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The Tumor-Suppressive Role Of Secreted Maspin Via The Exosomes

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A TUMOR-SUPPRESSIVE ROLE OF MASPIN SECRETED VIA THE EXOSOMES

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DEDICATION

This dissertation is specifically dedicated to my nephew, Jarrius, and my little brother, Nicholas. I hope I have been a positive example for you. I hope this inspires you to grow up to be positive, respectable, educated, successful men. Jarrius, you have a great spirit and kind heart. I love listening to you speak passionately about what you want to do when you grow up. I hope you achieve your goals. Do not let anyone stop you. As your mother has told you many times, you are my mini-me, which gives me confidence that you will fare well in this big and crazy world. Nicholas, I am so happy to have you be a part of my life. You are the definition of a fighter. From the moment you were born, you were determined to survive. I fell instantly in love with you the moment I saw you.

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PREFACE

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CHAPTER 1: Introduction and Hypothesis

Cancer Statistics

Prostate and breast cancers are the most commonly diagnosed cancers among men and women in the United States, respectively, and are ranked second for the highest death rates.[1] Lung cancer is the second most commonly diagnosed cancer but has the highest death rate compared to all other cancers.[1] When broadened globally, breast cancer is remains the most commonly diagnosed cancer in women, but increases in rank as the leading cause of cancer deaths.[2] This trend is mimicked in both developed and developing countries. Worldwide, prostate cancer falls to the second most commonly diagnosed cancer among men and is the sixth cause of cancer deaths.[2] Prostate cancer incidence further drops to the sixth most commonly diagnosed in developing countries, and it remains the sixth cause of cancer death. In contrast, prostate cancer is the most commonly diagnosed cancer in developed countries but is the third cause of cancer deaths among men. Globally, lung cancer remains the leading cause of cancer deaths in men but is the second leading cause of cancer deaths among women.[2] In incidence, lung cancer is the most commonly diagnosed in developing countries and is the second most commonly diagnosed in developed countries among men. For women, lung cancer ranks third in diagnosis for both developed and developing countries. Of note, these statistics exclude basal and squamous cell skin cancers.

Clinicians evaluate cancers using the TNM (tumor, node, and metastasis) staging system established by The American Joint Committee on Cancer (AJCC). Under the TNM system, the degree of local invasion is assessed in the primary tumor (T), the

presence or absence of local metastasis is assessed in the lymph nodes (N), and the presence or absence of distant metastasis is evaluated in the organs (M).[3] The combination of T, N and M is used to assign the tumor a stage (0 – 4). In prostate cancer, clinicians also use the Gleason grading system to establish a tumor grade to determine the aggressiveness of the tumor.[4] The Gleason grade is determined from the histology of prostate cancer tissue samples. The prognostic value of stage and grade are helpful, but has its limitations.

The Tumor Microenvironment: An Unhealable Wound

Cancer is a general term for a heterogeneous disease, in which the malignancy predominantly stems from metastasis.[5] Metastasis is characterized by the migration of aggressive tumor cells from one organ to another. In the continuum of metastasis, the vascular and lymph networks provide the means for tumor cells to escape their original microenvironment and colonize distant organs. Interestingly, metastasis does not seem to be a random process. Metastatic tumors undergo organ-specific metastasis, creating a pattern for metastasis.[6,7] For example, negative breast cancer cells often metastasize to the liver, bone, and brain.

Aggressive tumor cells often infiltrate nearby areas by disrupting the homeostasis of the microenvironment through the degradation of the extracellular matrix (ECM).[8,9] Extracellular matrix homeostasis is tightly maintained in the normal microenvironment during development and other physiological or non-malignant pathological processes. During wound healing in the normal microenvironment, platelets aggregate to form a plug and a fibrin clot is formed. Macrophages infiltrate the local area and secrete growth factors, which activate stromal cells such as fibroblasts. Fibroblasts secrete matrix

metalloproteases (MMPs), which degrade the ECM, and differentiate into myofibroblasts to facilitate wound contraction. Vascular endothelial cells are stimulated to undergo angiogenesis. Finally, epithelial cells increase cell motility in order to reform tissue. In contrast, homeostasis is disrupted in the tumor microenvironment with increased degradation of the ECM. Continuous degradation of the ECM promotes tumor invasion and metastasis. This phenomenon is analogous to an unhealable wound.[10] Similar to wound healing, tumor cells secrete growth factors that activate the stromal cells in the tumor microenvironment. Fibroblasts differentiate into myofibroblasts and vascular endothelial cells are induced to undergo angiogenesis. Tumor-associated reactive stromal cells are induced to constitutively secrete pro-collagen. Fibroblasts and macrophages secrete proteases, such as MMPs, which degrade the ECM. Excessive production of proteases at the invasive front prevents the collagen deposit from mounting to a physical barrier and forming scar tissue.[9,10] The imbalance between ECM deposition and ECM degradation gives the tumor microenvironment the characteristics of an unhealable wound.

Maspin: A Serine Protease Inhibitor

The discovery of maspin, through subtractive hybridization in nonmalignant and malignant breast cell lines, initiated the research of maspin as a tumor suppressor.[11] Maspin, a 42 kDa protein, is a clade B member of the serine protease inhibitor (serpin) superfamily. Hence, maspin (mammary serine protease inhibitor) is alternatively named SERPINB5. Maspin maps to chromosome 18q21.3 and shares high sequence homology with other serpins, such as equine neutrophil-monocyte elastase inhibitor

(43%), human neutrophil-monocyte elastase inhibitor (39%), human squamous cell carcinoma antigen (34%), human plasminogen activator inhibitor 2 (PAI-2; 31%), and chicken ovalbumin (31%).[11]

The tertiary structure of maspin consists of three anti-parallel β-pleated sheets and nine α-helices.[12] The reactive site loop (RSL), a common serpin structural feature, is located between the A and C β-pleated sheets. The RSL of maspin contains an arginine residue at its p_1 site, suggesting it is an arginine-specific protease inhibitor.[13] However, maspin's RSL does not undergo the stressed to relaxed transition, which is important for classic serpin inhibition. Unlike classical inhibitory serpins, the RSL of maspin does not have a conserved hinge region.[11] Typically, the RSL of an inhibitory serpin is cleaved and inserted into the center of the β-pleated sheet after the serpin has docked to its target, which creates an additional β-strand. Although maspin's RSL does not act like an inhibitory serpin, the RSL is necessary for maspin's tumor suppressive activity.[11,13] Immediately preceding the putative p_1p_1' site of maspin is a KDEL (lysine-aspartate-glutamate-leucine) sequence, which seems to be necessary in determining the subcellular localization and tumor suppressive function of maspin.[14] Out of the nine helices, the G-helix of maspin is able to undergo a novel and unprecedented conformational change, in which the G-helix switches between an open and a closed conformation.[12] Ravenhill *et al* (2010) showed that maspin modulates cell adhesion and migration via its G-helix.[15]

Maspin's expression is regulated by methylation for epithelial-specific expression in normal tissues. In non-epithelial tissues, the maspin gene is silenced through methylation.[16] Of note, maspin expression has been detected *in vitro* in human umbilical

vein endothelial cells (HUVEC) and prostate smooth muscle cells.[17,18] Therefore, maspin expression in non-epithelial cells may be due to *in vitro* cell culture conditions.

The Tumor-Suppressive Role of Maspin

Maspin is a class II (epigenetically-silenced) tumor suppressor gene. Clinical samples of normal epithelium tissues show that maspin is mainly expressed in the nucleus.[11] Maspin knockout was demonstrated to be embryonically lethal in an *in vivo* mouse model.[19] However, a recently published study reported viable embryos from maspin conditional knockout mouse models.[20] Teoh *et al* (2014) asserted that the neo selection cassette that was left in the maspin gene of the maspin knockdown mouse model of Gao *et al* (2004) may have resulted in the embryonic lethality. Furthermore, Teoh *et al* (2014) asserted that their data supports their hypothesis that maspin is not a tumor suppressor since they could not reproduce the results previously published by others. Recent evidence in the Sheng Laboratory may confirm that maspin is not embryonically lethal (unpublished observation). The Sheng laboratory will continue to investigate the embryonic lethality of maspin. Additionally, there is a wide array of data in the literature that supports the notion that maspin is a tumor suppressor. The Sheng laboratory and others have independently verified that maspin acts as a tumor suppressor.

In tumor cells, maspin was shown to be epigenetically silenced by aberrant (hyper)-methylation.[21,22] At the transcriptional level, maspin expression was shown to be positively regulated by the Ets element, which is active in normal epithelial cells, but inactive in tumor cells, of both breast and prostate.[23,24] In normal mammary epithelial

cells, the AP-1 element cooperates with the Ets to positively regulate maspin expression.[23] In normal prostate and prostate cancer cells, maspin expression was negatively regulated by the hormone response element (HRE), which was targeted by the androgen receptor but no other steroid receptor.[24] Of note, androgen ablation was shown to induce maspin promoter activity in prostate cancer cells.[25] The transcription factor, E2F1, was shown to mediate the up-regulation of the maspin gene in osteosarcoma cells.[26] Furthermore, re-expression of wild-type p53 was shown to activate maspin expression by binding directly to the p53 consensus-binding site present in the maspin promoter in both breast and prostate cancer cells.[27,28]

The loss of maspin has been associated with increased malignancy, with a less differentiated phenotype, and with increased angiogenesis. [11,25,29-31] Re-expression of maspin in breast and prostate cancer cells has been shown to push the cancer cells toward a more epithelial-like state *in vitro* and *in vivo*.[32-35] The better-differentiated phenotype was concurrent with decreased tumorigenicity, organized cell-cell interactions and concerted extracellular matrix remodeling. Maspin transfected prostate and breast carcinoma cells showed decreased tumor invasion and motility *in vitro*.[11] In *in vivo* mouse models of breast, prostate and lung cancers, tumors derived from maspin transfected cancer cells show decreased tumor growth and metastasis.[11,33,36] Additionally, maspin-expressing tumors showed a decrease in *in vivo* angiogenesis.[33,36] Re-expression or up-regulation of maspin has been shown to increase sensitivity to drug-induced apoptosis in breast and prostate cancer cells.[37-40]

Evidence suggests that secreted maspin acts as a tumor suppressor. Using recombinant maspin (rMas) to mimic secreted maspin, the Sheng laboratory has shown

that purified exogenous maspin inhibits prostate tumor cell invasion and motility *in vitro*.[41,42] Furthermore, rMas acts at the cell surface to retard cell detachment by altering the cellular distribution of phospho-FAK, and inhibit prostate tumor cell invasion and motility [32]. Treatment of breast cancer cells with rMas decreased *in vitro* invasion through basement membrane, which could be negated with anti-maspin antibody.[43] It was shown that subsequent internalization of rMas by breast cancer cells inhibits cell motility through inhibition of Rac signaling and promote cell adhesion through phosphoinositide 3-kinase (PI3K) signaling.[44] Additionally, rMas has been shown to exert an inhibitory effect on human umbilical vein endothelial cell (HUVEC) migration, strengthen HUVEC adhesion, and inhibit neovascularization.[17,36] The data generated using rMas provides a basis for a tumor suppressive role of secreted maspin.

Consistent with the observation that maspin localizes to the nucleus, cytoplasm and cell surface, maspin has been shown to interact with proteins in these subcellular compartments. In the nucleus, maspin has been shown to inhibit histone deacetylase 1 (HDAC1) in the nucleus.[34] In the cytoplasm, maspin has been shown to interact with several proteins, such as interferon gamma 6 (IRF6), heat shock protein 90 (hsp90) and glutathione-S-transferase π (GSTπ).[45,46] At the cell surface, extracellular maspin blocks the pericellular proteolysis mediated by urokinase plasminogen activator (uPA) at the cell surface of prostate cancer cells.[41,42] Interestingly, maspin partitions to those subcellular compartments despite the absence of any specific signal sequence. Therefore, the tumor suppressive activity of maspin may depend on maspin's subcellular localization and binding partners.

Maspin may function as a tumor suppressor through its inhibition of the activation of uPA, whose activation can initiate a proteolytic cascade by activating plasminogen to generate plasmin, a serine protease that activates many proteases and degrades non-fibrillar extracellular matrix (ECM). Urokinase plasminogen activator is secreted as an inactive zymogen, pro-uPA, which binds to its cell surface-anchored receptor, urokinase plasminogen activator receptor (uPAR), and undergoes specific proteolytic cleavage into an active two-chain serine protease. Extracellular uPA may remain bound to uPAR or may dissociate to become a soluble protein. Maspin was shown to have an affinity for uPAR-bound uPA, but not soluble uPA.[41,42] Furthermore, maspin has greater affinity for pro-uPA over active uPA.[32] Binding between maspin and uPA was shown to occur via their exosites.[47] Given that pro-uPA has been shown to act as a signaling ligand, maspin's interaction with pro-uPA may result in greater inhibiting capacity than maspin's interaction with uPA. Maspin has been shown to promote internalization of the uPA/uPAR complex via low density lipoprotein receptor-related protein-1 (LRP-1), which maybe one mechanism by which maspin may prevent ECM degradation and the proteolytic cascade as a tumor suppressor.[32]

High uPA expression is associated with a low disease-free and overall survival rate among breast cancer patients.[48] The tumor-promoting activity of uPA seems to depend on its ability to activate plasminogen, leading to the generation of plasmin. In addition to maspin, plasminogen activator inhibitor type I (PAI-1) is an inhibitor of uPA Interestingly, PAI-1 is up-regulated in many types of cancer and is associated with a low disease-free and overall survival rate. Maspin however is associated with better overall survival in many cancer types. Of note, uPA and its proteolytic target, plasmin, do not

directly cleave the fibrillar collagen matrix. However, they may degrade newly synthesized pro-collagen prior to its assembly into the fibrillar matrix. Additionally, maspin may prevent collagen I degradation by inhibiting uPA-mediated activation of plasminogen and, thus, pro-collagen I degradation.

The tumor suppressive activity of maspin in the nucleus may occur, in part, through inhibition of HDAC1, which was identified as an interacting partner of maspin through a yeast two-hybrid screen.[34] The Sheng laboratory has shown that maspin-mediated inhibition of HDAC1 leads to epigenetic changes and renewed expression signatures for a better-differentiated phenotype.[34,35,49,50] Of the genes down regulated, many were pro-tumor and of the genes up regulated, many were antitumor. Also, our lab has shown that a single point mutation of the aspartate to glutamate in the KDEL sequence (amino acid residue 346) resulted in a predominantly nuclear localization and stronger HDAC1 inhibition in comparison to the wild-type maspin.[14] Consistently, forced exclusion of maspin from the nucleus, using a nuclear exclusion signal, diminishes maspin-mediated inhibition of growth and metastasis of breast cancer cells.[51] Thus far, maspin is the only identified endogenous polypeptide inhibitor of HDAC1. Maspin's interaction with HDAC1 may be a mechanism by which nuclear maspin acts as a tumor suppressor.

Clinical Studies on Maspin's Expression

Using NCBI GEO microarray database, the Sheng laboratory observed that maspin mRNA expression was the highest in prostate hyperplasia and lowest in primary prostate cancers during tumor progression (unpublished data). At the protein level, maspin expression is up-regulated in pre-neoplastic lesions, which is consistent with the

NCBI GEO data.[29] Additionally, during tumor progression, the nuclear/cytoplasmic balance first shifts to a greater cytoplasmic expression.[29,52,53] Eventually, maspin expression is down regulated and often lost in invasive and metastatic carcinomas.[11,29,52] Maspin expression predicts a better prognosis for many cancers. Of note, high nuclear expression of maspin in patients showed better prognosis in comparison with high cytoplasmic maspin expression.[52,54,55] For example, nuclear expression of maspin is associated with a lower recurrence rate in squamous cell carcinoma of the larynx.[56]

Exceptions were reported with ovarian, gastric and thyroid cancers, where maspin expression correlates with a poor prognosis. In ovarian cancer, although overall survival was worse in patients with maspin expression, survival was greater when maspin is localized to the nucleus than when maspin is localized to the cytoplasm.[54] Similarly, in gastric cancers, patients with nuclear maspin showed better response to drug treatment with better overall survival than patients with cytoplasmic expression, although overall survival was worse in patients with maspin expression.[57] Strong cytoplasmic and weak nuclear maspin expression has also been noted in pancreatic cancer tissue specimens.[58,59] In contrast to other cancers, nuclear staining of maspin was not associated with better prognosis in pancreatic cancers, yet tissues with nuclear maspin staining were better differentiate.[59] In thyroid cancer, there are conflicting reports correlating maspin expression with prognosis. Bal *et al* (2008) reported strong cytoplasmic expression of maspin and completely negative nuclear expression of maspin in immunohistological stains of thyroid tissues.[60] The strong cytoplasmic expression of maspin was correlated with an adverse prognosis.

Interestingly, maspin is weakly expressed or not expressed in the normal tissues of the ovaries, pancreas and thyroid, yet maspin expression is high, and mostly cytoplasmic, in the tumor tissue.[54,58,60-62] Maspin may serve as an adverse prognostic indicator for ovarian, pancreatic and thyroid cancers.[54,63] The prognostic differences of maspin among cancers may reflect the cell origins of the tumor.

