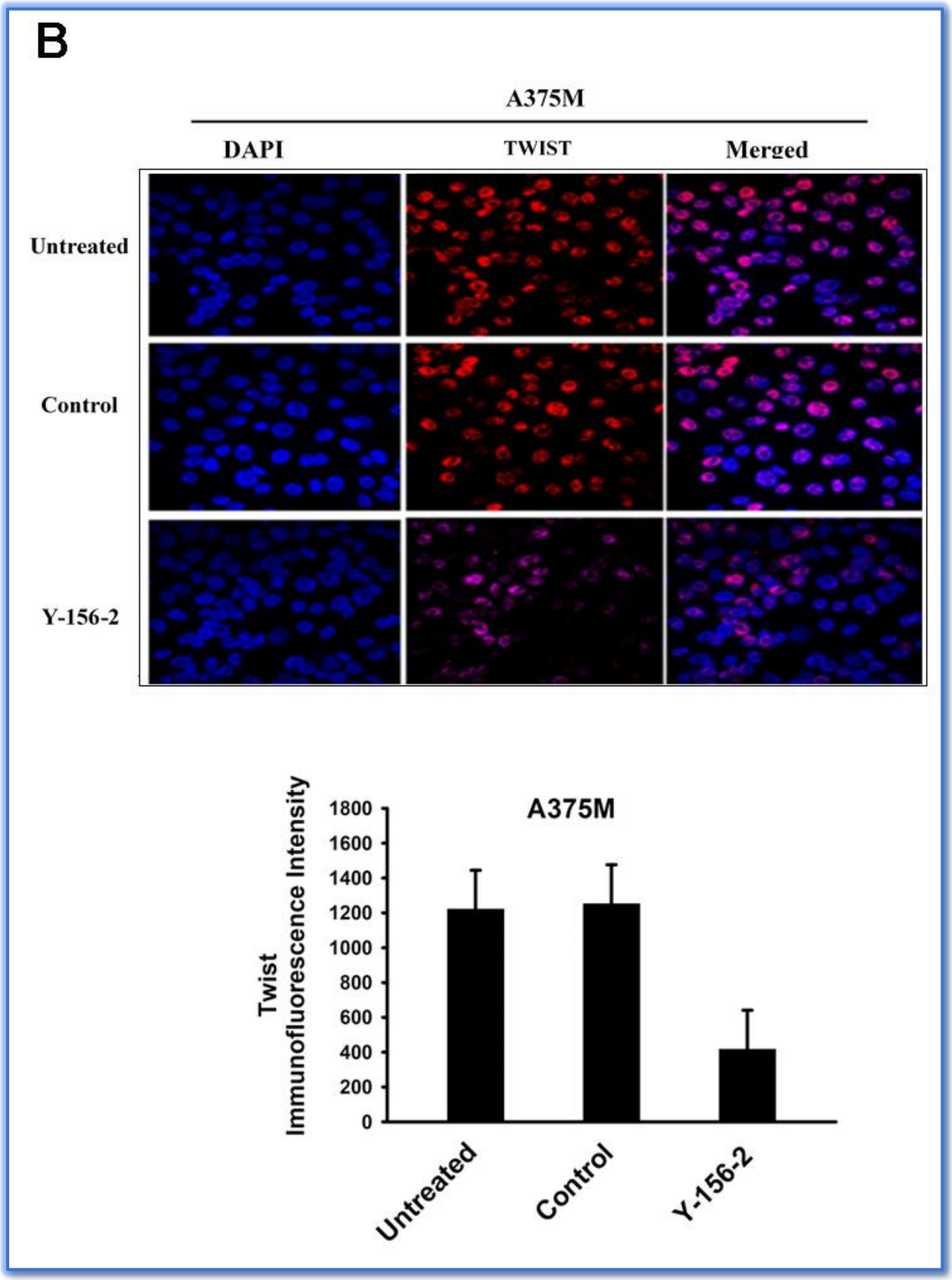
200
0
Untreated
Control
Y-156-2


B
A375M
DAPI
TWIST
Merged
Untreated
Control
Y-156-2
A375M
Twist
Immunofluorescence Intensity
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1600
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800
600
400
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0
Untreated
Control
Y-156-2