The Enigmatic Secretion of Maspin

Although one report described evidence that maspin is localized exclusively to the intracellular compartment [64], the compilation of published data highlights a majority consensus that maspin can act extracellularly to exert tumor suppressor activity. Upon initial discovery, immunohistochemical analysis revealed maspin protein within the luminal ducts of breast tissue, suggesting that maspin is secreted.[11] Additionally, maspin was reported to partition into secretory vesicles.[61] Maspin has been consistently detected in the conditioned media (CM) of maspin-expressing cells of normal and tumor breast, prostate and lung origin. Furthermore, extracellular maspin has been demonstrated to have anti-tumor effects.[32,42,65,66] Secreted maspin from maspin transfected prostate cancer cells inhibited degradation of collagen I (Col I).[33] Secreted maspin, derived from the CM of normal myoepithelial cells, was shown to inhibit breast carcinoma invasion without an effect on proliferation.[65] Secreted maspin was shown to exhibit anti-invasive and anti-angiogenic activity.[66,67] Either the addition of a maspin antibody into the CM or the depletion of maspin from the CM was able to abrogate invasion. The Sheng laboratory demonstrated that maspin associates with the uPA/uPAR complex at the cell surface, which may inhibit pericellular proteolysis.[42]

Taken together, the previous data suggest that secreted maspin acts in a paracrine manner to inhibit invasion and metastasis (regulate the tumor microenvironment).

The mode of maspin secretion is enigmatic because maspin's genetic sequence does not contain a hydrophobic N-terminal secretory signal peptide (SSP), which is an important feature for classical secretion of proteins.[11] Classical secretion requires an hydrophobic SSP at the N-terminus of the protein and are dependent on the endoplasmic reticulum (ER)-Golgi secretory pathway for expulsion from the cell.[68] Proteins with the SSP are trafficked to the ER and through the Golgi apparatus, packaged into secretory vesicles and are then secreted when the secretory vesicles fuse to the plasma membrane. The SSP is sequence motif that is analogous to an address that directs the protein to its destination. Each organelle has a specific sequence motif that directs proteins to that organelle. The location of the motif on the protein is also unique to that motif. For example, the SSP motif is located at the N-terminus of ER-Golgi dependent secreted proteins. Proteins without a localization signal sequence become cytoplasmic proteins by default. Not all secreted proteins contain the SSP and do not depend on ER-Golgi secretion pathway for expulsion from the cell. Secreted proteins that lack the SSP rely on non-classical secretory pathways, such as microvesicle shedding, exosome secretion, or direct translocation across the plasma membrane.

Exosomal Maspin: A New Frontier for the Tumor Suppressor

Interestingly, maspin has been reported as cargo of the exosomes, which highlights one of the secretion mechanisms of maspin. Maspin has been reported in the

exosomes of irradiated H460 lung adenocarcinoma cells, and maspin was absent from exosomes of the control cells (non-irradiated H460 lung adenocarcinoma cells).[69] In another publication, maspin was present in the exosomes of undifferentiated keratinocytes and absent in differentiated keratinocytes.[70] However, the authors reported the apparent molecular weight of maspin as 25 kDa in their proteomic analysis, instead of 42 kDa, which is the molecular weight of maspin.

The exosomes are cholesterol-rich and sphingomyelin-rich bilayer vesicles. They are packaged with biologically active mRNA, miRNA and proteins.[71-73] Additionally, DNA has also been identified in exosomes.[74,75] The exosomes range from 30 – 120 nm in size and are secreted upon the fusion of the endosomes to the plasma membrane.[71,73] The biogenesis of the exosomes starts with the formation of intraluminal vesicles (ILVs) from the invagination of the limiting membrane of the endosome.[76] The ILVs are termed exosomes once they are released into the extracellular space.

Interestingly, ILVs can fuse back to the endosome membrane, which is termed back-fusion. ILV formation, scission and back-fusion occur through endosomal sorting complexes required for transport (ESCRT) protein activity.[77] The ESCRT proteins also participate in the recruitment of cargo to the ILVs. The ESCRT proteins that are responsible for recruitment of cargo recognize ubiquitinated proteins.[77,78] Once encapsulated, proteins are de-ubiquitinated by de-ubiquitinating ESCRT proteins. If maspin is a bona fide cargo of the exosome, then the recruitment of maspin may depend on a particular set of ESCRT proteins.

Exosomes have been shown to mediate paracrine signaling. The mechanisms are unclear but there are three different hypotheses.[79] One hypothesis highlights the

receptor mediated interaction at the cell surface. Exosomes contain transmembrane proteins, as well as luminal proteins. Some of the transmembrane proteins may act as ligands that interact with cell surface proteins to initiate cell signaling. Another hypothesis focuses on the endocytosis of exosomes. Endocytosis of the exosomes may be initiated when the exosome docks to the cell surface. The exosomes may have certain transmembrane proteins for docking to the cell surface. It is also hypothesized that the exosomes may fuse to the plasma membrane. The exosome, like the plasma membrane, is a lipid bilayer. As a structure formed from the endosome limiting membrane, the exosome ultimately has a plasma membrane origin.

Exosomes are secreted by many cell types, such as immune cells, stem cells, endothelial cells and cancer cells.[80-83] Initial reports of exosomes claimed exosomes expelled unwanted cellular material.[84] Now, it is clear that exosomes are involved in important cellular physiology. As secreted extracellular vesicles, the exosomes can be used in paracrine signaling to regulate various biological processes. The function of the exosome depends on the molecular content, which is determined by the cell of origin. Under normal physiology, the exosomes have been shown to regulate biological processes, such as reticulocyte maturation, modulation of T cell signaling during pregnancy, and reprogramming hematopoietic progenitors.[85-87] In certain diseased states, exosomes have been shown to functions as positive mediators for processes, such as cardiac repair.[88] Exosomes may have prognostic value in evaluating preeclampsia during pregnancy.[89] Exosomes have also been shown to induce cytokine secretion in macrophages.[90] Exosomes may serve as prospective diagnostic markers of different diseased states, such as Alzheimer disease and ischemia/reperfusion.[91,92]

Furthermore, the exosomes may target either the local microenvironment or the distant microenvironment.[79]

Exosomes in Cancer

Tumor-derived exosomes have been shown to function as negative mediators to promote tumor activity, such as enhancing endothelial cell migration, promoting immune cell evasion, priming the metastatic niche, and increasing angiogenesis.[93-99] Melanoma-derived exosomes were shown to educate bone marrow progenitor cells toward pro-metastatic phenotype.[93] Prostate cancer-derived exosomes were shown to mediate $CD8^+$ T-cell apoptosis.[94] $CD8^+$ T cells can also be induced to suppress immune response by $CD4^+$ T cell-derived exosomes.[100] Transport of miR-150 monocyte-derived exosomes was shown to enhance endothelial cell migration.[95] $CD81^+$ exosomes secreted by cancer-associated fibroblasts were shown to stimulate breast cancer cell protrusive activity, motility and metastasis.[97] Tumor-derived exosomes were shown to communicate with immune cells to induce immune suppression.[99,101] Tumor cells were shown to respond to an acidic environment by increasing the secretion of exosomes.[102]

Hypothesis

To understand how different forms of secreted maspin may contribute to tumor suppression, it is critical to understand how maspin is regulated at the step of trafficking. The focus of my dissertation is to understand how maspin trafficking is regulated and how exosomal maspin affects tumor cell biology. The central **hypothesis** states that exosomal maspin may contribute to the tumor suppressive activity and may be

differentially regulated during tumor progression. In order to address the hypothesis, two specific aims were developed. Specific Aim 1 is to investigate the differential regulation of exosomal maspin in non-malignant cells and tumor cells. Specific Aim 2 is to investigate the biological function of exosomal maspin in the microenvironment. The results may provide novel insights into how the tumor microenvironment can be modulated with maspin-based therapies.

Chapter 2: Materials and Methods

Cell Lines, Cell Culture Media and Antibodies

The CRL2221 cell line is a non-malignant prostate cell line. The LNCaP cell line is a locally metastasized prostate cancer cell line collected from the lymph node. The PC3 cell line is a metastasized prostate cancer cell line collected from the bone. The MCF10A cell line is a spontaneously immortalized breast cell line. The SUM149 is a triple negative breast cancer cell line lacking the estrogen receptor (ER), progesterone receptor (PR) and Her2 receptor. The SUM159 is breast cancer cell line that does not express maspin. The BEAS-2B cell line is a non-malignant lung cell line. The NIH 3T3 mouse fibroblast cells are non-malignant stromal cells and were a gift from Dr. Hyeong-Reh Kim. CRL2221, PC3, LNCaP and BEAS-2B were purchased from American Type Culture Collection (Manassas, VA). The MCF10A cell line was a gift from Dr. Fred Miller. The SUM149 and SUM159 breast cancer cell lines were a gift from Dr. Stephen Ethier. LNCaP and PC3 cell cultures were maintained in RPMI 1640 medium supplemented with 5% v/v fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, L-glutamine (L-Glu; 2 mM), Hepes (10 mM), $NaHCO_3$ (1.5 mg/mL) and non-essential amino acids (NEAA; 1 mM). CRL2221 cell cultures were maintained in keratinocyte serum-free medium (KSFM) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin; additional supplements were provided with the KSFM. The MCF10A and NIH 3T3 cell cultures were maintained in DMEM/F-12 medium supplemented with 5% v/v FBS, L-Glu (2 mM), 100 U/mL penicillin,100 µg/mL streptomycin, amphotericin B (0.5 µg/mL), cholera toxin (100 ng/mL), hydrocortisone (HC; 1 µg/mL), epithelial growth factor (EGF; 10 ng/mL), and insulin (5 µg/mL). SUM149

and SUM159 cell cultures were maintained in Ham's F-12 medium supplemented with 10% v/v FBS, 100 U/mL penicillin,100 µg/mL streptomycin, insulin (5 µg/mL), and HC (1 µg/mL). BEAS-2B cell cultures were maintained in LHC-8 medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin.

The stable transfected cell lines, Neo and M7, were derived, by our laboratory, from the DU145 cell line, which is a metastasized prostate cancer cell line collected from the brain. The DU145 cells were purchased from American Type Culture Collection (Manassas, VA). Neo was transfected with an empty vector. M7 was transfected with a plasmid containing maspin. Neo and M7 cells are maintained in RPMI 1640 supplemented with 5% v/v fetal bovine serum, $NaHCO_3$ (1.5 mg/mL), G418/geneticin (0.3 mg/mL), 100 U/mL penicillin,100 µg/mL streptomycin, L-Glu (2 mM), Hepes (10 mM), and NEAA (1 mM).

All cell growth media, L-Glu, penicillin-streptomycin, Hepes, NEAA, and EGF were purchased from Life Technologies (Gaithersburg, MD). G418, $NaHCO_3$, insulin, hydrocortisone, amphotericin B and cholera toxin were purchased from Sigma-Aldrich (St. Louis, MO). All cell cultures were cultured in a humidified incubator at 37 °C with 5% CO_2.

Maspin mouse monoclonal antibody was purchased from BD Biosciences (San Jose, CA). Tsg101 mouse monoclonal antibody, LAMP-2 mouse monoclonal antibody, β-tubulin rabbit polyclonal antibody, PARP mouse monoclonal antibody, and GAPDH mouse monoclonal antibody were purchased from Abcam (Cambridge, MA). Alix mouse monoclonal antibody and Hsp90 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Dallas, Texas). Ribosomal L26 mouse monoclonal antibody and

Hsp70 rat monoclonal antibody were purchased from Cell Signaling (Danvers, MA). Anti-mouse secondary antibody and anti-rabbit polyclonal secondary antibody were purchased from GE Healthcare (Buckinghamshire, UK).

Exosome Isolation

Cells were cultured in 10 mm cell culture dishes. At seventy percent confluence, culture media was removed and cells were washed thrice with PBS. Cells were incubated in defined keratinocyte serum-free media (DKSFM) purchased from Life Technologies (Gaithersburg, MD) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin; additional supplements were provided with the DKSFM. After 24 h, the conditioned media (CM) was collected and centrifuged at 4,000 x g for 10 min, at room temperature, to pellet dead cells and cell debris. The supernatant (CM) was concentrated with 15 mL Millipore 10 kDa centrifugal filter units (Darmstadt, Germany) until the CM reached the desired volume of 1 mL (100X concentrated). The CM was collected and centrifuged at 16,000 x g for 30 min at 4 °C to deplete the CM of high molecular weight microvesicles. To isolate exosomes, the CM was centrifuged at 4 °C and 100,000 x g for 24 h. The supernatant was saved and labeled vesicle-depleted conditioned media (VDCM). The protein concentration of the VDCM was measured. The pellet (exosomes) was washed twice by re-suspending exosomes in PBS and centrifuged at 200,000 x g for two hours at 4 °C. For immunoblot analysis, the exosomal proteins were resolved on 10% SDS-PAGE gel. For cell viability and motility assay, exosomes were re-suspended in 1 mL of DKSFM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin.

Atomic Force Microscopy (AFM)

Atomic force microscopy imaging was conducted using Multimode IIIa (Digital Instrument) and Dimension 3100 (VEECO). Exosomes were re-suspended in 50 µL of PBS after isolation. Twenty microliters of exosomes were immobilized on cleaved mica for detection. Briefly, 50 µL of 10 mM manganese chloride solution was incubated on cleaved mica (1 cm x 1 cm) for 30 s. Excess manganese chloride was removed by blowing dry with filtered compressed air. The exosomes were placed on mica and incubated for 2 min, followed by rinsing (with ddH_2O) and drying with filtered compressed air. To image exosomes, tapping mode in air was performed using silicon probes (Vistaprobe) with a nominal radius of curvature of 10 nm and cantilever spring constant of 48 N/m as provided by the manufacturer. The surface was imaged continuously at an average rate of 1−2 Hz on a 1×1−5×5 μm^2 area. The ranges of frequency, amplitude, integral, and proportional gains used were 7.5-8.5 kHz, 0.5−1 V, 0.5−2, and 0.75−3 respectively. All AFM images were analyzed using Nanoscope software version 5.12b (VEECO). Grade 5 muscovite mica was purchased from Ted Pella and hand cleaved just before use. Manganese chloride (99%) was purchased from Sigma-Aldrich (St. Louis, MO).

Dynamic Light Scattering (DLS) for Size Distribution and Zeta Potential

The size of the exosomes was measured by dynamic light scattering in terms of hydrodynamic diameter (D_H) with the Nanosizer ZS purchased from Malvern Instruments (UK). Briefly, exosomes were re-suspended in 50 µL of PBS after isolation and transferred to a low-volume, quartz microcuvette (ZEN0040, Malvern instrument).

The backscattering angle Θ was fixed at 172° with a laser wavelength λ = 633 nm. The size measurement range was from 1 nm to 6 μm. D_H is a function of the diffusion coefficient (D), temperature (T), and viscosity (η) according to the Stokes-Einstein equation: $D_H = \frac{kT}{3\pi\eta D}$, where k is Boltzmann constant and T is the temperature at 25 °C. D is obtained from autocorrelation function via the cumulant fitting. For measuring the surface charge, exosomes were re-suspended in 1 mL of PBS. The zeta potential of the exosomes was measured with a combination of laser Doppler velocimetry and phase analysis light scattering in a disposable capillary cell (DTS1070, Malvern Instrument).

Electron Microscopy

MCF10A derived exosomes were isolated and re-suspended in primary fixative (4% v/v paraformaldehyde in 0.1 M sodium phosphate buffer). Exosomes were incubated for 24 h in primary fixative. Then, 5 μL of exosomes were allowed to adsorb onto carbon-coated, 400 mesh, nickel grids purchased from Electron Microscopy Sciences (Hatfield, PA). After adsorption, the exosomes were incubated in either 0.3% saponin or Tween-20 diluted in PBS for permeabilization. Exosomes were washed once with PBS and thrice with glycine. Afterward, the exosomes were incubated in blocking buffer (1% w/v cold-water fish gelatin in PBS) for 45 min. For primary antibody labeling, exosomes were incubated for 1 h in 1:10 dilution of maspin, Tsg101 or mouse IgG. After washing with 0.1% cold-water fish gelatin six times, exosomes were incubated in 1:10 dilution of conjugated gold mouse secondary antibody for 30 min. Exosomes were washed six times in PBS followed by incubation in 1% v/v glutaraldehyde in 0.1M phosphate buffer for 30 min. Then, the exosomes were washed in ddH_2O. Negative

staining was conducted in 2% uranyl oxalate (pH 7.0) for five minutes. The exosomes were quickly rinsed once in ddH_2O and allowed to air dry. Of note, incubations and washings were carried out by transferring the grids with adsorbed exosomes onto droplets of solutions/buffers. Exosomes were visualized using the JEOL 2010 FasTEM instrument at 200 kV.

Immunoblot

Lysate: Cells were washed with PBS and incubated in 0.25% trypsin purchased from Life Technologies (Gaithersburg, MD). Detached cells were re-suspended in the appropriate media and pelleted by centrifugation at 2,000 x g for 5 min. Cells were re-suspended in PBS and pelleted again by centrifugation at 2,000 x g for 5 min. Cells were lysed with cold RIPA lysis buffer (20 mM Hepes, 100 mM NaCl, 0.1% SDS, 1% NP-40/IGEPAL-CA630, 1 mM deoxycholic acid, 1 mM Na_3VO_4, 1 mM EGTA, 50 mM NaF, 10% Glycerol, 1X Protease Inhibitor Cocktail, 1 mM EDTA). Cell lysates were collected, as the supernatant, by centrifugation at 16,000 x g for 30 min at 4 °C. Protein concentration was measured using the Pierce/ThermoScientific BCA Protein Assay Reagent Kit (Rockford, IL). Proteins were resolved on 10% SDS-PAGE and, then, transferred overnight to a PVDF membrane using electrophoresis. The membrane was incubated in blocking buffer (5% nonfat dry milk in PBS with 0.1% Tween-20) for one hour and incubated in a 1:1000 dilution of primary antibody overnight. The membrane was washed and incubated in a 1:5000 dilution of secondary antibody for 1 h followed by washing. Membranes were developed on film for visualization of proteins.

Conditioned media (CM) and Vesicle-depleted Conditioned Media (VDCM): The conditioned media was isolated and concentrated as described in "Exosome Isolation" section. Conditioned media, in which microvesicles were removed, were termed "vesicle-depleted CM" (VDCM). Protein concentration was measured. Proteins of the CM were processed for immunoblot as described for lysate proteins.

Isolation of Cytoplasmic Ribosomes (Polysomes)

CRL2221 cells were cultured in 10 mm cell culture dishes. At seventy percent confluence, cells were collected with trypsin. Briefly, cells were incubated in 0.25% trypsin for 1 min. Detached cells were pipetted into 1.5 mL collection tubes and centrifuged at 2,000 x g for 5 min. Trypsin was removed by aspiration and cells were re-suspended in PBS. Cells were washed by centrifugation at 2.000 x g for 5 min. Cells were re-suspended in cold homogenization buffer (40 mM Tris pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, and 0.5 mg/ml heparin) and transferred to a 3 mL Dunce homogenizer. Cells were lysed using a Dounce homogenizer for 1 min on ice. Lysates were centrifuged at 4 °C and 16,000 x g to remove the nuclei and mitochondria from the homogenates. Then, the lysates were centrifuged at 4 °C and 100,000 x g to remove microsomes (rough endoplasmic reticulum; RER) from the homogenates. 500 µL of the supernatant was layered over a discontinuous sucrose gradient containing 1 ml each of 1.0 M (top), 1.5 M, 2.0 M and 2.5 M (bottom) sucrose in polysome buffer. The supernatant was centrifuged at 4 °C for 2 hours at 100,000 x g. After centrifugation, 500 µL fractions were collected starting at the bottom (2.5 M sucrose). Thirty microliters of the fractions were mixed with sample buffer and loaded into the wells of a 10% SDS-

PAGE gel. Proteins were resolved and transferred by electrophoresis to a PVDF membrane for immunoblot analysis.

Immunofluorescence Labeling

The CRL2221 cells were seeded at a density of 4,000 cells/mL and incubated in an 8-well chamber slide purchased from Thermo Fisher Scientific (Hudson, NH). After 24 h, the media were removed and cells were washed twice with PBS. The cells were then incubated in 4% paraformaldehyde for 15 min followed by methanol fixation for 10 min at -20 °C. Cells were incubated with 1% Triton-X 100 and then with 10% normal goat serum in PBS for 1 hr. The cells were incubated in 1:100 maspin primary antibody and 1:100 LAMP-2 primary antibody. Cells were washed and incubated for 1 h in 1:500 dilution of Alexa Fluor 488 and Alexa Fluor 594 fluorescent secondary antibodies. The nucleus was stained with 4´,6-diamidino-2-phenylindole (DAPI) for 1 min. Cells were visualized under a LSM-510 confocal microscope at 400X magnification.

Secretion-Targeted Drug Treatments

The CRL2221 cells were cultured in 10 mm cell culture dishes until seventy percent confluence. Cells were washed thrice with PBS. Cells were incubated for 24 h in DKSFM with or without brefeldin A (BFA), ionomycin (IONO, methylamine (MA), or chloroquine (CQ) at the concentrations indicated in the figure legends. After 24 h, the CM was removed and saved for immunoblot analysis. In order to determine whether the drug treatments affected trafficking of exosomal maspin, the media were removed and processed for the isolation of the exosomes from the CM as described in the “Exosome

Isolation" section. The cells were washed and collected after incubation with 0.25% trypsin. The cell lysates, exosomes and VDCM were processed for immunoblot analysis as described in the "Immunoblot" section. The VDCM was also used for zymography as described in the "Zymography" section. Drugs were purchased from Sigma-Aldrich (St. Louis, MO).

Zymography

CM (containing exosomes) and VDCM (depleted of exosomes) were collected from CRL2221 cells treated for 24 h with or without BFA, IONO, MA, or CQ in DKSFM. The drug concentrations are indicated in the figure legends. The CM was concentrated using 15 mL Millipore 10 kDa centrifugal filter units. Twenty microliters of CM was diluted with β-mercaptoethanol free sample buffer and loaded onto a zymogram gel. Proteins were resolved by electrophoresis. Recombinant matrix metalloprotease 9 (MMP9), gifted by Dr. Rafael Fridman, was loaded onto the zymogram gel as a positive control. Following electrophoresis, the gel was incubated in re-naturing buffer (2.5% Triton X-100 in ddH_2O) for 30 min. Then, the gel was washed in ddH_2O for 10 min. The gel was transferred to developing buffer (50 mM Tris 9, pH 8.0), 5 mM $CaCl_2$, 200 mM NaCl, and 0.02% v/v Brij-35) and incubated for 30 min. The developing buffer was replaced with new developing buffer and incubated at 37 °C for at least 16 hours. The gel was stained in 0.5% w/v Coomasie blue solution (Coomasie blue brilliant R-250 in 5% v/v acetic acid, and 10% v/v methanol) and incubated at room temperature with gentle shaking for 30-60 min. The gel was then incubated in de-staining solution (5% v/v

acetic acid and 10% v/v methanol in ddH_2O) until bands were visualized. Images of the zymogram gel were captured using Bio-Rad Gel Doc XR+ system (Hercules, CA).

Membrane Stripping Assay

To remove peripheral proteins at the membrane surface, pelleted CRL2221-derived exosomes were re-suspended and washed twice with 12.5 mM, 25 mM, 50 mM or 100 mM KCl in PBS at 4 °C and 100,000 x g for 2 h. The control (no KCl) was re-suspended and washed twice in PBS at 4 °C and 100,000 x g for 2 h. The supernatant was removed and the exosomes were washed two additional times with PBS at 4 °C and 100,000 x g for 2 h to remove residual KCl. For immunoblot analysis, the exosomes were re-suspended in 1X sample buffer after the final wash, and the exosomal proteins were resolved on 10% SDS-PAGE gel and transferred to PVDF membrane.

Maspin Knockdown and Stable Transfection

Stable transfected MCF10A cells lines were established with the pGIPZ shRNA-mir lentiviral plasmid system (Thermo Scientific, Asheville, NC) according to the manufacturer's instructions. Briefly, MCF10A cells were seeded in 10 mm cell culture plates. At fifty percent confluence, cells were transfected with either a mixture of maspin shRNA plasmids (RHS4430-98895314, RHS4430-99297939, RHS4430-99139485) or the noncoding shRNA plasmid (RHS4346) using the X-treme GENE 9 DNA transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Stable transfected clones were selected with 10 µg/mL puromycin. The clones were maintained in DMEM/F-12 medium containing 5% FBS. To

verify maspin knockdown, cell lysates of maspin knockdown clones and noncoding clones were processed for immunoblot analysis as described in the “Immunoblot” section.

Exosome Labeling

Exosomes were isolated as described in the “Exosome Isolation” section. After the centrifugation at 100,000 x g, the exosomes were washed once with PBS and labeled with the PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich) according to the manufacturer’s protocol. The exosomes were centrifuged at 100,000 x g to remove excess PKH26 and re-suspended in 1 mL of DKSFM. The NIH 3T3 mouse fibroblast cells were incubated in a 1:100 dilution of labeled exosomes for 24 h. Live cell imaging of NIH 3T3 cells after exosome treatment was performed using the Leica Fluorescence microscope.

Cell Viability Assay

Drug Treatment: The CRL2221 cells were seeded into 96-well plates at a density of 20,000 cells/mL and allowed to attach to the plate for 24 h. The CRL2221 cells were treated with or without BFA, IONO, MA or CQ at the concentrations indicated in the figure legends (Figures 4 and 9) for 24 h. Cell viability was assessed with the WST-1 Reagent (Roche Diagnostics) according to the manufacturer’s instructions. All drugs were purchased from Sigma-Aldrich (St. Louis, MO). For statistical analysis, one way analysis of variance (ANOVA) was performed using the SigmaPlot software (Chicago, IL).

Exosome Treatment: Cell viability of the NIH 3T3 cells was assessed after treatment with various concentrations of the exosomes as indicated in figure 13. The NIH 3T3 cells were seeded at 20,000 cells/mL in 96-well plates. Cells were allowed to attach to the plate for 24 h. The exosomes were isolated as described in "Exosome Isolation" section. The exosomes were re-suspended in 1 mL of DKSFM and dilutions of isolated exosomes were prepared from the suspended exosomes. Then, cells were treated for 24 h with or without exosomes derived from the MCF10A stable transfected clones. WST-1 Reagent (Roche Diagnostics) was added according to the manufacturer's instructions.

Cell Lysis (LDH Assay)

The CRL2221 cells were seeded into 96-well plates at a density of 20,000 cells/mL and allowed to attach to the plate for 24 h. The CRL2221 cells were treated with or without BFA, IONO, MA or CQ at the concentrations indicated in the figure legend (Figures 4 and 9) for 24 h. 100 µL of the conditioned media of the CRL2221 cells were transferred to a new 96-well plate. Lactate dehydrogenase (LDH) cytotoxicity assay was used to assess LDH activity according to the manufacturer's instructions. The LDH cytotoxicity assay was purchased from Cayman Chemical (Ann Arbor, MI). For statistical analysis, one way analysis of variance (ANOVA) was performed using the SigmaPlot software (Chicago, IL).

Motility Assay

NIH 3T3 cells were seeded into 6 well plates and allowed to attach to the plate for 24 h. The exosomes were re-suspended in 1 mL of PBS and administered to the NIH 3T3 cells at a dilution of 1:100 dilution. The NIH 3T3 cells were treated with the exosomes for 24 h. NIH 3T3 cells were collected and seeded into the chambers of Corning transwell plates (Sigma-Aldrich). Motility was assessed after 24 h. For statistical analysis, one way analysis of variance (ANOVA) was performed using the SigmaPlot software (Chicago, IL).

Quantitative Real Time-PCR (qRT-PCR)

Total RNA was isolated using the Qiagen RNeasy Mini kit (Valencia, CA,). The RNA was converted to cDNA using the Bio-Rad iScript cDNA synthesis kit (Irvine, CA). The mRNA levels of the following genes were quantified using the indicated forward and reverse primer pairs: GAPDH (forward: 5´ - ATC ACC ATC TTC CAG GAG CGA - 3´ and reverse: 5´ - GCC AGT GAG CTT CCC GTT CA - 3´); Vimentin (VIM ; forward: 5´ - GCT CCT ACG ATT CAC AGC CA- 3´ and reverse: 5´ - CGT GTG GAC GTG CTG ACA TA- 3´); uPA forward: 5´ - CAT CCA TCC AGT CCT TGC GT - 3´ and reverse: 5´ - ACG CAT AC ACCT CCG TTC TG - 3´); α-smooth muscle actin (SMA; forward: 5´ - GAG CCC AGG CAT TGC TGD CA - 3´ and SMA reverse: 5´ - GAG GCG CTG ATC CACA AA AC - 3´); p21 forward: 5´ - CAG GCA CCA TGT CCA ATC CT- 3´ and p21 reverse: 5´ - AAT CTG TCA GGC TGG TCT GC - 3´); collagen I (Col I; forward: 5´ - TCT CCA CTC TTC TAG TTC CT- 3´ and reverse: 5´ - TTG GGT CAT TTC CAC ATG

C - 3´). GAPDH was used as an internal reference for normalization. For statistical analysis, ANOVA was performed using the SigmaPlot software (Chicago, IL).

Chapter 3: Results

Chapter 3A: Investigating the Differential Regulation of Exosomal Maspin in Non-Malignant Cells and Tumor Cells

Exosomes have distinct physical properties

The exosomes were isolated from a panel of maspin-expressing non-malignant cells and tumor cell lines of the breast, prostate and lung. Then, the exosomes were analyzed by atomic force microscopy (AFM) and dynamic light scattering (DLS) through the collaboration with laboratory of Guangzhao Mao, Ph.D. at Wayne State University. The height and amplitude (the first derivative of the height) were measured using AFM. The images from AFM confirmed the isolation of the secreted nanoparticles from non-malignant cells (Figure 1A) and tumor cells (Figure 1B). The nanoparticles were spherical in morphology, which is consistent with the description of exosomes reported in the literature.[71] Additionally, the morphology of the exosome is described as cup-shaped in some reports.[103] In our hands, only a few of the nanoparticles visualized by AFM seem to exhibit a cup shape. The original morphological description of the exosomes was determined from electron microscopy (EM) images produced. Therefore, it is unknown whether the cup shape is due to the EM preparation or if it is the bona fide shape of exosomes. The data presented in Figure 1 show a representative visualization of the exosomes by AFM. Heterogeneity of the exosomes was observed in the AFM images, irrespective of the cell type, as well as some degree of aggregation. The degree of aggregation seemed to vary among the cell lines. In addition, various amounts of exosomes were observed in the AFM images. The amount of exosomes

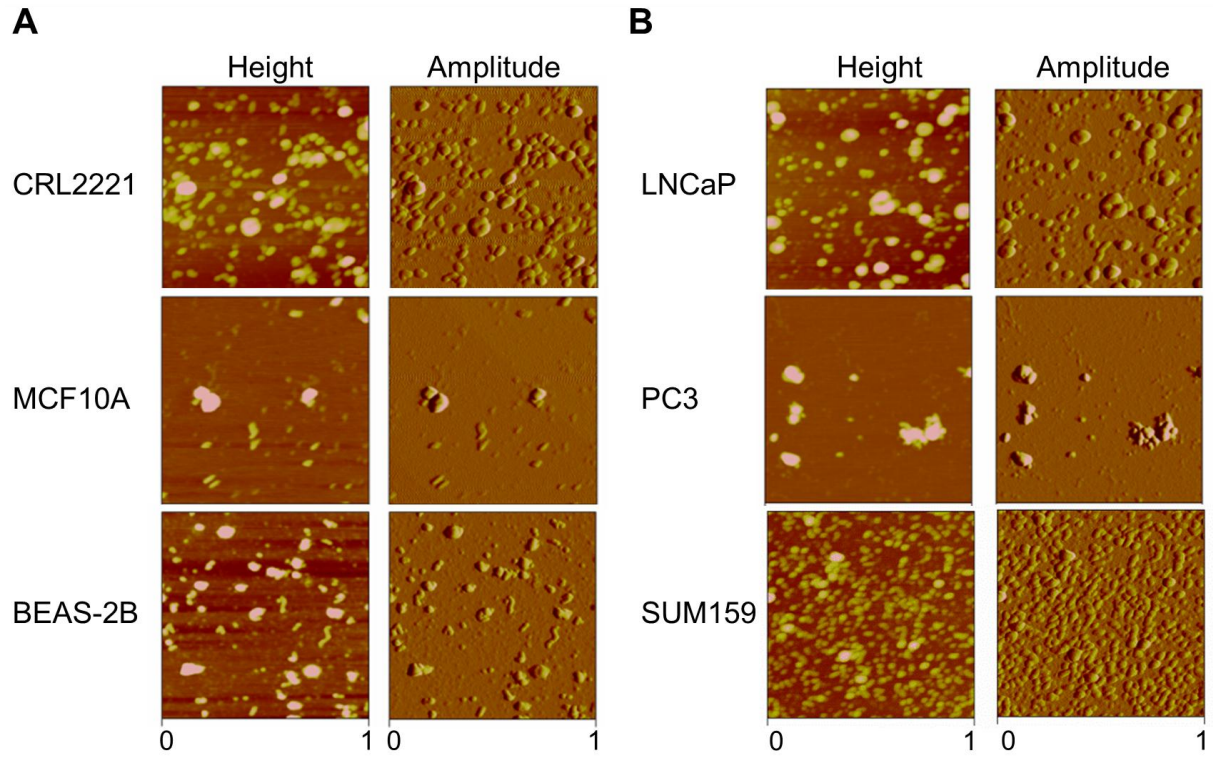

Figure 1 – The physical characteristics of the exosomes. Images were produced using atomic force microscopy. (A) Representative images of exosomes derived from non-malignant cell lines. (B) Representative images of exosomes derived from tumor cell lines. Scale is 1 um.

detected did not appear to differ between non-malignant and tumor cells. However, there is not yet a reliable method to quantify exosomes because of their size. FACS sorting has been used to try to quantify the number of exosomes, but requires antibody linkage of the exosomes to magnetic beads, which may result in multiple beads attaching to one exosome.[104]

Atomic force microscopy allowed the visualization of the exosomes but does not provide quantitative data. In order to quantify the size of the exosomes, DLS was used to measure the size distribution. DLS is advantageous in that it measures the size distribution of particles that are in the nanometer range. The exosome fraction derived

Table 1: Average Size Distribution of the Exosomes			
Cell Type	**Cell Line**	**Size Distribution (± standard deviation)**	**n**
Normal	CRL2221	53.6 ± 12.8; 367.6 ± 144.8	5
	MCF10A	92.9 ± 42.4; 337.7 ± 60.4	7
	BEAS-2B	41.2 ± 8.8; 305.0 ± 85.6	3
Tumor	LNCaP	55.4 ± 5.3	4
	PC3	87.2 ± 10.1	3
	SUM149	91.1 ± 1.8	3