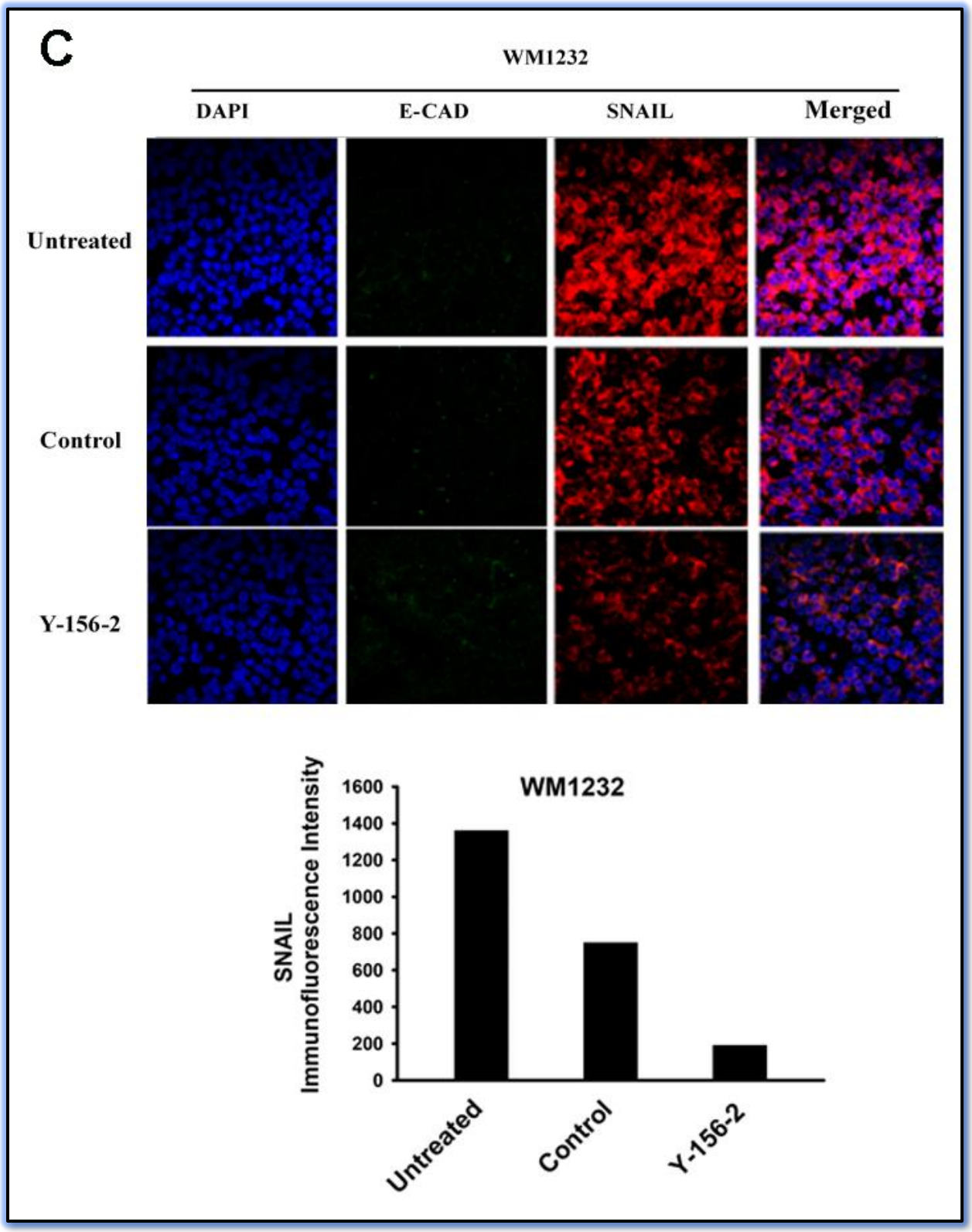

C
WM1232
DAPI
E-CAD
SNAIL
Merged
Untreated
Control
Y-156-2
WM1232
SNAIL
Immunofluorescence Intensity
1600
1400
1200
1000
800
600
400
200
0
Untreated
Control
Y-156-2

Figure 3-11 KiSS1/GPR54 receptor agonist regulates EMT markers in melanoma.

A&B) Representative confocal immunofluorescence images and means ±SEM of fluorescence intensities of Snail (red), Twist (red) and nuclei (DAPI, blue) in A375M melanoma cells untreated, or stimulated for 72 hours with control peptide (10 nM) or Y-156-2 peptide (10 nM). (N=3).
C) Representative confocal immunofluorescence images and fluorescence intensities of Snail (red) and E-Cadherin (green) in WM1232 melanoma cells untreated or stimulated for 72 hours with control peptide (10 nM) or Y-156-2 peptide (10 nM). (N=1).

3.3.4 KiSS1/GPR54 receptor agonist trends to decrease epithelial-mesenchymal transition markers in melanoma.

To verify the role of the KISS1/GPR54 receptor agonist in the regulation of EMT processes in melanoma cells, SNAIL protein expression was assessed using western blot analyses in WM1232 and A375M melanoma cells. SNAIL protein levels decreased in both cell types in response to KiSS1/GPR54 receptor agonist treatment, suggesting an important role for the KiSS1/GPR54 receptor agonist in the regulation of EMT in melanoma cancer cells (Figure 3-12).

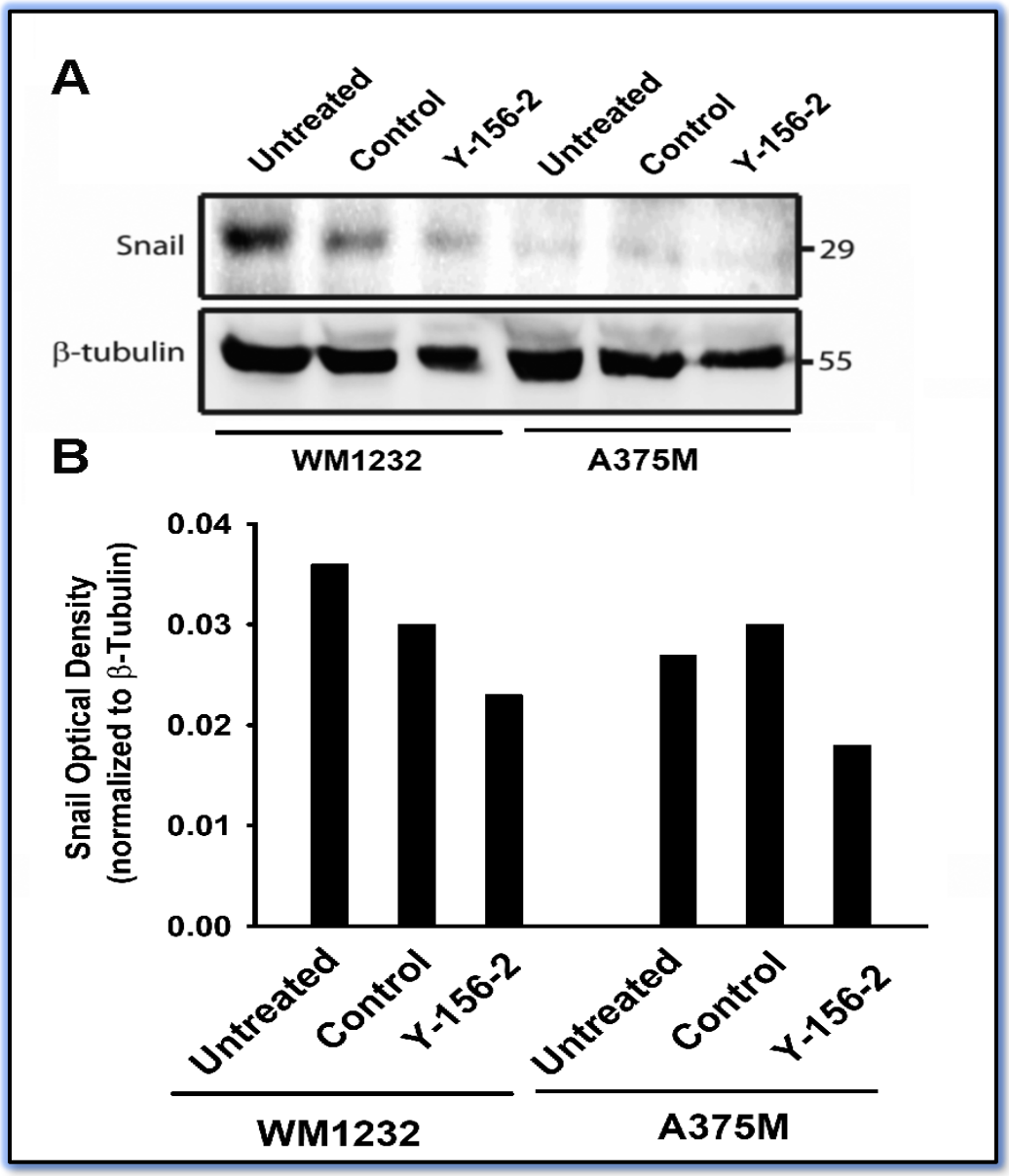


Figure 3-12 KiSS1/GPR54 receptor agonist trends to modulate the expression of Snail in melanoma cells.

WM1232 and A375M melanoma cells were left untreated or stimulated with control peptide (10 nM) or Y-156-2 peptide (10 nM) for 72 hours. Cells were then lysed, and immunoblotting was performed for Snail and β-Tubulin proteins using specific antibodies. Panel A shows Snail and β-Tubulin immunoblots while panel B shows the optical densities of Snail protein normalized for β-Tubulin. N=1.

3.4 Prostate cancer

3.4.1 KiSS1/GPR54 receptor agonist regulates migration of prostate cancer cells depending on the cell type.

We assessed the role of the KiSS1/GPR54 receptor agonist in prostate cancer cell migration using LNCAP and PC3 cells. In LNCAP cells, the KiSS1/GPR54 receptor agonist and control negative peptide had similar effects on cell migration. In PC3 cells, the KiSS1/GPR54 receptor agonist decreased wound healing compared to control peptide (Figure 3-13).

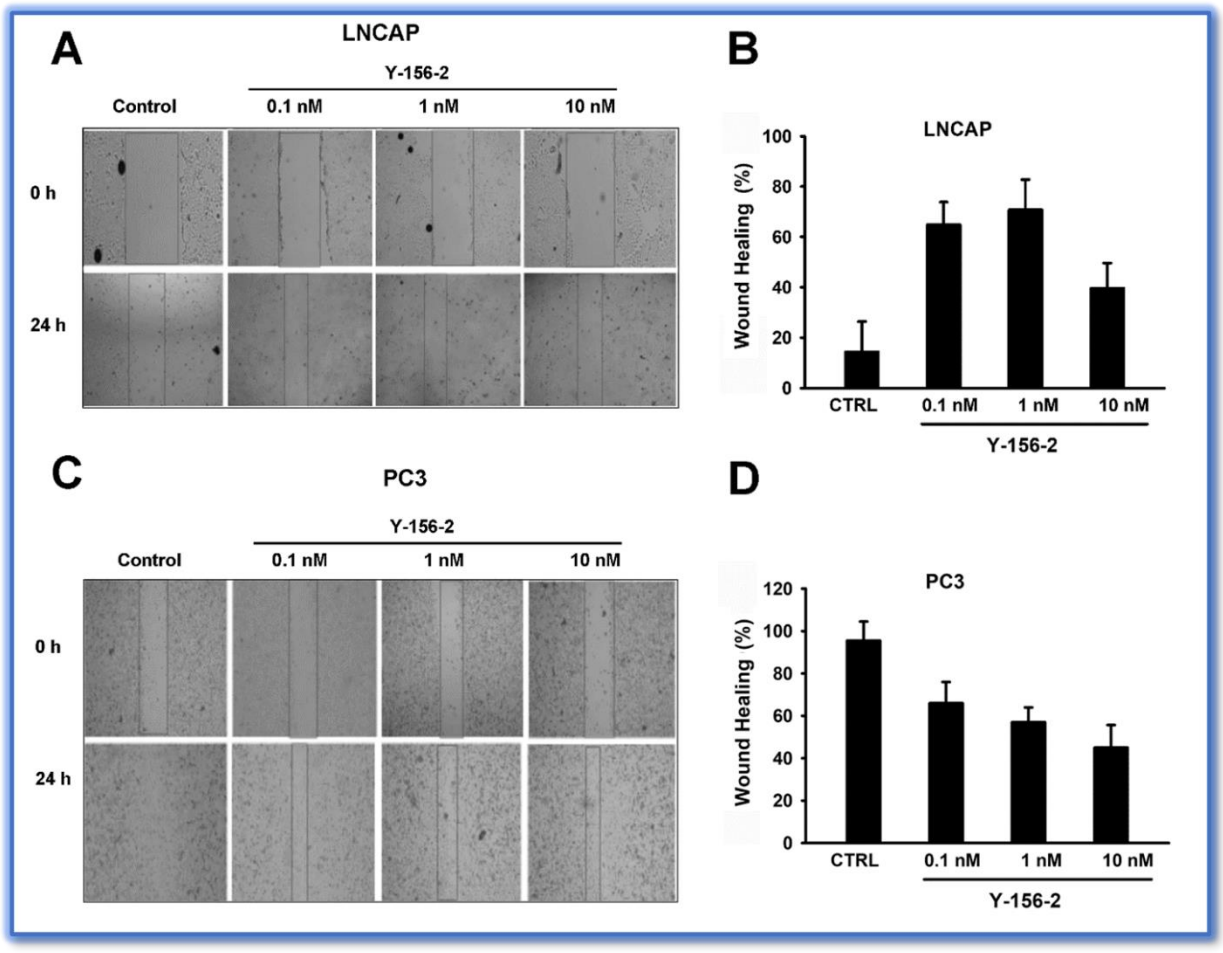


Figure 3-13 KiSS1/GPR54 receptor agonist has no effect on cell migration in LNCAP while it exhibits a suppressive effect in PC3 wound closure.

A & C) LNCAP and PC3 prostate cancer cells were subjected to a wound healing assay (cell migration) and were treated with control peptide Y-20 of 10 nM or three different concentrations of Y-156-2 peptide. Wound healing was analyzed 24 hours after wounding.
B&D) Means ± SE of wound healing (expressed as percent of maximum). (N=3).

3.4.2 KiSS1/GPR54 receptor agonist does not regulate prostate cancer cell viability.

To investigate the role of KiSS1/GPR54 receptor agonist in prostate cancer cell viability, MTT assay was performed using the LNCAP cell line. The KiSS1/GPR54 receptor agonist had no effect on LNCAP cell viability (Figure 3-14).