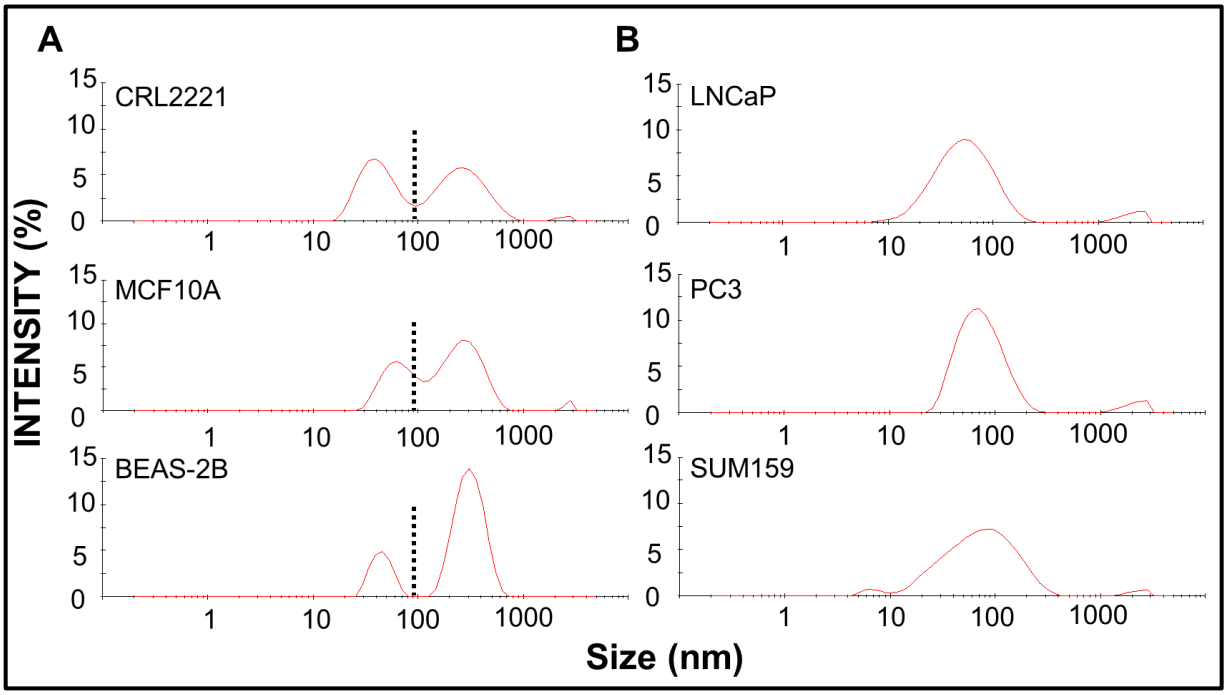

Figure 2 – Size distribution of exosomes measured by DLS. (A) Representative graphs of exosomes derived from non-malignant cell lines. (B) Representative graphs of exosomes derived from tumor cell lines.

from the non-malignant cell lines contained two populations of nanoparticles (Table 1 and Figure 2). The first population identified from the non-malignant cell line corresponds to the reported size range of the exosomes. The second population

identified from the non-malignant cell line is above the reported size range of the exosomes. In contrast, the tumor cell lines secreted one population of nanoparticles that were in the reported size range of the exosomes (Table I). Of note, the same exosome preparations were used for DLS and AFM. Therefore, the more detailed physical properties revealed by DLS were not due to variations in exosomal preparation.

MCF10A-derived exosomes were also visualized by electron microscopy, which was conducted through a collaboration with Dr. James Granneman of Wayne State University School of Medicine, Department of Psychiatry and Behavioral Neuroscience. Consistent with the literature, the MCF10A-derived exosomes were spherical in shape (Figure 3). A few exosomes exhibited the “cup” shape that has been described by some of the literature (Figure 3A, left image, and 3C, left image). Of note, it is unknown whether the “cup” shape is due to the preparation process for EM. The cup shape was not observed in the AFM images of the exosomes. The exosomes were immunolabeled with either maspin (Figure 3B) or Tsg101 (Figure 3C). Intact exosomes were observed with a defined border. Maspin was not labeled in the intact exosomes. However, maspin congregated in areas where the exosomes are not well-defined. This data suggest that some of the exosomes may have been sensitive to the permeabilization process and ruptured. The ruptured exosomes exposed the luminal proteins and maspin was easily accessible. The intact exosomes may have not been permeabilized, which may be why maspin was not detected in the intact exosomes. The Tsg101 was detected in intact exosomes but also congregated in areas where the exosomes were less defined (Figure 3C, middle and left images). There were also exosomes that did not contain Tsg101 (Figure 3C, left image). Consistent with the AFM images, single particles and

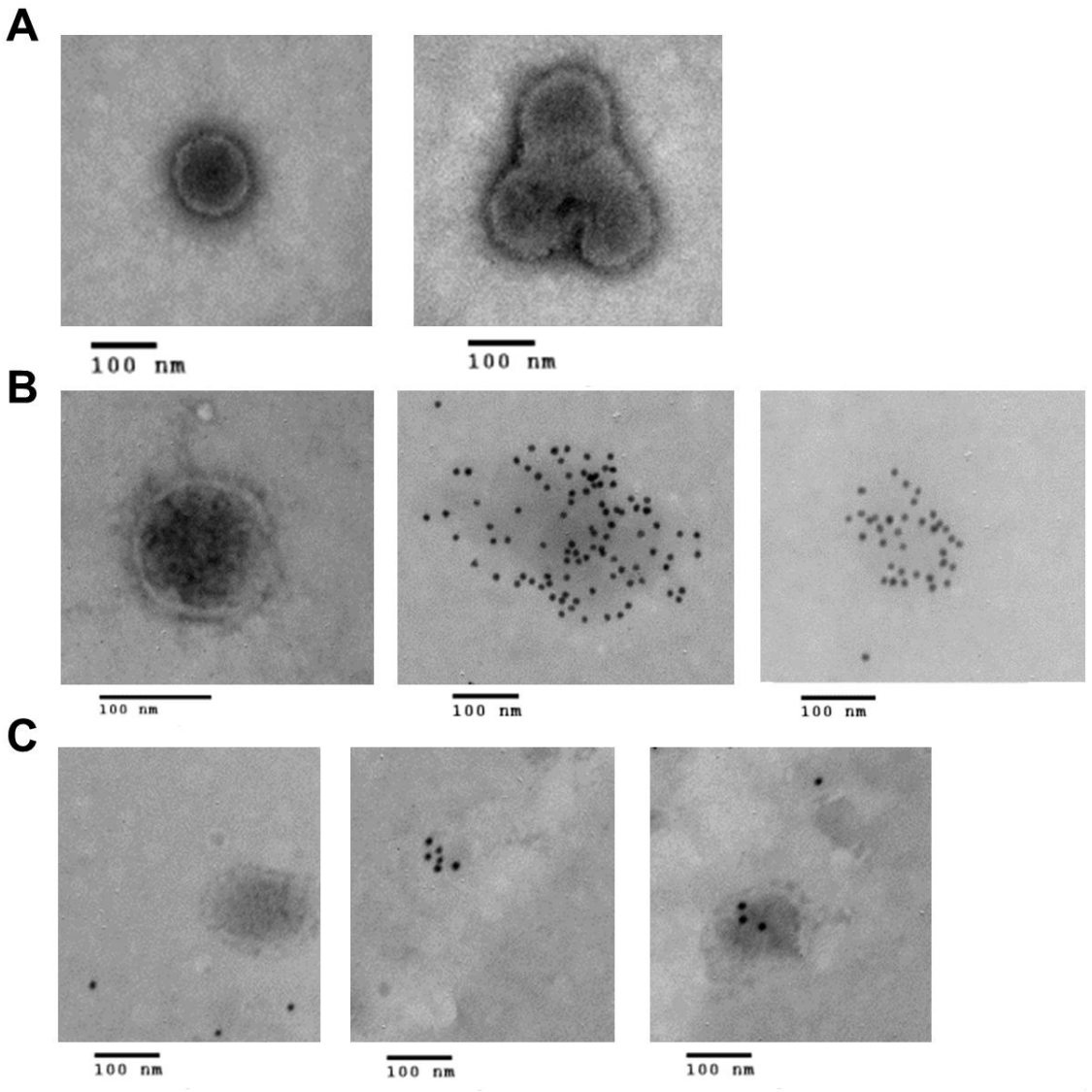

Figure 3 – Electron Microscopy of the exosomes. Representative images of the MCF10A-derived exosomes. Exosomes were isolated and permeablized for immunolabeling. (A) Exosomes were incubated with IgG control (B) Exosomes were incubated in maspin primary antibody (C) Exosomes were incubated in Tsg101 primary antibody. 100 nm scale bar.

aggregated particles were detected in the exosome fraction. The data from the EM images further support the speculation that the second peak detected in the normal-derived exosome fraction is most likely due to a high degree of aggregation.

The exosomes of non-malignant cells and tumor cells were further analyzed by DLS for membrane surface charge by measuring the zeta potential. The zeta potential for the exosomes of all of the cell lines was negative (Table 2). Thus, exosomes across various cell lines maintained an average negative membrane surface charge. Of note, the zeta potential represents an average of the surface charge, which does not rule out the possibility that positively charged exosomes exist. Given that the charge of a bilayer membrane depends on the associated proteins, the consistent average negative surface charge suggests that the exosomes of the cell lines share common transmembrane and/or peripheral proteins.

Table 2: Average Zeta potential of the Exosomes

Cell Type	Cell Line	Zeta Potential	n
Normal	CRL2221	-13.8 ± 0.666	3
	MCF10A	-12.6 ± 1.85	3
	BEAS-2B	-13 ± 0.907	3
Tumor	LNCaP	-11.2 ± 0.611	3
	PC3	-11 ± 0.681	3
	SUM149	-11.7 ± 0.473	3

Maspin Trafficking and Secretion is Independent of the ER-Golgi

The primary sequence of maspin does not reveal an N-terminal secretory signal peptide (SSP), which is necessary for ER-Golgi dependent (classical) secretion. Therefore, maspin may be secreted by a non-classical mechanism. Software has been developed to predict the chance of a non-classical secretion by a protein that lacks the

Table 3: Prediction for Non-Classical Secretion of Maspin

CENTERFO RBIOLOGI CALSEQU ENCEANA LYSIS CBS

Secretome 2.0 Server - prediction results

Technical University of Denmark

Non-classically secreted proteins should obtain an NN-score exceeding the normal threshold of **0.5**, but not at the same time be predicted to contain a signal peptide.

```
# SecretomeP 1.0f predictions

# Name          NN-score  Odds   Weighted   Warning
#                                 by prior
# ================================================================================
Sequence          0.499    0.997      0.002     -
# ================================================================================
```

Explain the output. Go back.

SSP. Proteins with an NN-score above 0.5 are strongly predicted to be secreted by a non-classical mechanism.[105] Interestingly, maspin received an NN-score of 0.499 from the software, which is 0.001 below the predictive threshold (Table 3). Maspin's score may be below the threshold due to the fact that maspin also functions as an intracellular protein, which may not be taken into account by the software.

The secretion of a protein may depend on where the protein is synthesized and what particular pathways are involved. Proteins that are secreted by the classical secretory pathway are thought to be translated by the ribosomes on the rough ER (RER), where they can be trafficked through the ER and Golgi apparatus for secretion[68]. In contrast, non-classically secreted proteins are thought to be translated by the free ribosomes. Maspin sequence lacks the SSP necessary for ER-Golgi dependent secretion, which suggests that maspin is most likely translated by free ribosomes. Ribosomes have the ability to translate mRNA to proteins when attached to or

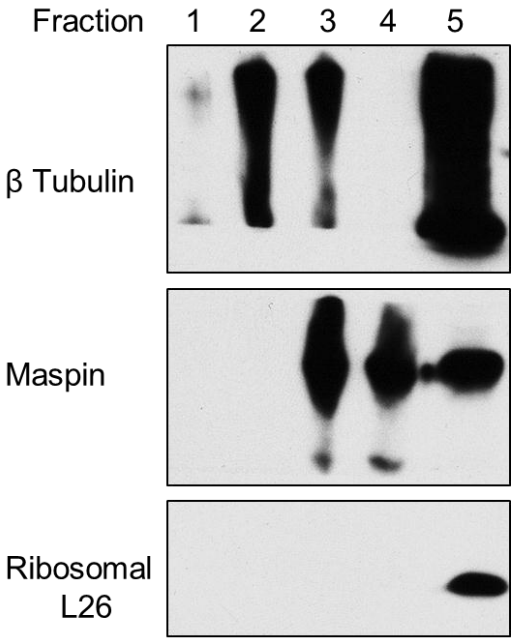

Figure 4 – Cytoplasmic ribosomes translate maspin mRNA. Cytoplasmic ribosomes were isolated from lysates of the CRL2221 cells by a sucrose density gradient. Proteins were resolved on a 10% SDS-PAGE for Western blot analysis.

unattached to the rough ER (RER). Free cytoplasmic ribosomes are physically identical to ribosomes attached to the RER with identical translation efficiency.[68] Free ribosomes mainly translate cytoplasmic proteins and other intracellular proteins that do not need the ER or Golgi for post-translational processing. In concordance, many of the non-classically secreted proteins are cytoplasmic proteins.[68] In order to determine whether maspin is translated by the free ribosomes, the free ribosomes were isolated from the cytoplasm and floated on a sucrose density gradient for purification. Fractions were collected from the gradient and proteins were detected by immunoblot analysis (Figure 4). The free ribosome fraction (fraction 5) was verified with L26, a ribosome marker. β tubulin was used as a positive control for cytoplasmic proteins. Maspin was also detected in fraction 5, which suggests that maspin is translated in the cytoplasm by free ribosomes (Figure 4).

To further investigate the trafficking of maspin, CRL2221 cells were treated with BFA for 24 h. BFA targets retrograde transport from the Golgi to the ER, which causes the intracellular accumulation of ER-Golgi dependent secretory proteins, such as matrix metalloprotease 9 (MMP9). Treatment with BFA did not inhibit the secretion of maspin,

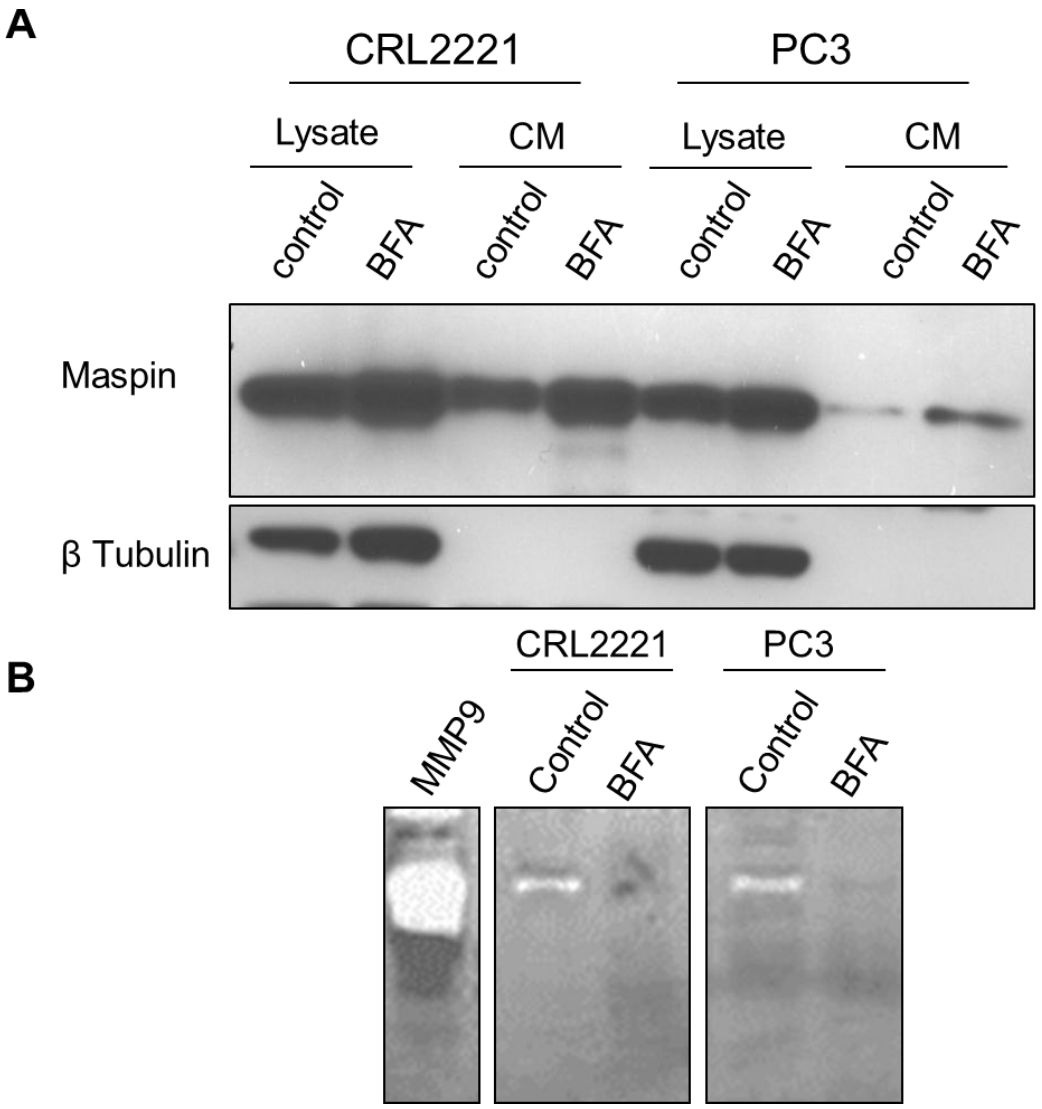

Figure 5 – Maspin is not secreted through the ER-Golgi secretory pathway. (A) Immunoblot analysis of lysates and CM from CRL2221 cells after 24 h treatment with 18 µM of BFA. (B) Zymogram of the CM from CRL2221 cells after 24 h treatment with 18 µM of BFA.

which suggests that maspin secretion occurs by an ER-Golgi independent mechanism (Figure 5A). When compared to untreated cells, the secretion of maspin increased as a result of BFA treatment. There was also a slight increase in intracellular maspin. The increase in maspin in both lysates and CM may be due to general cellular stress caused

by the treatment. Interestingly, maspin secretion has been shown to increase under cellular stress conditions.[106] MMP9 is secreted via the ER-Golgi secretory pathway and the primary amino acid sequence of MMP9 contains an N-terminal SSP. Thus, MMP9 was used as a positive control to verify that BFA inhibited classical protein secretion. The CM of both the CRL2221 cells and PC3 cells were assessed for MMP9 by zymography. The secretion of MMP9, as detected by zymography, was significantly inhibited by the treatment of BFA, which indicates that BFA inhibited ER-Golgi dependent secretion (Figure 5B). In contrast, secreted MMP9 was detected in untreated cells.

Confocal microscopy of the CRL2221 prostate cells revealed co-localization between maspin and lysosome-associated membrane protein 2 (LAMP-2), a late endosome and lysosome marker (Figure 6A, white arrows).[107,108] Additionally, LAMP-2 has also been detected in the exosomes.[102,108-110] The data suggest that maspin may be trafficked to the late endosome, which is consistent with the notion that maspin is associated with the exosomes. Of note, the CRL2221 cells are heterogeneous in maspin subceullar localization, in which some cells have mostly nuclear maspin while some cells have mostly cytoplasmic maspin. Figure 6B shows the subcellular fractionation of a heterogeneous poopulation of CRL2221 cells. Unlike the normal tissue epithelium, which has high nuclear maspin staining, the CRL2221 cells have more cytosolic maspin. Our lab has also shown that maspin can be mostly localized to the nucleus in CRL2221 cells.[14] The difference in maspin nuclear:cytoplasmic ratio in the CRL2221 cells may be due to unidentified variations in cell culture conditions. Additionally, it is unknown whether maspin localization changes during certain cellular

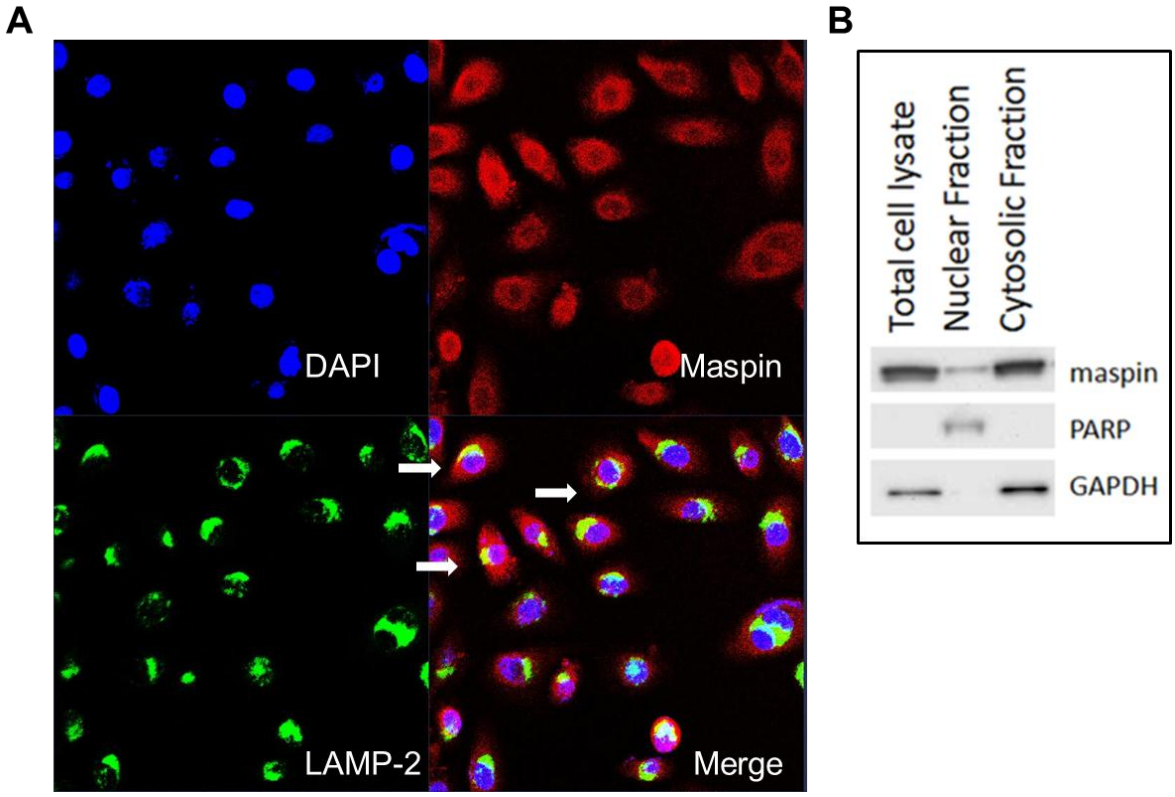

Figure 6 – Co-localization of maspin with the endo-lysosomal protein, LAMP-2. (A) Representative confocal microscopy images of CRL2221 cells after immunolabeling with maspin and LAMP-2 antibodies. Maspin co-localizes with LAMP-2 (white arrows). 400X magnification. (B) Subcellular fractionation of CRL2221 cells.

states, such as cell cycle progression, or under certain processes, such as cell stress. These unknown factors may contribute to the difference I observed in maspin subcellular localization compared to what was previously reported. Of note, the CRL2221 cells are an immortalized cell line. Clinical tissue samples consistently show evidence of maspin nuclear localization in normal cells as discussed in the introduction.

Exosomal maspin in non-malignant cells and tumor cells

It is unknown how the level of maspin in the exosomes is regulated in response to tumor progression. The exosomal cargo is recruited to the intraluminal vesicles (ILVs)

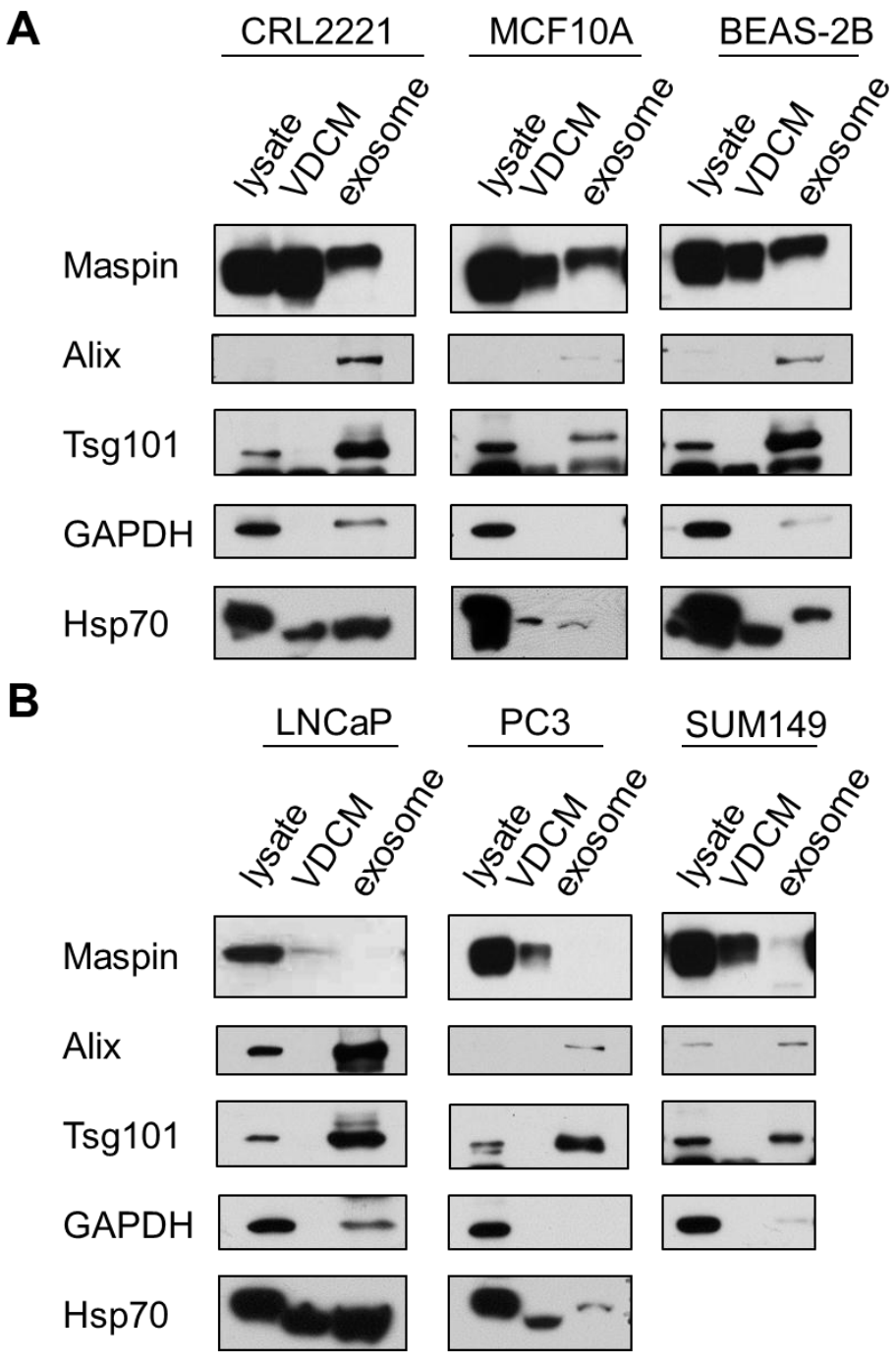

Figure 7 – Exosomal maspin is secreted by non-malignant cells. Immunoblot analysis of the lysates, VDCM and exosomes from non-malignant cells (CRL2221, MCF10A and BEAS-2B) and tumor cells (LNCaP, PC3 and SUM149). Immunoblot membranes were probed for various proteins that have been reported in the exosomes. Alix and Tsg101 are markers of the exosome.

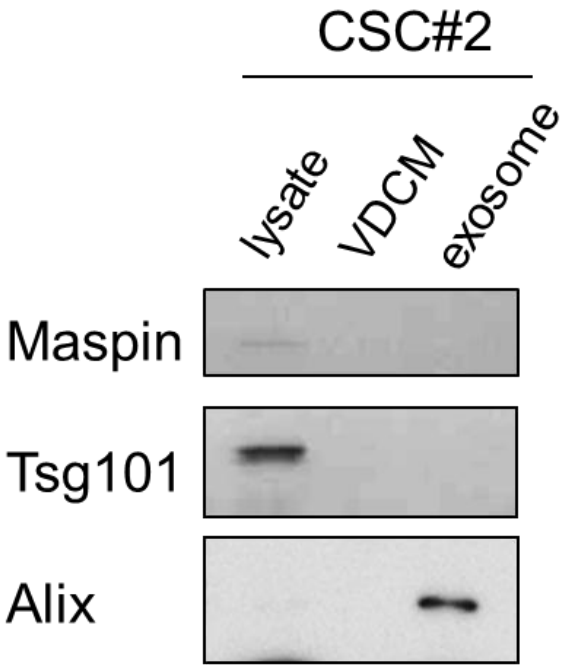

Figure 8 – Loss of maspin expression results in absence of exosomal maspin. Immunoblot analysis of the lysates, VDCM and exosome fractions of the CSC#2 lung cancer cells. Tsg101 and Alix are markers of the exosome.

during formation. Since maspin co-localizes with an endosome marker, maspin may be recruited to the exosome as a cargo protein. The levels of maspin in the exosomes isolated from the non-malignant cells and tumor cells grown under exponential cell culture conditions were compared. The PVDF membranes were overexposed in order to determine the presence of maspin. As shown in figure 7, the non-malignant cells (CRL2221, MCF10A and BEAS-2B) contain high levels of exosomal maspin. In contrast, the amount of exosomal maspin in the tumor-derived exosomes (LNCaP, PC3, and SUM149) is reduced. Judging from the detection of the exosomal markers, Alix and Tsg101, the exosomal preparation was highly enriched and distinct from the vesicle-depleted conditioned media (VDCM). Interestingly, soluble maspin was detected in the VDCM of the non-malignant cells. The absence of the exosomal markers, Alix and Tsg101, and cellular (cytoplasmic) markers, GAPDH and tubulin, from the VDCM validate that the VDCM was not contaminated with cellular or vesicular components. Therefore, maspin's expression in the VDCM was not due to contamination. The level of soluble maspin was most abundant in the VDCM of the non-

malignant cells. These data demonstrate that the isolation of the exosomal maspin does not deplete maspin from the VDCM. In comparison, despite the expression of intracellular and VDCM (by the tumor cells), little to no exosomal maspin was detectable by the tumor cells. The evidence suggests that maspin is secreted by two mechanisms that results in both a soluble protein and exosomal protein. Moreover, the presence of both soluble maspin and exosomal maspin in the CM may indicate dual mechanisms of secretion. The variation of exosomal maspin between non-malignant cells and tumor cells indicate that the recruitment of maspin to the endosome and, subsequently, the exosome may be differentially regulated in tumor progression.

Additionally, CSC#2, a cancer cell line developed from the BEAS-2B cells using smoke condensate, was analyzed for exosomal maspin. The lysate, VDCM and exosomes were isolated from the CSC#2. The proteins were resolved on the same gel as the BEAS-2B fractions from figure 6 for immunoblotting on the same membrane. As shown in figure 8, the CSC#2 does not express very much maspin in the lysate and maspin is undetectable in the CM and exosome fractions. The data from this study suggests that during tumor progression, exosomal maspin is lost even though the tumor cells continue to express intracellular maspin and secreted soluble maspin, but not exosomal maspin.

Re-expression of maspin in DU145 cells does not induce exosomal maspin

During tumor progression, maspin expression can be lost. Using the DU145 prostate cancer cell-derived M7 (maspin-expressing) and Neo (mock control) cell lines, the Sheng laboratory has shown a tumor suppressive role for maspin at the step of

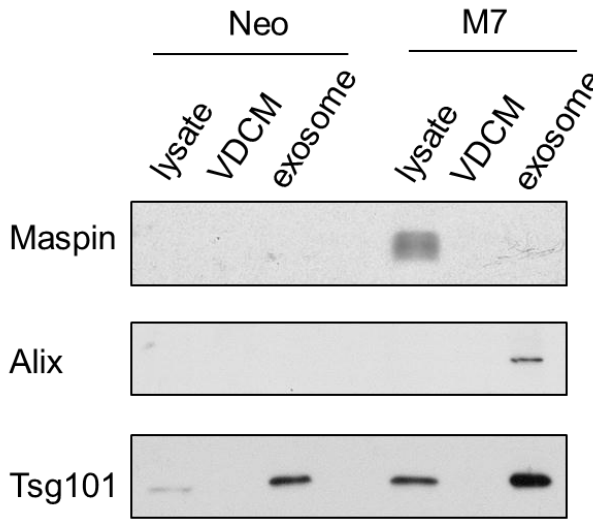

Figure 9 – Maspin re-expression does not induce exosomal maspin secretion. Western blot analysis of the lysate, VDCM and exosome from the Neo cells and the M7 cells.

tumor invasion, motility, metastasis, and overall epithelial de-differentiation. In the current study, exosomes were isolated from the M7 cells and the Neo cells in order to determine whether re-expression induces secretion of exosomal maspin. As expected, maspin was detected in the lysate of the M7 cells, but not in the lysate of the Neo cells (Figure 9). Maspin was not detected in either the VDCM or exosomes of the Neo or M7. The detection of Tsg101 and Alix were used in combination to verify the isolation of the exosomes. OF note, different cell lines express different levels of the markers, which is why the markers were both used. Interestingly, the expression of Tsg101 and Alix are stronger in the M7 cells than in the Neo cells, yet maspin is not detected in the exosomes of the M7 cells. The level of maspin in the transfected cells may not be sufficient to support exosomal maspin. It is possible that cancer cells that still express endogenous maspin may have lost the ability to efficiently recruit maspin to the exosomes for detection, which further indicates that exosomal maspin is dysregulated in the tumor cell.