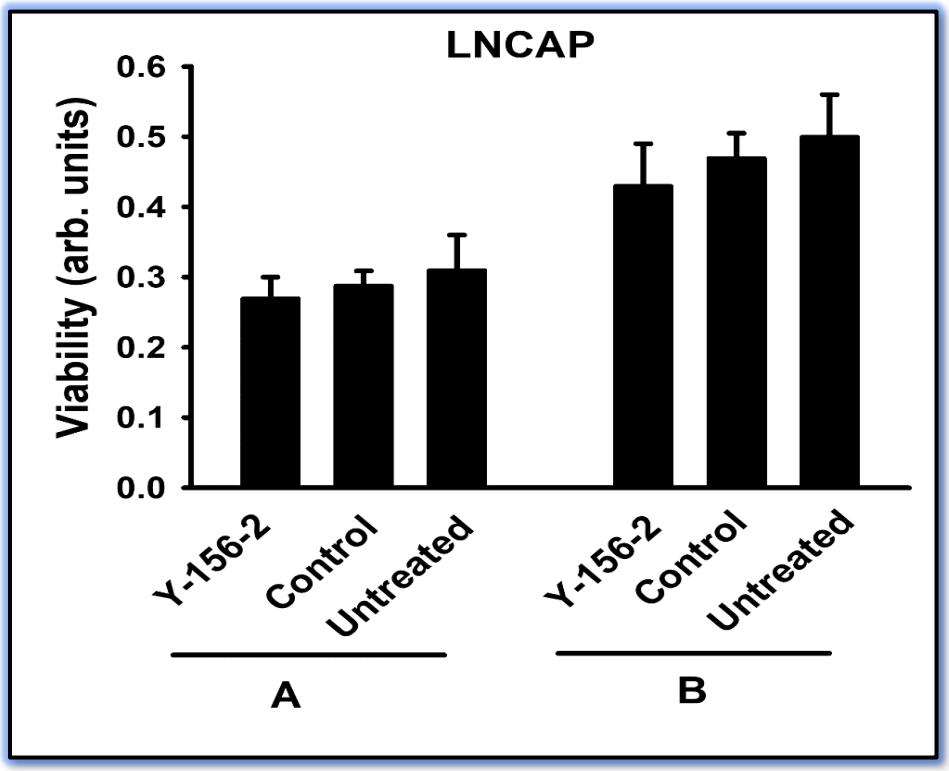
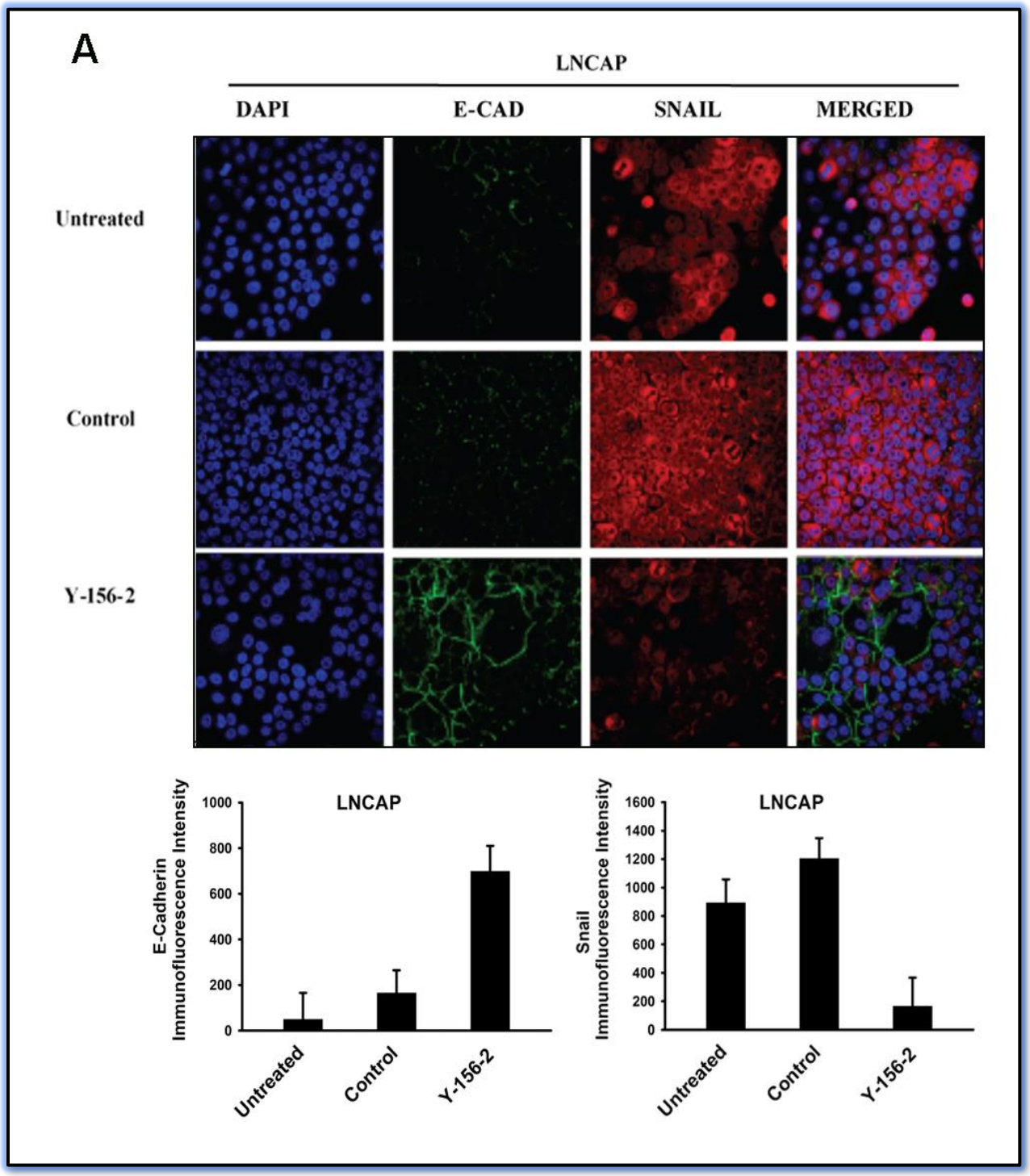


Figure 3-14 KiSS1/GPR54 receptor agonist does not affect prostate cancer cell viability.

LNCAP prostate cancer cells were stimulated for 24 hours with Y-156-2 peptide (10 nM), control peptide Y-20 (10 nM) or left untreated. In condition A, 2500 cells were used, while 5000 cells were used in condition B. Data are means ± SEM. Arb. Units refers to arbitrary units. (N=3).

3.4.3 KiSS1/GPR54 receptor agonist suppresses epithelial-mesenchymal transition markers in prostate cancer.

We assessed the role of the KiSS1/GPR54 receptor agonist in regulating EMT processes in prostate cancer cells and we evaluated Snail, Twist, and E-cad protein levels in LNCAP cells using immunofluorescence analyses. The KiSS1/ GPR54 receptor agonist decreased Snail and Twist protein levels compared to control peptide and untreated condition while increasing the expression of E-Cadherin. These results suggest the KiSS1/ GPR54 receptor agonist suppresses EMT in prostate cancer (Figure 3-15).