Maspin Secretion Occurs by Dual Mechanisms

The two forms of secreted maspin, soluble and exosomal proteins, may be secreted either dependently, or independently of each other. Various drugs that have been shown to target specific pathways of secretion were used to help delineate the secretion mechanism of maspin (Table 4). BFA targets the ER-Golgi trafficking pathway by inhibiting retrograde transport from the Golgi to the ER.[111] IONO is a calcium ionophore that inhibits ER-Golgi trafficking by disrupting ER calcium levels.[112,113] Since many cellular processes are calcium dependent, ionomycin is may have non-specific consequences. MA is a weak base that raises the luminal pH of the acidic organelles, such as the endosome and lysosome.[114-116]

Table 4: Inhibitors of Secretion

Drug	Targeting Organelle
Brefeldin A (BFA)	ER/Golgi
Ionomycin (IONO)	ER/Golgi
Methylamine (MA)	Endosome
Chloroquine (CQ)	Endosome

The mechanism of methylamine is unclear and may affect other cellular processes. CQ, an antimalarial drug, is a weak base that raises the luminal pH of acidic organelles.[114,117,118] MA and CQ were used to target endosome trafficking and subsequently exosome secretion. The CRL2221 cells were treated with or without BFA, IONO, MA or CQ for 24 h. The lysates, CM, and exosomes were collected and analyzed with a immunoblot (Figure 10A). Consistent with figure 5, maspin secretion into the VDCM was not inhibited by BFA. Under BFA treatment, maspin secretion into the VDCM increased. Moreover, maspin levels in the exosome also increased. In order to

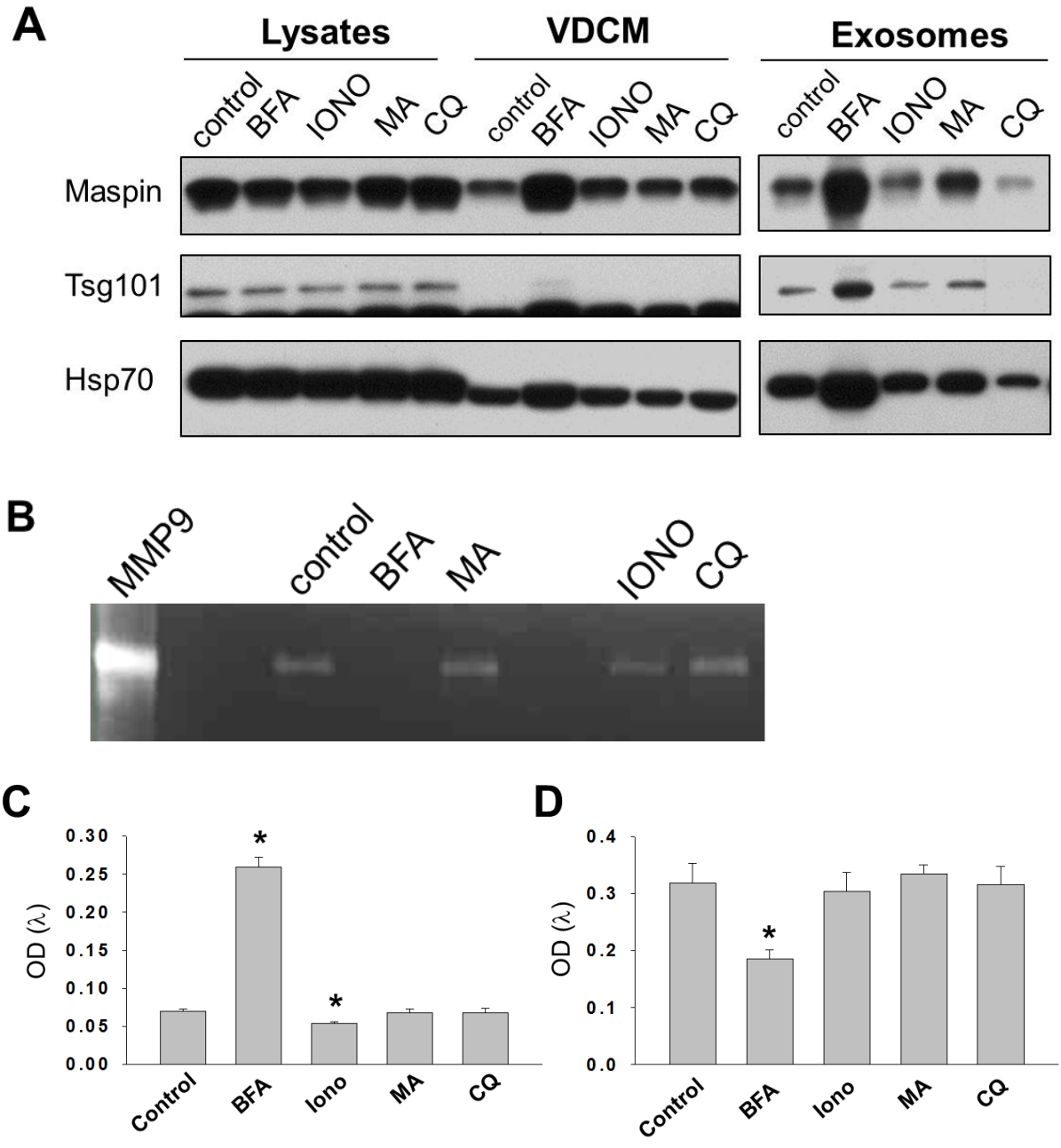

Figure 10 – Exosomal maspin and soluble maspin are secreted independently. CRL2221 cells were treated with or without 5 µM BFA, 0.1 µM IONO, 1 mM MA or 5 µM CQ for 24 h. (A) Immunoblot analysis of the lysates, VDCM and exosome of the CRL2221 cells. (B) Zymogram of the CM of the CRL2221 cells. (C) LDH activity assay in the CM of the CRL2221 cells. Cells treated with BFA and IONO showed significantly different changes in LDH activity. (*p = <0.001) (D) Cell viability was assessed with the WST-1 reagent. Cells treated with BFA showed a significant decrease in viability. (*p = <0.001)

confirm the effect of drug treatment, zymography was used to analyze the CM of the drug-treated cell (Figure 10B). Zymography of the CM revealed that the BFA was effective at inhibiting the ER-Golgi dependent secretion of MMP9. In contrast, targeting ER-Golgi secretory pathway with ionomycin was less effective and did not inhibit MMP9 secretion. Under IONO treatment, neither the levels of soluble maspin nor exosomal maspin was affected. MA has been shown to inhibit exosome secretion by some but was not an effective inhibitor for others.[115,116] Under MA treatment, maspin secretion was not inhibited. On the contrary, maspin secretion slightly increased in the VDCM and noticeably increased in the exosomes. Under CQ treatment, secretion of soluble maspin was not affected. However, secretion of exosomal maspin decreased. LDH activity assay was performed to monitor cell lysis under drug treatment (Figure 10C). Cell lysis only increased under BFA treatment. Therefore, the presence of maspin in the VDCM or exosomes was not due to cell lysis. Additionally, WST-1 assay was also performed in order to monitor cell viability under drug treatment (Figure 10D). BFA treatment elicited cell stress as judged from the increase in LDH activity and reduced cell viability. However, BFA was effective at inhibiting classical secretion as judged by the absence of MMP9 in the CM of BFA treated cells. IONO treatment resulted in a decrease in LDH activity, which may have been due to the off-target effects of the calcium ionophore.

It is possible that the soluble maspin may bind to a protein at the surface of the exosome. It is also possible that maspin is in the lumen of the exosome. To further investigate the two possibilities, exosomes derived from CRL2221 cells were stripped of peripheral proteins at the exosome surface with various concentrations of potassium

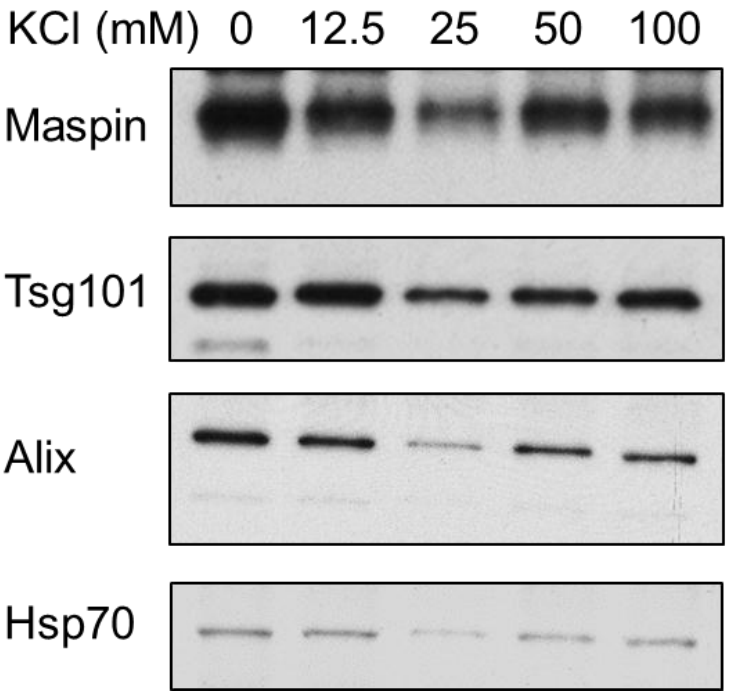

Figure 11 – Maspin is not at the membrane surface of the exosome. Immunoblot analysis of the exosomes derived from CRL2221 cells after treatment with various concentrations of KCl. Alix and Tsg101 are markers of the exosome.

chloride (KCl). Low levels of KCl were used as not to disrupt the exosome membrane. The levels of maspin slightly decreased under KCl treatment (Figure 11). However, the exosomal markers also decreased. Treatment with 25 mM KCl showed the biggest reduction in maspin, Alix and Tsg101. This may be due to the treatment conditions since a new batch of exosomes was prepared for each condition. The reduction may be due to the loss of exosomes during the isolation process. The data suggests that maspin is not associated with the surface of the exosomes but rather the lumen. This is additionally supported by the observation, from EM, that maspin was easily detected when the lumen of ruptured exosomes was exposed (Figure 3B). In contrast, maspin was not observed at the surface of the exosomes visualized by EM. Further confirmation of maspin within the lumen can be given with experiments such as a protease protection assay.

Chapter 3B: The biological function of exosomal maspin in paracrine communication in the microenvironment

Maspin Knockdown in MCF10A Cells

Previous literature on exosomes has shown that the molecular content associated with the exosome is functional molecules.[80,83,95] The Sheng laboratory showed the impact on tumor expression of maspin on inhibiting stromal reactivity that may specifically involve endothelial cells.[33] To test whether the secreted exosomal maspin contribute to this tumor suppressive effect, isogenic exosomes with and without maspin were generated through the knockdown of maspin in the MCF10A spontaneously immortalized breast cell line, which secretes an abundance of exosomal maspin. Stable transfected cell lines were generated as described in Materials and Methods. The level of maspin knockdown in the lysates of the clones was assessed by immunoblot analysis. The noncoding clones were generated as controls. MCF10A maspin shRNA clones 3, 7, 8, 9 and 11 showed a reduction in maspin levels in the lysate (Figure 12A). The levels of maspin in the noncoding clones were comparable to the parental cell line. Analysis of the VDCM of noncoding clone 1 and 5 revealed secretion of maspin in clone 1 (NC1) only, which was chosen for application in the functional assay (Figure 12B). In contrast, secreted maspin was not detected in maspin shRNA clones 3, 7, 8, and 11. Analysis of the exosomes revealed that exosomal maspin was detected in the noncoding clone 1 (NC1) but not noncoding clone 5 or maspin shRNA clones 3, 7, 8, and 11 (Figure 12C). MCF10A maspin shRNA clone 8 (siMas8) was chosen for application in functional assays. The data demonstrate that

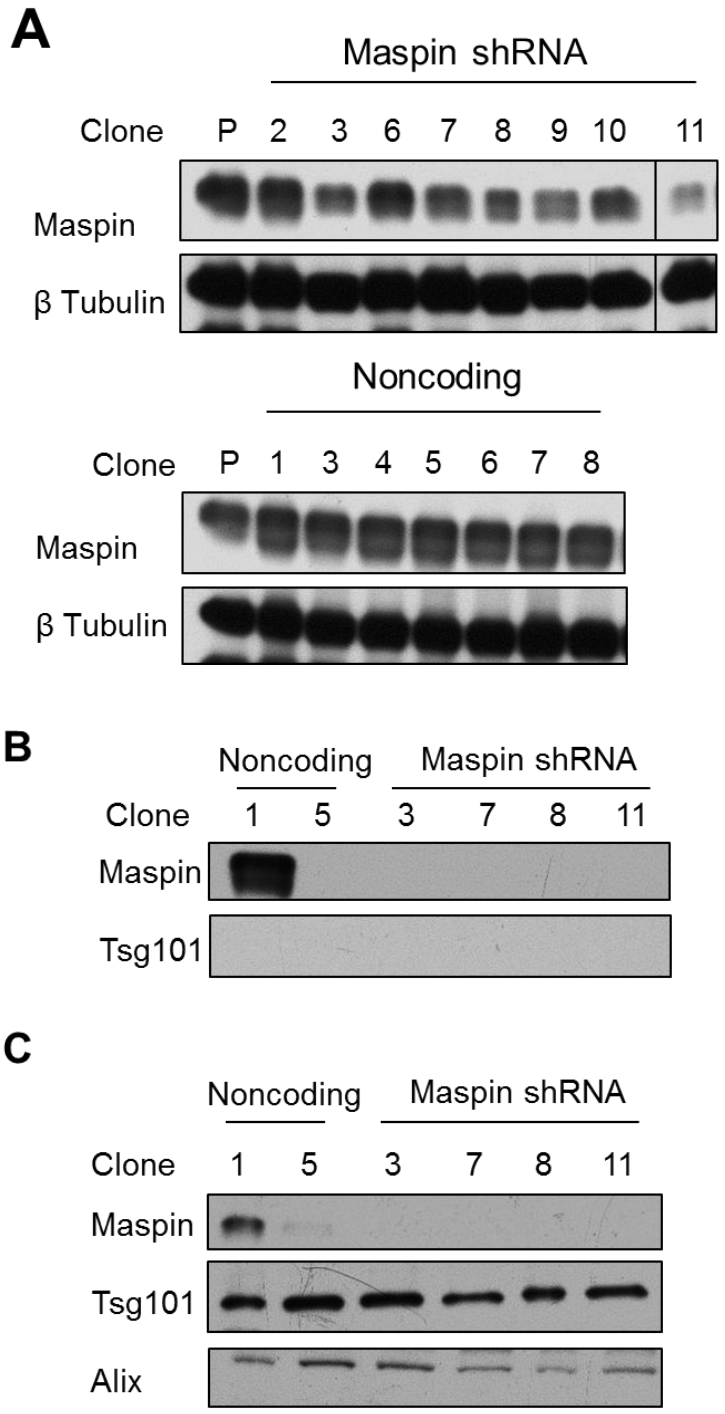

Figure 12 – Knockdown of intracellular maspin affects maspin secretion. Maspin was knocked down in the MCF10A cells and the levels were determined by immunoblot analysis of the lysates (A), VDCM (B), and the exosomes (C). The amount of exosomal maspin was determined by immunoblot analysis. β-tubulin was used as a loading control. Clone number is indicated above each lane. P = parental MCF10A cell line

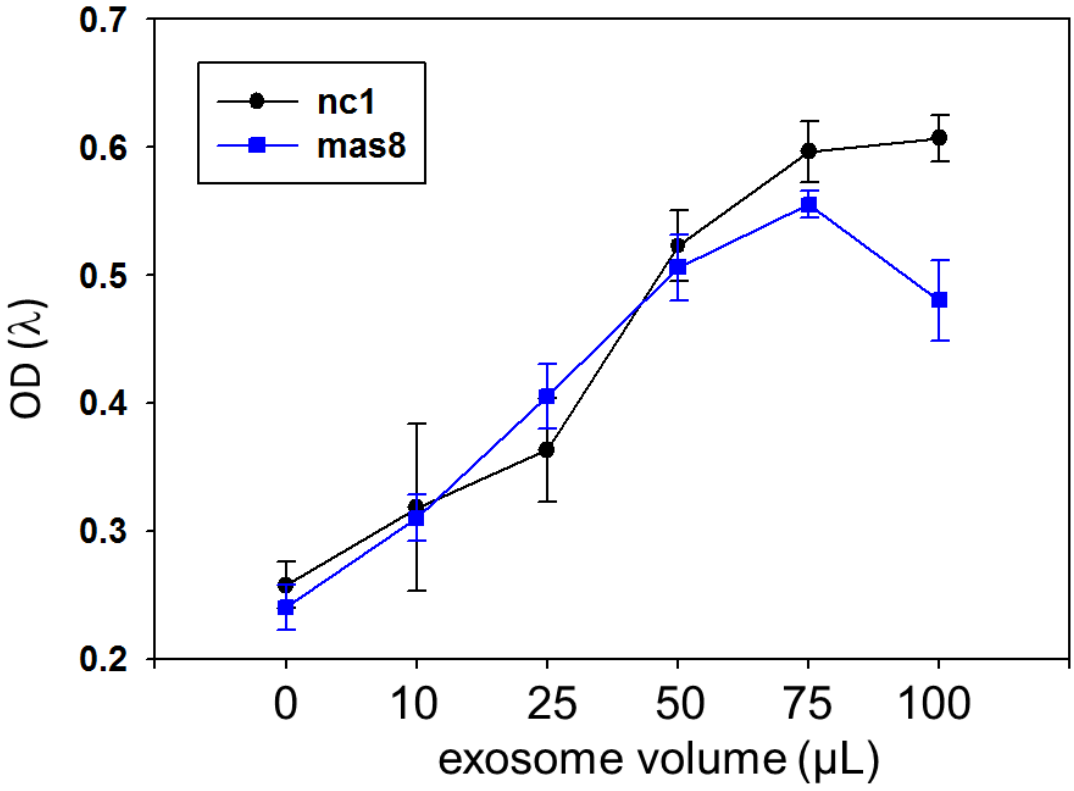

Figure 13 – Cell viability of the NIH 3T3 cells after exosome treatment. The NIH 3T3 cells were treated with various concentrations of exosomes for 24 h. The cell viability was measured with a WST-1 assay.

knockdown of the maspin gene leads to loss of exosomal maspin. The data further emphasizes that maspin is a bona fide exosomal protein.