A
LNCAP
DAPI
E-CAD
SNAIL
MERGED
Untreated
Control
Y-156-2
LNCAP
E-Cadherin Immunofluorescence Intensity
1000
800
600
400
200
0
Untreated
Control
Y-156-2
LNCAP
Snail Immunofluorescence Intensity
1600
1400
1200
1000
800
600
400
200
0
Untreated
Control
Y-156-2

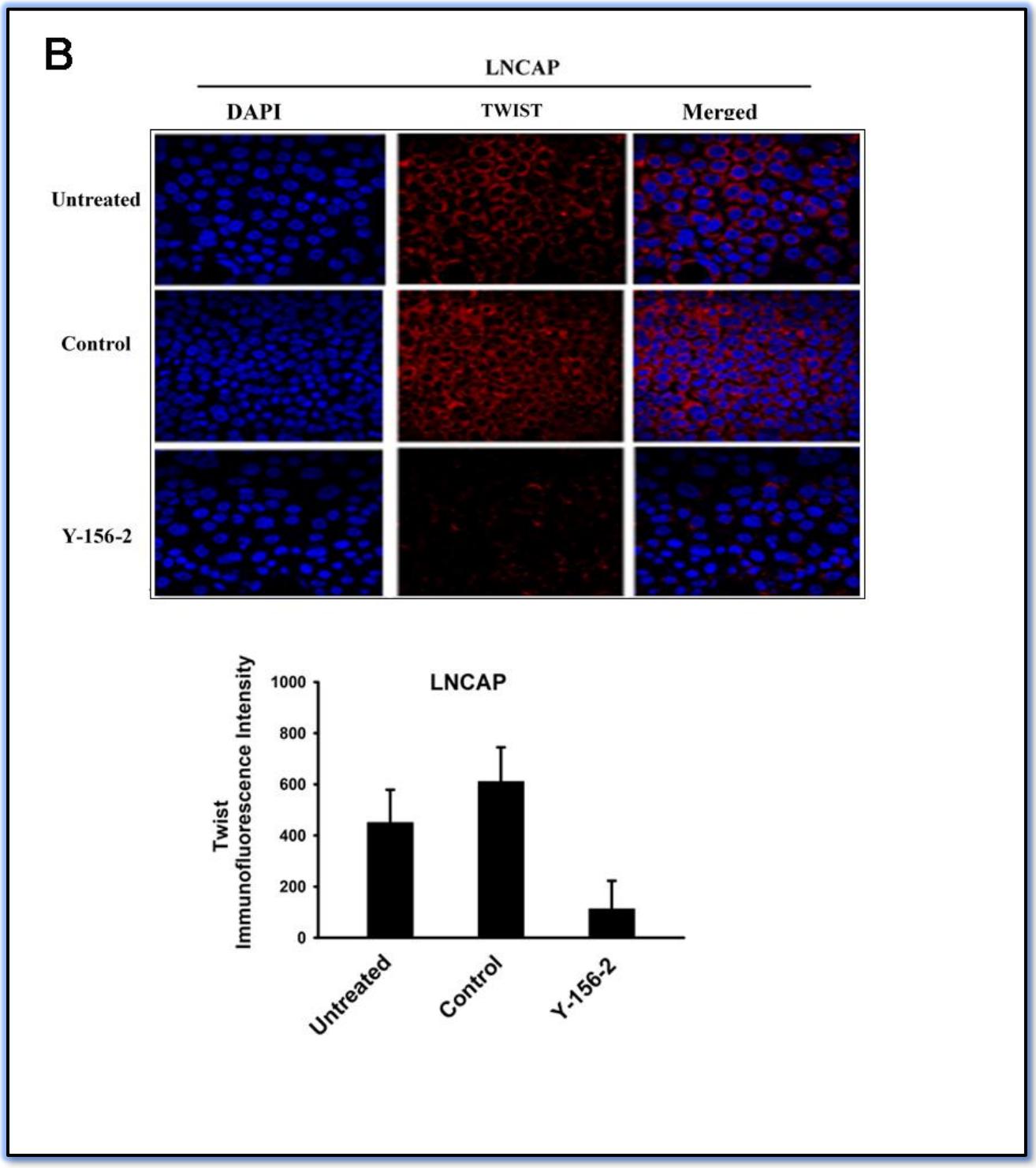

B
LNCAP
DAPI
TWIST
Merged
Untreated
Control
Y-156-2
LNCAP
Twist
Immunofluorescence Intensity
1000
800
600
400
200
0
Untreated
Control
Y-156-2

Figure 3-15 KiSS1/GPR54 receptor agonist regulates EMT in prostate cancer cells.

A) Representative confocal immunofluorescence images and means ±SEM of fluorescence intensities of E-Cadherin (green) and Snail (red) in LNCAP prostate cancer cells untreated or stimulated for 72 hours with control peptide (10 nM) or Y-156-2 peptide (10 nM). Nuclei are shown in blue. (N=3).
B) Representative confocal immunofluorescence images and means ±SEM of fluorescence intensities of Twist (red) in LNCAP prostate cancer cells untreated or stimulated for 72 hours with control peptide (10 nM) or Y-156-2 peptide (10 nM). (N=3).

3.4.5 KiSS1/GPR54 receptor agonist trends to induce E-cadherin expression in prostate cancer.

To confirm the induction of E-Cad protein levels by the KiSS1/GPR54 receptor agonist observed with immunofluorescence, we measured E-Cadherin protein levels using western blotting of LNCAP and PC3. E-Cadherin protein levels increased in response to KiSS1/ GPR54 receptor agonist treatment compared to control peptide and untreated conditions (Figure 3-16).

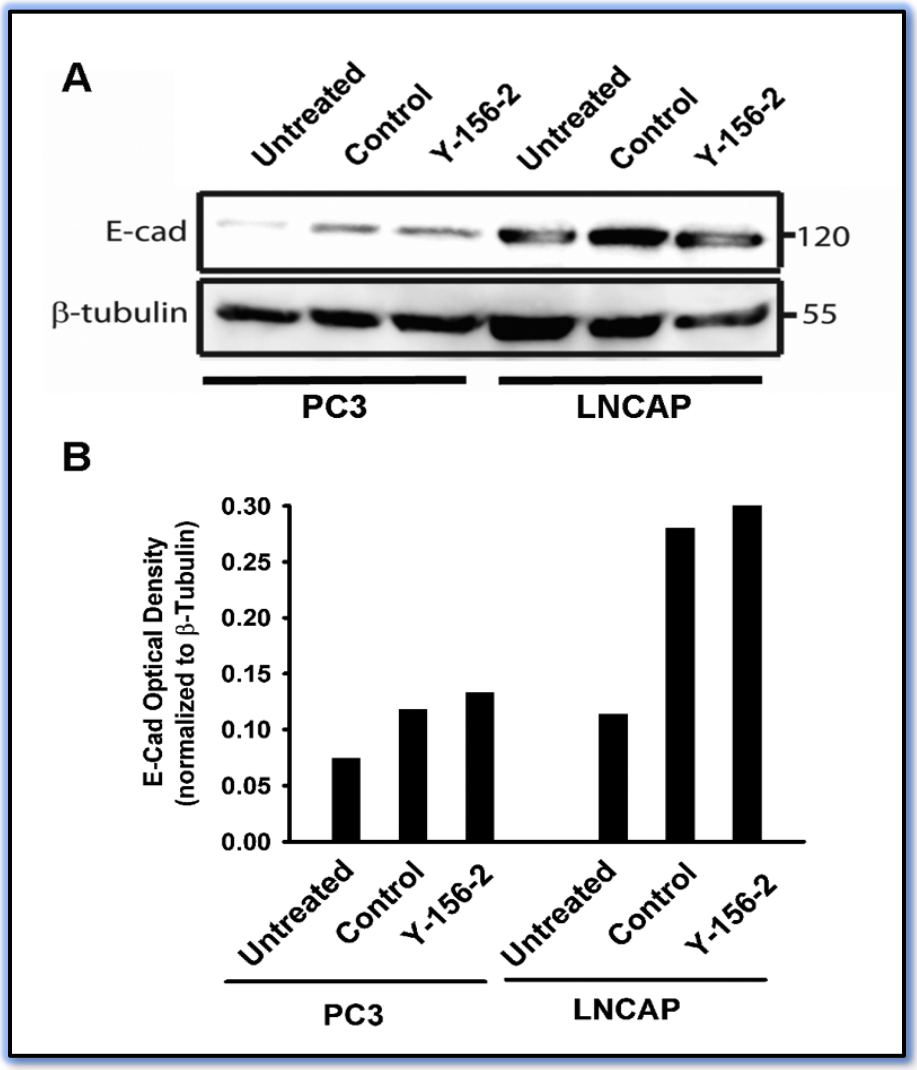


Figure 3-16 KiSS1/GPR54 receptor agonist trends to upregulate E-cadherin levels in prostate cancer cells.

PC3 and LNCAP prostate cancer cells were left untreated or stimulated with control peptide (10 nM) or Y-156-2 peptide (10 nM) for 72 hours. Cells were then lysed, and western blotting was performed for E-cad and β-Tubulin proteins using specific antibodies. Panel A shows E-cad and β-Tubulin immunoblots while panel B shows the optical densities of E-cad normalized for β-Tubulin. N=1.

Chapter Four: DISCUSSION

4.1 Discussion

Despite all new modalities in treating cancer metastasis in recent years, the survival rates of patients with metastasis remain relatively low. Metastasis remains the greatest challenge of managing cancer patients and the driving factor of high mortality rates with approximately 90% of cancer patients dying from complications of metastasis. Many factors contribute to poor outcomes for metastatic cancer patients. Metastasis is a complex process and many factors such as the site and type of the primary tumors and the type of extracellular matrix near the primary tumors play significant roles in the degree of metastasis. The multiple challenges of late-stage clinical trials for testing potential anti-metastatic drugs discouraged the pharmaceutical industry from prioritizing the development of anti-metastatic drugs. Despite the sizeable number of potential anti-metastatic pathways being targeted and the discovery of new drugs that target these pathways, little progress has been made in either treating or avoiding metastasis. Furthermore, redefining the clinical development paradigm requires a high level of cooperation between researchers, drug developers, regulatory agencies as well as statisticians in order to boost the development of high-potential oncology drugs candidates (Fidler and Kripke 2015; Steeg 2016; Vreeland, Clifton, et al. 2016).

To date, around twenty-three genes responsible for metastasis suppression have been identified. Over the years, academic researchers and drug-developing agencies have been working to design drugs to restore the lacking effect of these tumor suppressive genes, with hopes to regenerate their biological and pharmacological functions within cancer cells. One of these potential molecules is the KiSS1 receptor agonist (Y-156-2) that has been designed and developed

by ShangPharma Innovation, Inc. Our team has been working on identifying target genes that promote tumorigenicity or suppress metastasis downstream of TGF-β in breast cancer. In a recent study, we have found that KiSS1 promotes invasive capacity of triple negative breast cancer (Tian, Al-Odaini, et al. 2018). These results contradict original findings and conclusions that KiSS1 acts as a tumor-suppressive gene in melanoma (Lee, Miele, et al. 1996). Our current results, along with previous reports, highlight the complex and context-dependent mechanisms behind the suppressive and promoter effects of KiSS1 on cancer metastasis. Experiments included in this thesis were designed to investigate the effect of the KiSS1 receptor agonist on multiple biological processes of different cancer cells such as cell viability, motility, and EMT. Our current findings suggest that the KiSS1 receptor agonist has no effect on cell viability in four types of cancer cells. This finding is in accordance with previous report of Wang et al. showing that KiSS1 had no effect on cell proliferation in prostate cancer cells (Wang et al. 2012).

We also found that the KiSS1 receptor agonist had variable effects on cancer cell migration. The agonist had no effect on migration of pancreatic cells and triple negative breast cancer cells. The effect of the KiSS1 receptor agonist on prostate cell migration and melanoma cells is highly dependent on the type of the cancer cell line. We observed an inhibitory effect of KiSS1 receptor agonist on migration of A375M cells but no effect on WM1232 cells. Also, we observed an inhibitory effect of KiSS1 receptor agonist on migration of PC3 cells (androgen-insensitive) but no effect on migration of LNCAP (androgen-sensitive) cells. Based on these results, I speculate that in treating prostate cancer patients who have androgen-sensitive cells, a combinatorial therapy involving KiSS1 receptor agonist along with androgen-blocking agents should be considered and may likely prevent the refractoriness (Gleave, Goldenberg, et al. 1998; Ideta, Tanaka, et al. 2008).

We also investigated the effect of the KiSS1 receptor agonist on epithelial mesenchymal transition (EMT) markers. We found that the KiSS1/GPR54 receptor agonist upregulated EMT markers in triple-negative breast cancer (TNBC) such as Twist and Snail1. These results are consistent with our pervious findings that KiSS1 promotes tumor metastasis in TNBC (Tian, Al-Odaini, et al. 2018). Moreover, this finding is in accordance with a study showing that high levels of KiSS1 and its receptor are associated with poor prognosis in breast cancer patients (Marot, Bieche, et al. 2007). Furthermore, KiSS-1 expression has been reported to be elevated in human breast cancer, and is significantly elevated in patients with aggressive tumors and with mortality (Martin, Watkins, et al. 2005). It should be emphasized that the stimulatory effect of the KiSS1 receptor agonist on EMT in breast cancer cells was not applicable to other types of cancers. In fact, we observed inhibitory effects of the KiSS1 receptor agonist on EMT markers including Snail and Twist in pancreatic, prostate and melanoma cancer cells.