In order to investigate the role of exosomal maspin in paracrine communication, the cell viability of the NIH 3T3 fibroblasts was analyzed (Figure 13). The concentration of exosomes derived from NC1 and siMas8 were optimized as described in the Material and Methods. NIH 3T3 mouse fibroblast cells, which do not express maspin, were treated with various concentrations of the exosomes. After 24 h of treatment, cell viability of the NIH 3T3 cells was assessed with the WST-1 assay. Cell viability

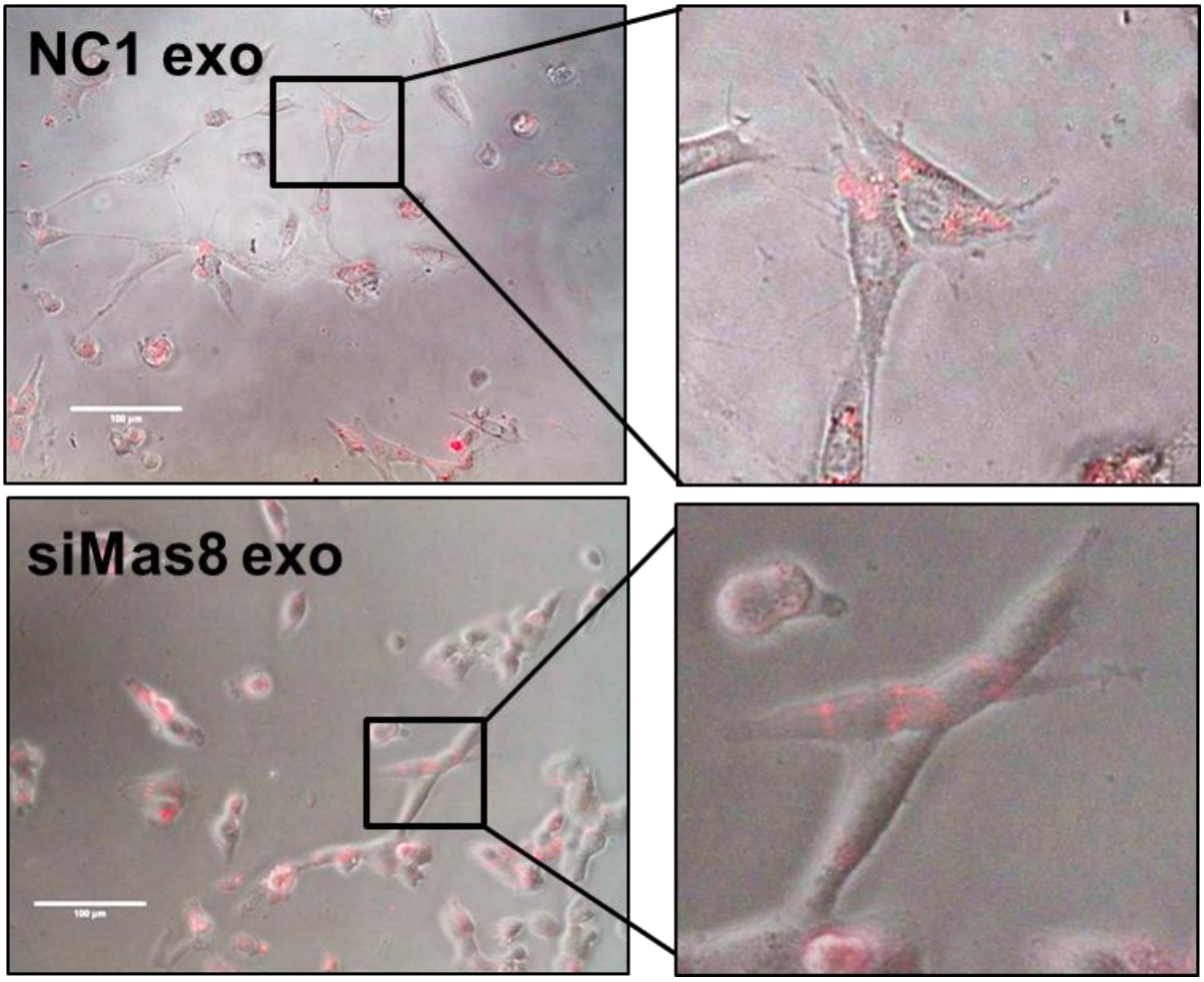

Figure 14 – Exosome uptake by NIH 3T3 fibroblasts. NIH 3T3 cells were treated with PKH26 labeled exosomes for 24 h and visualized with fluorescence microscopy. The red fluorescent images were overlaid with phase contrast images of the cells.

decreased as exosome concentration increased (Figure 13). The optimal concentration of exosomes chosen was at 1:100.

In order for molecular content to exhibit function, the exosomes must be received by a cell. Next, the uptake of the NC1-derived exosomes and the siMas8-derived exosomes were verified. First, the exosomes were labeled with pKH26, a membrane dye, as described in the Material and Methods section. Then, the NIH 3T3

cells were treated with PKH26 labeled exosomes isolated from either NC1 or siMas8 at a 1:100 dilution. Labeling of the exosomes with PKH26 is described in the Material and Methods. NIH 3T3 cells were incubated with the labeled exosomes for 24 h. NIH 3T3 cells accepted the labeled exosomes as judged by the red fluorescence (Figure 14). The underlying mechanism of exosome uptake is unknown. As discussed in the introduciton, there are multiple potential mechanisms for exosome uptake.

The Sheng laboratory has shown that exogenous (rMas) maspin inhibits motility and invasion of cancer cells.[32,41,42] In order to determine the function of exosomal maspin, I evaluated the effect of exosomal maspin on fibroblast motility. NIH 3T3 cells were incubated in a 1:100 dilution of exosomes, in DKSFM, that were derived from either NC1-derived exosomes (NC1 exo) or siMas8-derived exosomes (siMas8 exo) for 24 h. Then, the NIH 3T3 cells were seeded into the upper chamber of transwell plates and incubated for 24 h. The NIH 3T3 cells that were treated with siMas8-derived exosomes (maspin negative exosomes) showed a statistically significant increase in motility compared to the control cells and cells pre-treated with exosomes with maspin (Figure 15A). Interestingly, motility of NC1 exosome pre-treated cells was comparable to the control. Representative images of the cells that migrated through the membrane pores ware shown in Figure 15B. Thus, the loss of maspin seems to stimulate the motility of the fibroblasts. The data further suggest that the normal epithelial-derived exosomes may prevent maspin from directly inhibiting fibroblast motility. In order to determine whether the exosome prevents direct inhibition by maspin, the effect of soluble maspin on motility is under investigation by the Sheng laboratory. Additionally,

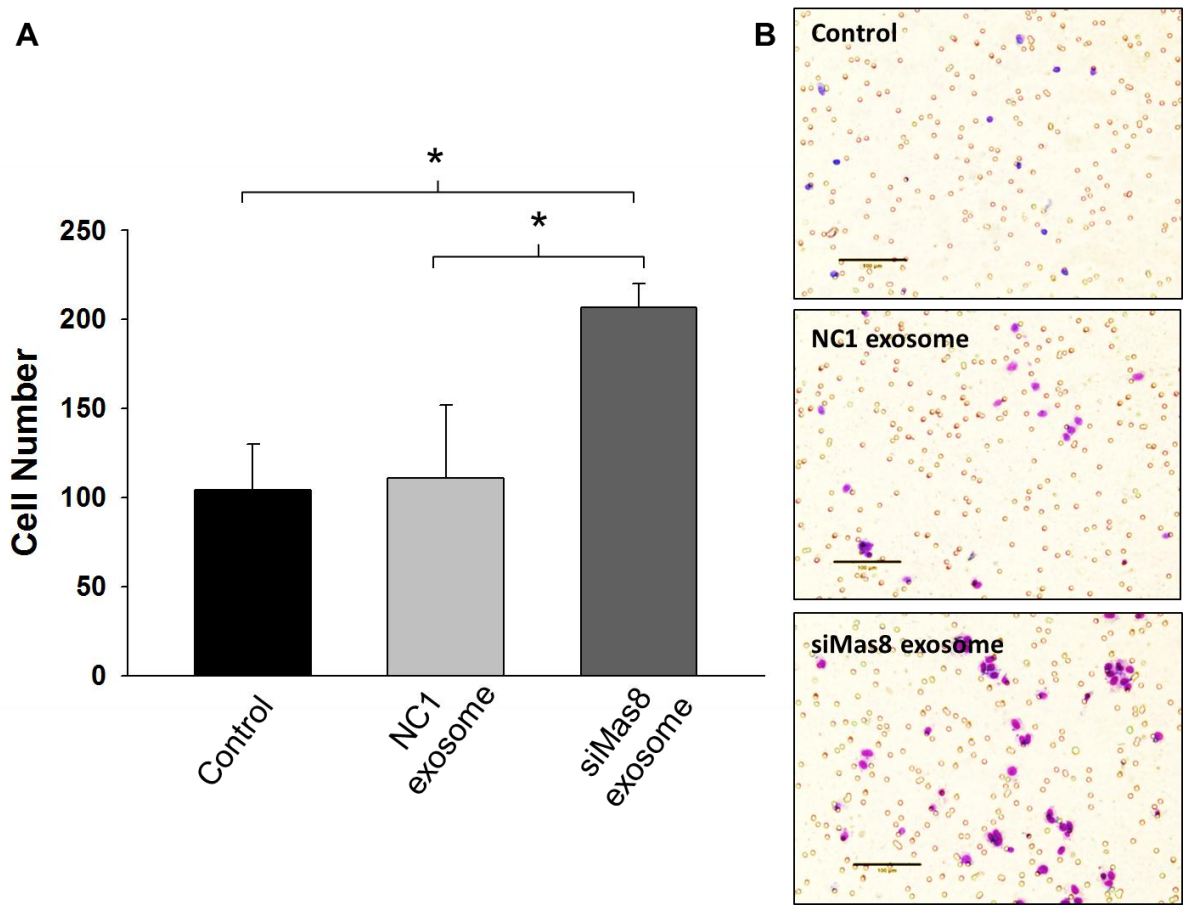

Figure 15 – Loss of exosomal maspin results in increased motility for NIH 3T3 cells. (A) NIH 3T3 cells were pre-treated with or without exosomes derived from the noncoding clone or maspin knockdown clones. Motility of the NIH 3T3 cells was assessed after 24 h. (B) Representative images of the motility of the NIH 3T3 cells after 24 h.

the Sheng laboratory is also testing a second maspin knockdown clone (siMas11) to verify that the loss of maspin stimulates NIH 3T3 fibroblast motility.

Based on the evidence that the exosomes were internalized by NIH 3T3 cells and that maspin is a cargo of the exosome, it is possible that maspin acts inside the recipient cell once the exosome is internalized. In particular, exosomal maspin may function in an epigenetic capacity. To test the possibility, qRT-PCR measurements of

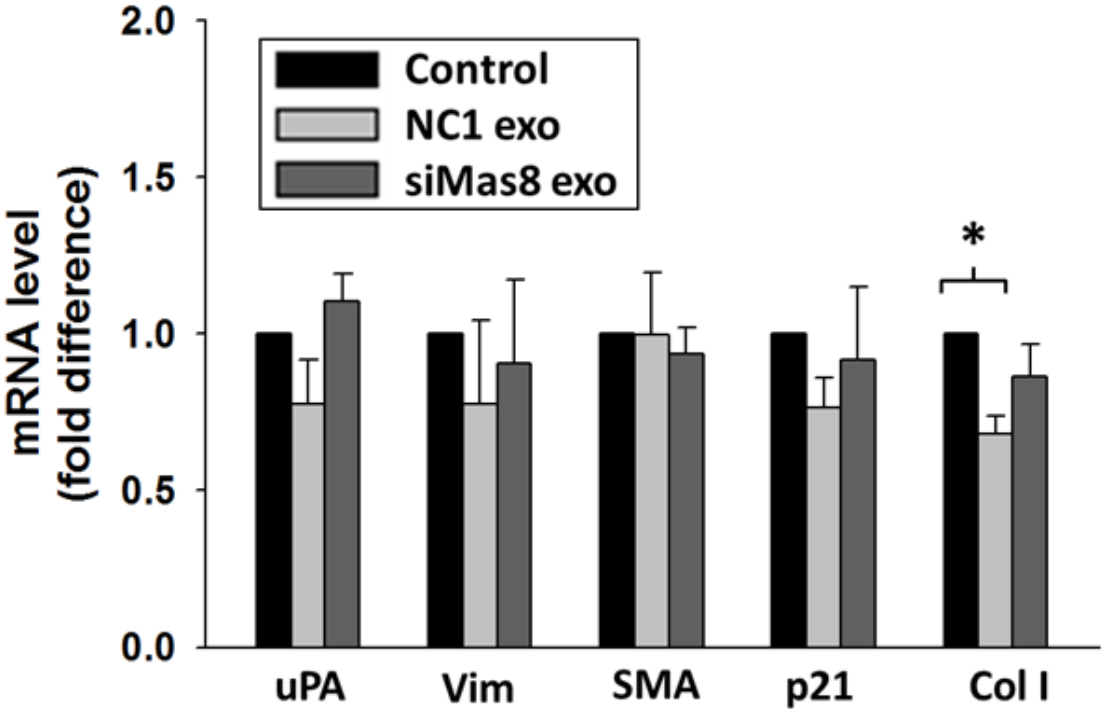

Figure 16 - Messenger RNA levels of various genes in the NIH 3T3 cells after treatment with exosomes derived from either noncoding clone or maspin knockdown clones. Qualitative RT-PCR was performed to measure the levels of mRNA of genes in the NIH 3T3 cells. Analysis of the variance was performed for statistical data. *$p < 0.05$ indicates statistical significance.

several HDAC1 target genes (p21, uPA, Col I and vimentin), along with genes implicated in fibroblast reactivity (Vim and SMA). As shown in figure 16, there was a statistically significant decrease in collagen 1 in cells pre-treated with NC1 exo (maspin positive). This suggests that exosomal maspin may specifically suppress the wound healing activity of the fibroblasts at the step of collagen 1 expression. There was not a statistical difference in mRNA levels of Col I between NIH 3T3 cells treated with NC1 exo and siMas8 exo. In parallel, although not statistically significant, there was a trending decrease in p21, an HDAC1 target gene. Thus, internalized maspin may indeed inhibit HDAC1. Although there was a decrease in uPA in cells treated with NC1

exo, the change was not statistically significant. Overall, the exosomal maspin seem to have a limited capacity in causing epigenetic changes. This may be due to the amount of maspin available via the exosomes.

Chapter 4: Discussion

Hypothetical Model

This study suggests that the exosome acts as a barrier between maspin and the extracellular microenvironment, which gives exosomal maspin a fate that is distinct from the fate of soluble maspin. Different modes of secretion thereby segregate the functions of soluble and exosomal maspin. As described in Figure 17, secreted soluble maspin can interact locally with cell surface proteins, such as its molecular partner, uPA. Cell surface activity of soluble maspin may be immediate and short-lasting, since soluble maspin is exposed to the extracellular environment. In contrast, encapsulation of

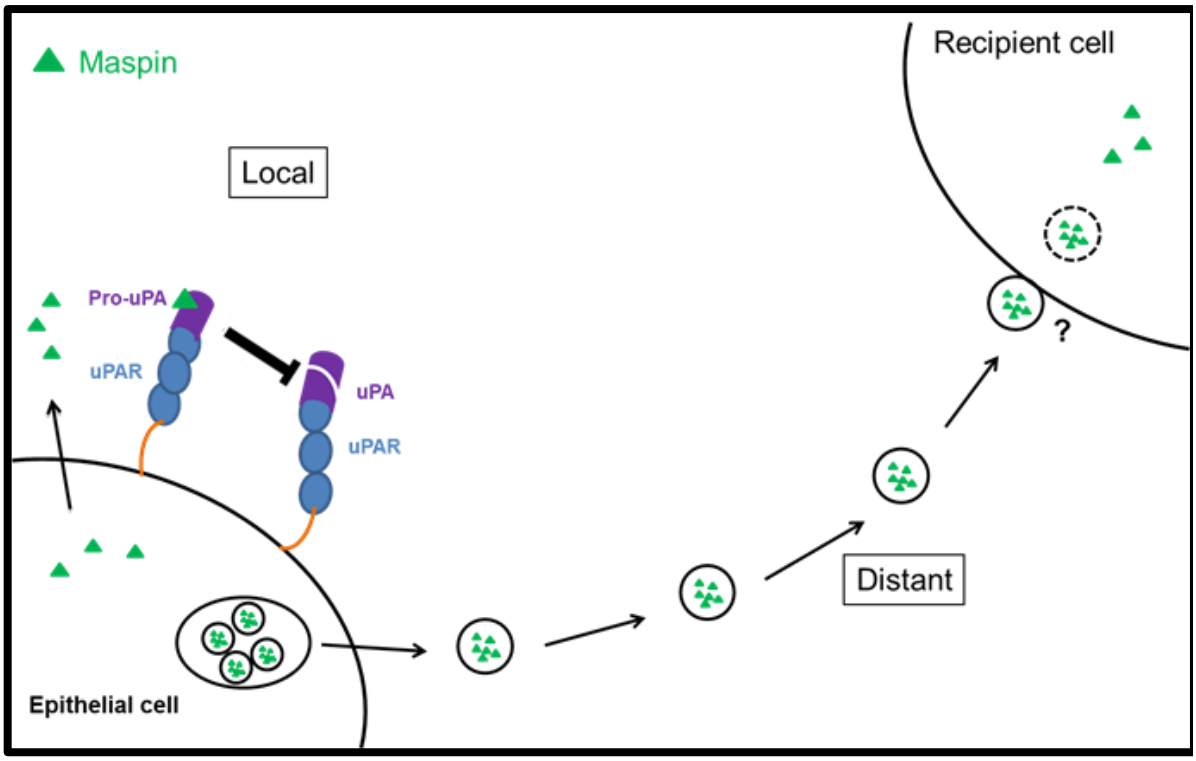

Figure 17 - Hypothetical Model of the Extracellular Activity of Soluble and Exosomal Maspin. Soluble maspin acts at the cell surface and has local activity. The exosomal maspin, because of encapsulation, cannot act the cell surface. However, exosomal maspin can travel to distant locations to communicate with target (recipient) cells such as the stromal cells.

exosomal maspin may serve as a protective barrier from the extracellular environment. Exosome encapsulation allows maspin to bypass cell surface activity with uPA. Unlike soluble maspin, the exosomal maspin may have delayed activity because the exosome has to reach a target cell and be internalized.