In some of the immunofluorescence assays, the number of cells appear to decrease in response to KiSS1 receptor agonist treatment even though this agonist doesn't decrease cell viability. This observation could be attributed to the fact that immunofluorescence measurements were done after three days of peptide exposure while viability measurements were made one day post agonist stimulation. Based on these observations, we propose that in future experiments the effects of KiSS1 receptor agonist on cell viability and cell proliferation should be measured up to three days post stimulation to accurately assess whether this peptide regulates cell viability and proliferation of various cancer cells.

Several reports have investigated the effects of KiSS1 on pancreatic cell viability and migration. For instance, Masui et al. (Masui, Doi, et al. 2004) reported that KiSS1 had no effect on proliferation of PANC-1 pancreatic cancer cells, but significantly inhibited migration of these cells. They suggested this inhibitory effect on migration was mediated by ERK1 pathway activation. It should be noted that PANC-1 cells are overly aggressive and poorly differentiated human pancreatic cancer cells. In addition, Wang and colleagues (Wang, Qiao, et al. 20160) reported that PANC-1 cell lines express relatively low levels of KiSS1 mRNA and protein but relatively high levels of GPR54 compared to highly differentiated BxPc-3 human pancreatic cancer cells. These authors also found that exogenous KiSS1 has no effect on prostate cell proliferation and that over-expression of KiSS1 using an exogenous vector resulted in significant inhibition of invasiveness of PANC-1 cells (Wang, Qiao, et al. 2016). In the current thesis, we only examined the effect of exogenous KiSS1 receptor agonist in two types of highly differentiated pancreatic cancer cells, namely, HPAF2 and BXPC3. We found that KiSS1 receptor agonist had no effect on migration and viability of these cells although cell migration tended to decline in response to 1 nM of KiSS1 receptor agonist (Figure 3-5). The contradictory results of our study with the findings of Masui et al. and Wang and colleagues regarding the effects of KiSS1 receptor agonist on pancreatic cell migration might be related to the type of pancreatic cells under study. In addition, it should be noted that Wang et al. used an exogenous vector to increase KiSS1 levels that might well have been much larger than the 0.1 to 10 nM concentration of KiSS1 receptor agonist used in our study.

Finally, as expected, we found that the KiSS1/GPR54 receptor agonist reduced EMT markers in melanoma cancer cells as indicated by the downregulation of two mesenchymal

markers, Twist1 and Snail. These findings are in accordance with results of Lee et al. (Lee, Miele, et al. 1996) who was the first to report that KiSS1 may act as an inhibitor of metastatic characteristics in melanoma.

4.2 Conclusion

Taken together, the results presented in this thesis indicate important roles of the KiSS1 receptor agonist in cancer metastasis. The KiSS1 receptor agonist has dual contextual roles, whereby it suppresses the mesenchymal phenotype of cancer cells in melanoma, prostate and pancreatic cancer, while it promotes the mesenchymal phenotype in breast cancer. In the light of these results, it will be interesting, in future studies to investigate the potential therapeutic use of such KiSS1 peptide agonists in treating metastatic melanoma, prostate and pancreatic cancers in pre-clinical models of these types of cancer.

4.3 Limitation & areas of improvement

We must point out that we found the negative control peptide (Y-20) to have biological effects in some cell migration and EMT marker assays employed in the current thesis. It is difficult for us to investigate whether these effects are mediated through stimulation of the KiSS1 receptor or through a yet to be determined mechanism. This is due to the fact, that we have limited information about the structure of this peptide due to patent-related issues. Another weakness of our experimental design was the lack of an untreated group in a few of the cell migration experiments. This is due to technical limitations at the time of the experiment, and future work will need to repeat these experiments with the appropriate additional controls. Lastly, I would like to emphasize that the lack of experimental repetitions, particularly related to some of the western

blots and immunofluorescence measurements, are a direct result of my experiments stopping as a result of the COVID-19 pandemic. The laboratory in the Research Institute of McGill University Health Centre was inaccessible to complete these experiments. Future work will be needed to repeat these experiments.

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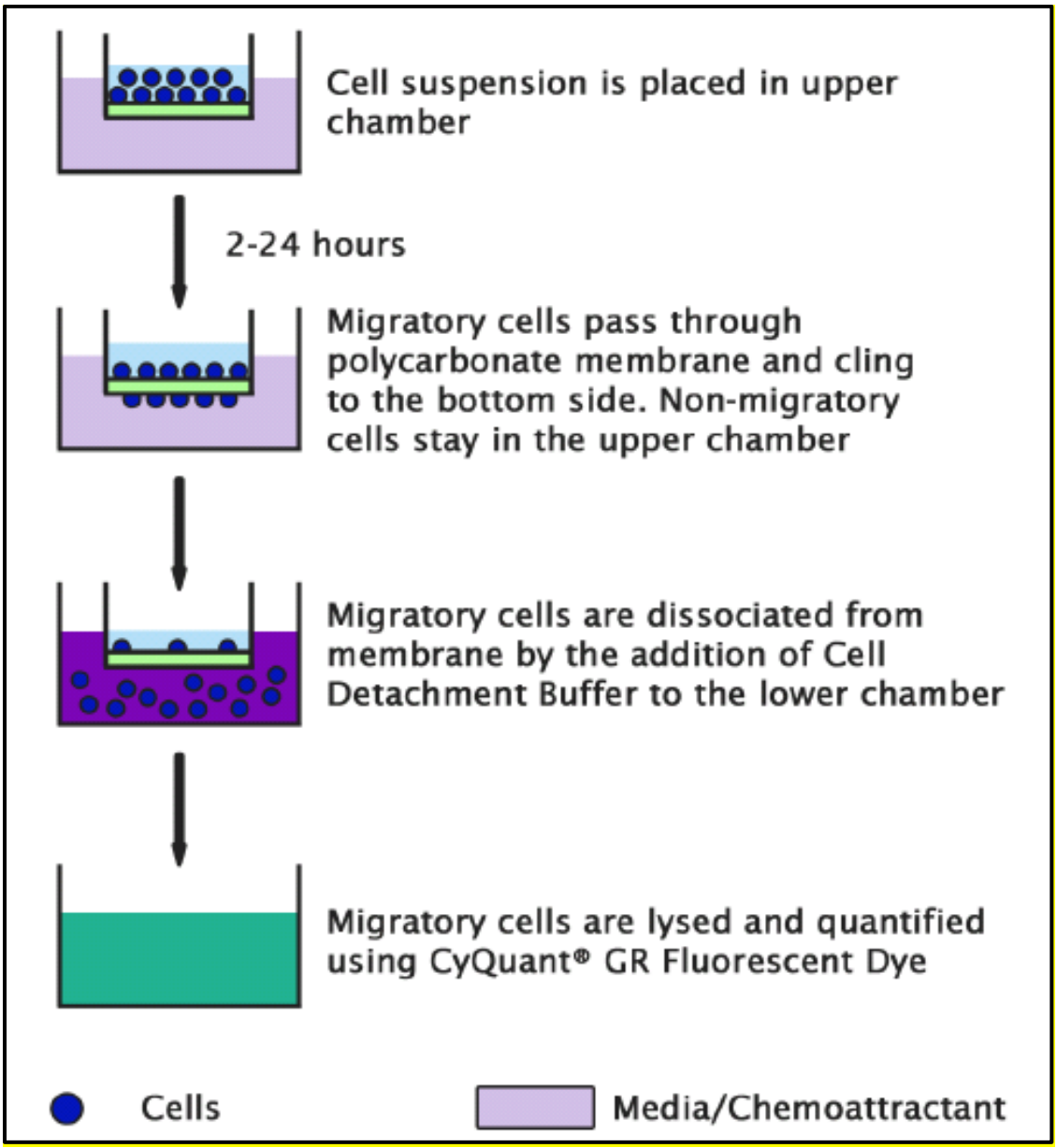
SUPPLEMENTARY MATERIALS

Provided by ShangPharma Inc.

Validation Assays for KissKiSS1 receptor agonist Y-156-2

Migration Assay



Assay Protocol

1. Allow the 96-well Migration Plate to warm up at room temperature for 10 minutes.
2. Prepare a cell suspension containing 0.5-5.0 x 10^6 cells/ml in serum free media. Agents that inhibit or stimulate cell migration can be added directly to the cell suspension.
   (Note: Overnight starvation may be performed prior to running the assay)
3. Under sterile conditions, separate the cover and membrane chamber from the 96-well Migration Plate.
4. Add 150 µL of media containing 10% fetal bovine serum or desired chemoattractant(s) to the wells of the feeder tray.
5. Place the membrane chamber back into the feeder tray (containing chemoattractant solution). **Ensure no bubbles are trapped under the membrane.**
6. Gently mix the cell suspension (without chemoattractant) from step 2 and add 100 µL to the membrane chamber.
7. Finally, cover the plate and transfer to a cell culture incubator for 2-24 hours.
8. Just prior to the end of the incubation, pipette 150 µL of prewarmed Cell Detachment Solution into wells of the clean, 96-Well Cell Harvesting Tray (provided).
9. Carefully remove the 96-well Migration Plate from the incubator. Separate the membrane chamber from the feeder tray.
   Note: Retain the feeder tray for step 12.
10. Remove the cells/media from the top side of the membrane chamber by aspirating or inverting. Place the membrane chamber into the Cell Harvesting Tray containing 150 µL of Cell Detachment Solution (step 8). Incubate 30 minutes at 37°C.
11. Completely dislodge the cells from the underside of the membrane by gently tilting the membrane chamber several times in the Cell Detachment Solution.
12. In a clean 96-well plate (not provided), combine 75 µL of media from the feeder tray (step 9) with 75 µL of the detachment solution (step 11).
13. Prepare sufficient 4X Lysis Buffer/CyQuant® GR dye solution for all samples by diluting the dye 1:75 in 4X Lysis Buffer (for example, add 5 µL dye to 370 µL of 4X Lysis Buffer).
14. Add 50 µL of 4X Lysis Buffer/CyQuant® GR dye solution to each well (already containing 150 µL of Cell Detachment Solution). Incubate 20 minutes at room temperature.

15. Transfer 150 µL of the mixture to a 96-well plate suitable for fluorescence measurement. Read the fluorescence with a fluorescence plate reader at 480 nm/520 nm.

Primary Assay

Beta Lactamase Reporter Assay using CHO-K1 cells expressing *h1R*

Agonist Assay Quick Reference Guide

	Unstimulated Wells	Stimulated Wells	Cell-free Wells	Test Compound Wells
Step 1 Plate cells, incubate	32 µl cells in Assay Medium (10,000 cells/well)	32 µl cells in Assay Medium (10,000 cells/well)	32 µl Assay Medium (no cells)	32 µl cells in Assay Medium 10,000 cells/well)
	Incubate cells for 16-20 hrs. at 37°C/ 5%CO_2			
Step 2 Change Media	Remove Plating Medium and replace with 32 µL of Assay Medium.			
Step 3 Add Agonist or Test Compounds	8 µl Assay Medium with 0.5% DMSO	8 µl 5X agonist in Assay Medium with 0.5% DMSO	8 µl Assay Medium with 0.5% DMSO	8 µl 5X Test Compounds in 0.5% DMSO
Step 4 Incubate cells	Incubate in a humidified 37°C/5% CO_2 incubator for 4 hours			
Step 5 Prepare 6X Substrate Mix	6 µl of 1 mM LiveBLAzer™-FRET B/G (CCF4-AM) Substrate + 60 µl of solution B, mix. Add 904 µl of Solution C, mix. Add 30 µl of Solution D, mix.			
Step 6 Add Substrate Mixture	8 µl per well			
Step 7 Incubate Substrate Mix. + cells	2 hours at room temperature in the dark			

KiSS1R Agonist Validation Assays

Table 2: Summary of KISS Receptor Agonist Validation Assays

Study	Result	Comments
Primary Assay	EC50 = 0.33 nM	Beta lactamase reporter assay with CHO-K1 cell line expressing hKiss1R
Secondary Assay	IC50 = 0.008 nM	Cell migration assay; GPR54-expressing CHO cells
Plasma Stability (Rat, Mouse, Human)	T1/2 = 44.38 hr; 28.84 hr; 115.80 hr	
Plasma Protein Binding (Fraction Unbound)	0.6 % unbound	
PAMPA; Log D	LogPe = -8.86; Log D = 3.42	LogPe value <-5 shows low permeability
HLM Metabolic Stability	T1/2 = 21.83 min; Cl = 79.64 mL/min/kg)	Prone to liver metabolism
Solubility of TFA Salt Form (0.9% Nacl; 5% Glucose; Water)	0.63 mg/mL; 21.17 mg/mL; 27.0 mg/mL	
Mouse PK	T1/2 = 2.99 hr; CL = 0.0688 L/h/kg; urine fraction 0.167	IV dose at 1 mg/kg in male C57BL6 mice; low kidney excretion
In Vivo Efficacy	TGI = 75% w/80 nmol/kg TID and TGI = 85% w/160 nmol/kgTID ; significant tumor weight reduction	Good anti-tumor efficacy with 80 nmol/kg and 160 nmol/kg TID dosing in s.c. x1LnCaP mouse efficacy model