The cell can internalize both soluble maspin and exosomal maspin. Internalization may be the limiting step for exosomal maspin to elicit its function inside the recipient cell. The actions of soluble maspin may be more immediate after secretion since it is not encapsulated by the exosome. Once inside of the cell, exosomal maspin may interact with the same target proteins as internalized soluble maspin. For example, rMas has been shown to inhibit breast cancer cell motility through inhibition of Rac signaling and promote cell adhesion through PI3K signaling.[44] Therefore, the effect of maspin on Rac and PI3K signaling is a feasible future direction. Through the exosomes, maspin is involved in paracrine signaling. It is equally possible that the exosomes can be involved in autocrine signaling (not depicted in hypothetical model). The exosomes may also be taken up by neighboring cells and the cell that secreted the exosomes.

Dual and Independent Mechanisms of Secretion of Maspin

This study offers new insights into the secretion of maspin. Since the discovery of maspin, its secretion has been observed and shown to have tumor suppressive activity. Additionally, maspin has been reported as an exosomal protein. Maspin was first demonstrated to be in the exosomes of irradiated H460 lung adenocarcinoma cells.[69] The H460 lung adenocarcinoma cells express intracellular maspin but do not secrete exosomal maspin unless irradiated. This observation is consistent with my data, which

shows that tumor cells secrete little to no exosomal maspin. Secretion of exosomal maspin was also shown to differ between undifferentiated and differentiated keratinocytes.[70] Maspin was detected in the exosomes of the undifferentiated keratinocytes and reported as a 25kDa protein, although the molecular weight of maspin is 42 kDa. Neither of the previous reports addressed the coexistence of both exosomal maspin and soluble maspin.

This study is the first to show that exosome-secreted maspin is distinct from the secreted soluble maspin, since exosomal maspin can be isolated from the conditioned media (CM), while soluble maspin remains in the CM. The secretion of maspin as either a soluble or exosomal protein is not mutually exclusive, but concurrent. The discrimination of exosomal maspin between normal cell lines and tumor cell lines may indicate maspin secretion via the exosomes is a naturally occurring, regulated process perturbed in tumor cells. This study suggests that the routes of secretion for soluble maspin and exosomal maspin are independent and differ mechanistically. Furthermore, neither soluble maspin nor exosomal maspin depend on the ER-Golgi secretory pathway.

Exosomal maspin depends on endosome trafficking for secretion. Endosomes are organelles formed from endocytosis at the plasma membrane.[71] After endocytosis, the endosome undergoes maturation, in which the luminal pH becomes increasingly acidic as it goes from the early endosome to the late endosome. The endosomes form intraluminal vesicles (ILVs) to become multivesicular bodies (MVBs). When the endosome fuses to the plasma membrane, the ILVs are released as exosomes. During the formation of the ILVs, proteins, such as maspin, are recruited to the endosome

membrane. The recruitment of proteins may depend on transient ubiquitination of the protein, followed by de-ubiquitination of the protein after recruitment.[77,78] Previously, our laboratory has shown that maspin was degraded by the proteasome, which is dependent on ubiquitination.[68,119,120] Therefore, it is possible that maspin may be ubiquitinated and recruited to the endosome membrane during ILV formation. Inhibition of endosome trafficking with chloroquine (CQ), an anti-malarial drug that increases the pH of the endosome, subsequently inhibits exosome secretion and exosomal maspin levels. However, it is unclear at which point of the endosome trafficking process (endosome plasma membrane fusion, ILV formation, exosome secretion, etc.) is targeted by CQ to affect exosomal maspin secretion.

This study demonstrates that inhibiting the secretion of exosomal maspin does not affect the secretion of soluble maspin. Currently, the mechanism of secretion of soluble maspin is not clear. Efforts to ascertain the mechanism revealed that drugs commonly used to inhibit various secretion pathways did not inhibit the secretion of soluble maspin. In contrast, secretion of soluble maspin increased under drug treatment, as well as general cellular stress. Fusion of the endosome to the plasma membrane may release exosomes, as well as soluble proteins. Soluble maspin is most likely not secreted by the endosomes since drugs targeting endosome trafficking did not inhibit soluble maspin, but by an alternative non-classical pathway. Non-classical secretion may occur by different mechanisms, which may be vesicular or non-vesicular. Microvesicles, such as exosomes, membrane blebs, and apoptotic bodies, represent vesicular methods of secretion. There are also several non-vesicular routes of non-

classical secretion, such as direct translocation across the plasma membrane. Further investigation into the mechanism of secretion of soluble maspin is warranted.

Exosomal Maspin and Tumor Progression

This study is the first to demonstrate that fibroblast motility is stimulated by exosomes that have loss maspin. Interestingly, although maspin has been shown to inhibit cell motility, treatment with NC1 exo, which contain maspin, did not inhibit the motility of the NIH 3T3 cells. Our data raised the possibility that normal cell-derived exosomes may be naïve but may become tumor promoting when maspin is down regulated. The loss of exosomal maspin may result in early gain of function for stromal cells in the tumor microenvironment. Alternatively, exosomal maspin may be sufficient to block the activation or activity of mesenchymal cells, which is lost when maspin is down regulated. It was previously reported that extracellular maspin readily inhibits tumor cell motility and invasion *in vitro*.[121] While further studies are underway to dissect the differences between soluble maspin and exosomal maspin, internalization of exosomal maspin may have tumor-suppressive activity.

At the molecular level, the Sheng laboratory identified maspin as the first polypeptide inhibitor of HDAC1 thus far.[49,50] Data from this study suggests that exosomal maspin may exert its biological effects without significantly altering the expression of many HDAC1 target genes. In order to validate our observations, a wider array of HDAC1 target genes needs to be tested. It was noted that the presence of exosomal maspin resulted in a decrease in collagen I mRNA in NIH 3T3 cells, while the absence of exosomal maspin resulted in an increase in NIH 3T3 cell motility. Thus, the

underlying mechanism for the effect of maspin on mesenchymal cells may differ from that on epithelial cells. The data are the first to show that the biological functions of exosomal maspin may be differentially regulated in the continuum of tumor progression.

Proteins undergo aberrant trafficking in tumor cells as a response to transformation and the challenges of tumor microenvironment. The secretion of maspin may be subjected to differential regulation by tumor cells in response to epithelial transformation and changes within the tumor microenvironment. The data show that soluble maspin is secreted at a lower level in maspin-expressing tumor cell lines than normal cell lines. In comparison, exosomal maspin was barely detectable in tumor cell lines that express various levels of intracellular maspin. This is the first evidence that exosomes produced by normal and tumor cells have distinct features. This data is consistent with Yu *et. al.* (2006) who showed that exosomes derived from H460 lung adenocarcinoma cells did not express maspin when cells were grown under exponential growth conditions. In contrast, maspin expression was induced in the exosomes through irradiation of the H460 cells. While the loss of secreted soluble maspin may be coupled with the down-regulation of intracellular maspin, the loss of exosomal maspin may occur before the down-regulation of intracellular maspin. This may be due to early dysregulation of exosome machinery in tumorigenesis, since the molecular content of the normal cell-derived and tumor cell-derived exosomes differ. During biogenesis of the exosomes as ILVs, cytoplasmic proteins are recruited to the invaginated endosome membrane. Cytoplasmic maspin levels increase during tumor progression at the stage of pre-neoplastic lesions.[29,52] Therefore, we speculate that an increase in cytoplasmic maspin may increase the amount of maspin recruited to the endosome. This study is

the first to show that exosomal maspin may also be down regulated during tumor progression. Further investigation is needed to determine whether biphasic expression of intracellular maspin affects exosomal maspin.

Equally possible, an increase in cytoplasmic maspin may not affect the amount of maspin recruited to the endosome. The ESCRT proteins are responsible for the recruitment of proteins, as well as ILV formation, scission, and back-fusion.[77,78] In line with the function of the ESCRT proteins, recruitment of maspin most likely depends on a particular set of ESCRT proteins. Loss of exosomal maspin may occur if those ESCRT proteins are down regulated in tumor. Further investigation is necessary to determine whether loss of maspin in tumor cell-derived exosomes occurs at the recruitment stage of ILV formation.

The data reported here are the first evidence that the exosomes derived from normal cells have a distinct size distribution pattern from the exosomes derived from tumor cells. Dynamic light scattering revealed two distinct populations of particles enriched from the normal-derived exosome fraction by differential centrifugation. The first population is below 100 nm in diameter, with an average size consistent with exosome size range, which suggests the first population is the exosomes. In contrast, the second population was absent in the exosome fraction isolated from tumor cells. Therefore, the second population is a novel population of particulates secreted by the normal cells. Despite the presence of the second population, the zeta potential of both normal- and tumor-derived exosome fractions was negative. Given these observations, further investigation is necessary to evaluate the significance of the second population detected in the normal-derived exosome fraction.

The quality of our subcellular fractionation and exosomal purification was ascertained by multiple approaches. My experiments were performed under exponential cell growth condition, without detectable cell lysis or cell death. The tumor cell-derived exosomes lost maspin, which was disproportional to the overall changes of maspin expression. Considering the earlier report that maspin was detected in exosomes of H460 lung adenocarcinoma cells only when the cells were irradiated, it seems necessary to caution that irradiation may damage the integrity of cellular structure and lead to contamination of the exosomal fraction with other microvesicles and lytic cell debris.[69]

Diagnostic Potential of Exosomal Maspin

Combined with the previous literature showing that exosomes circulate in bodily fluids, my novel observations indicate that exosomal maspin status may be a potential diagnostic marker. Our study is the first to demonstrate that maspin is consistently expressed in exosomes produced by normal cell lines, but not in tumor-derived exosomes. This expression pattern of exosomal maspin is distinct from the expression patterns of soluble maspin and intracellular maspin. Exosomal maspin may be lost before intracellular maspin is lost. The loss of exosomal maspin may pinpoint an early stage in tumor progression, which would advantageous for the early detection of aberrant cells. As a diagnostic marker, the status of maspin in the exosome could indicate whether or not a patient has aberrant cells present in the body. More specifically, the lack of maspin in the exosomes may indicate the presence of aberrant

cells. The presence of maspin in the exosomes may indicate a normal state of the cells in the body.

In order to establish exosomal maspin as a diagnostic tool, a major limitation would have to be addressed. The limitation is that bodily fluids contain a mixture of exosomes derived from various cellular origins. Although the exosomes can be isolated from the bodily fluids, the presence of many different exosomes may dilute the amount of exosomal maspin. The limitation can be addressed by creating diagnostic tools that are specific and sensitive to epithelial exosomes and exosomal maspin. Maspin is expressed solely in epithelial cells and, as a result, epithelial-derived exosomes. Therefore, an assay may be developed to isolate epithelial-derived exosomes with an antibody targeting an exosomal transmembrane protein of epithelial origin. Then, the epithelial-derived exosomes may be analyzed for exosomal maspin and compared to a baseline level of exosomal maspin. Since many solid tumors are of epithelial origin, the epithelial-derived exosomes may be a mixture of normal cell-derived exosomes and tumor-derived exosomes. In conjunction with isolating the epithelial-derived exosomes, the tumor-derived exosomes may be further isolated from the normal-derived exosomes with an antibody specific to a transmembrane protein tumor-derived exosomes. Then, the status of exosomal maspin may be determined. Of note, the exosome field is investigating the molecular content of tumor-derived exosomes in order to establish reliable, specific tumor-derived exosome markers. Further investigation is needed to determine whether maspin's status in the exosome may be a beneficial diagnostic marker.

The diagnostic potential of the exosomes is of interest because the exosomes may provide a non-invasive method for cancer diagnosis. The feasibility of using exosomes as diagnostic markers is already being explored in the science industry. For example, Exosome Diagnostics, Inc. and Exosome Sciences, Inc. are two companies that are developing assays to use exosomes as diagnostic tools. These companies have a particular interest in exosomal miRNA for diagnostics and therapeutics. My research suggests that exosomal maspin may provide a basis for a novel protein-based diagnostic tool.

Prognostic Potential of Exosomal Maspin

Correlation of intracellular maspin expression to patient outcome differs across various cancer types differs. Additionally, not all cancer cells lose intracellular maspin expression. Of note, strong nuclear expression of maspin was shown to correlate with increased overall patient survival in comparison to strong cytoplasmic expression and loss of intracellular maspin.[52,54,55] However, there are limitations in determining clinical outcome based on nuclear maspin expression. Our data indicates maspin positive aberrant cells of various origins lose exosomal maspin. In addition, my data show that forced re-expression of maspin in maspin negative tumor cells does not automatically reinstate exosomal maspin. Yu *et al* (2006) showed that subjecting cancer cells to radiation induced the presence of exosomal maspin. Our data indicate that exosomal maspin may have tumor suppressive activity. However, we do not know whether induction of exosomal maspin in tumor cells would correlate with a better clinical outcome. The prognostic value of exosomal maspin may rely on establishing a

correlation between induced exosomal maspin after therapeutic drug treatment and better overall survival. If induction of exosomal maspin correlates to a positive therapeutic drug response, then exosomal maspin would serve as a novel prognostic marker. Further investigation is necessary to determine the prognostic value of exosomal maspin after therapeutic drug treatment.

Therapeutic Potential of Exosomal Maspin

The tumor-suppressive activity of maspin makes it a protein of therapeutic interest. Re-expression of maspin in tumor cell lines resulted in sensitivity to drug-induced apoptosis. In our model, we showed that re-expression of maspin was not sufficient to restore exosomal maspin. The exosomes represent a natural mechanism for delivering molecular materials. Currently, synthetic liposomes are being developed as drug delivery systems. Maspin may have therapeutic use as a tumor suppressor by packing maspin into synthetic liposomes. Combined with data that exosomal maspin has tumor suppressive activity, delivery of maspin via synthetic liposomes may be beneficial for anti-tumor response.

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ABSTRACT

A TUMOR-SUPPRESSIVE ROLE OF MASPIN SECRETED VIA THE EXOSOMES

by

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This dissertation highlights several novel findings. Maspin has been consistently detected in the conditioned media of maspin-expressing cells of normal and tumor breast, prostate and lung origin. Furthermore, extracellular maspin has been demonstrated to have anti-tumor effects. Interestingly, maspin has been reported as cargo of the exosomes, which highlights one of the secretion mechanisms of maspin. Maspin secretion as an exosomal molecule was verified by electron microscopy, atomic force microscopy, light scattering dynamic analysis and immunoblot analysis.

The data showed that exosomes derived from the non-malignant cell lines have two distinct populations that do no overlap in their size distributions. Based on the size distribution and the electron microscopy analysis, it is likely that exosomes derived from the non-malignant cells are aggregated exosomes.. In contrast, tumor cell-derived exosomes comprised a population of broader size distribution.

To understand how secreted maspin may contribute to tumor suppression, it is critical to understand how maspin is regulated at the step of protein trafficking. The data showed that maspin is secreted by dual mechanisms, as free and exosomal protein,

respectively. These two mechanisms seem to be independent. While tumor cells are capable of secreting maspin as a free molecule, albeit at a lower level as compared to that by normal epithelial cells, they do not secrete exosomal maspin.

Loss of maspin in exosomes from derived non-malignant cells conferred a stimulatory effect on the motility of fibroblasts, suggesting a biological function of exosomal maspin in suppressing the stromal reactivity in the tumor microenvironment. These novel findings highlight a new role for exosomal maspin as a tumor suppressor.

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Journal Publications

1. Sijana Dzinic, Alexander Kaplun, Xiaohua Li, Margarida M. Bernardo, Yonghong Meng, Ivory Dean, David Krass, Fulvio Lonardo, Shijie Sheng**. Identification of an Intrinsic Determinant Critical for Maspin Subcellular Localization and Function.** PLoS One. 2013, 8(11): e74502
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