Metastasis and Cathepsin B

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By

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ABSTRACT

Metastasis and Cathepsin B

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Cancer is one of the leading causes of mortality in the United States as well as the developing world. Metastasis is responsible for about 90% of cancer deaths, and is a cause of morbidity in those who continue to battle it. Most cancers can be attributed to genetic mutations as well as unhealthy behaviors, but a tumor can metastasize from its primary site into the blood and to distant organs if the tumor microenvironment is supportive. A number of endogenous proteases, embedded in a complex protease network, are shown to be upregulated in cancers and aid in metastasis. This study examines cathepsin B, which is reported to play an important role in tumor progression and metastasis, as a target for drug therapy. It is usually tightly regulated, but in metastasis it cleaves the extracellular matrix of cancer cells to aid in invasion. Inhibition of cathepsin B has been shown to limit metastasis, although ultimately, any attempt to curtail the metastatic process through proteases must consider the relationships of the protease network.

TABLE OF CONTENTS

Chapter 1: Cancer

A. Cancer has high mortality and incidence rates in the United States
B. Cancer in the United States is a multi-layered problem
C. Cancer is a problem of developed as well as developing nations
D. Tumor grade and cancer stage describe the progress of cancer in the human body
E. Statement of Purpose

Chapter 2: Metastasis

A. Metastasis is the cause of 90% of cancer deaths
B. The Invasion-Metastasis Cascade is a useful and accepted way of illustrating the transition of a tumor cell from primary tumor to metastasis.
C. The tumor cell can proceed toward metastasis via the epithelial-mesenchymal transition, amoeboid migration, or both.

Chapter 3: Proteases

A. Whether or not tumor cells metastasize depends greatly on the tumor microenvironment and its constituents.
B. MMPs and Cathepsins are the two most important protease families in the tumor microenvironment.

Chapter 4: Cathepsin B

A. Current treatments of metastasis, a health concern of both mortality and morbidity, have limited efficacy
B. Structure-based exogenous inhibitors of Cathepsin B have been useful in determining pathologies stemming from abnormal expression of Cathepsin B
C. A number of cancers implicate cathepsin B-dependent pathways in tumor progression and metastasis. Inhibiting cathepsin B has been shown to limit metastasis *in vivo*
D. Discussion and Conclusions

ABBREVIATIONS

ACS= American Cancer Society
ADAM= a disintegrin and metalloproteinase
ADAMT= a disintegrin and metalloproteinase with thrombospondin motifs
Bmp7= bone morphogenetic protein 7
Ca2+= calcium ion +2 charge
CMM= cutaneous malignant melanoma
CML= chronic myeloid leukemia
CTC= circulating tumor cell
ECM= extracellular matrix
EMT= epithelial-mesenchymal transition
GTP= Guanosine-5'-triphosphate
HMWK= high molecular weight kininogens
LWWK= low molecular weight kininogens
MAT/AMT= mesenchymal-amoeboid transition/Amoeboid-mesenchymal transition
MET= mesenchymal-epithelial transition
MMP= Matrix-metalloproteinase
MLC= myosin II regulatory light chain
MLCK= myosin light chain kinase
MLCP= myosin light chain phosphate
NCI = National Cancer Institute
NK cell= natural killer cell
Pax2= paired box 2
PP1= protein phosphatase 1
Rho: ras homolog gene
ROCK= rho-associated protein kinase
TAM= tumor-associated macrophage
TGFβ = transforming growth factor-β
TIMP= tissue inhibitors of metalloproteinases
TNM = tumor, node, metastasis (staging system)
TrkB= neurotrophic tyrosine kinase receptor, type 2
Wt1= wilms tumor 1
ZEB1, 2= zinc finger e-box-binding homeobox

CHAPTER ONE

Cancer

Cancer has high mortality and incidence rates in the United States.

One in four deaths in the United States is caused by cancer; and metastasis, or the spread of cancer to distant sites, is the leading cause of cancer death. Cancer closely follows heart disease as a lifetime leading cause of death in both sexes, with men being more likely (about 1 in 2) to develop an invasive cancer than women (about 1 in 3). Over half a million people are estimated to die of cancer in 2013 in the United States (mortality: 178.7 per 100,000 people) (Siegel et al, 2013).

Table1: Probability of cancers by age and sex in the United States (2007-2009)

		BIRTH TO 39	40 TO 59	60 TO 69	70 AND OLDER	BIRTH TO DEATH
All sites†	Male	1.46 (1 in 69)	8.79 (1 in 11)	16.03 (1 in 6)	38.07 (1 in 3)	44.81 (1 in 2)
	Female	2.20 (1 in 46)	9.19 (1 in 11)	10.39 (1 in 10)	26.69 (1 in 4)	38.17 (1 in 3)
Urinary bladder‡	Male	0.02 (1 in 4,924)	0.37 (1 in 272)	0.92 (1 in 109)	3.69 (1 in 27)	3.81 (1 in 26)
	Female	0.01 (1 in 12,663)	0.12 (1 in 864)	0.24 (1 in 410)	0.98 (1 in 106)	1.15 (1 in 90)
Breast	Female	0.50 (1 in 202)	3.78 (1 in 26)	3.56 (1 in 28)	6.65 (1 in 15)	12.38 (1 in 8)
Colorectum	Male	0.08 (1 in 1,212)	0.94 (1 in 106)	1.40 (1 in 71)	4.19 (1 in 24)	5.17 (1 in 19)
	Female	0.08 (1 in 1,236)	0.75 (1 in 134)	0.98 (1 in 102)	3.80 (1 in 26)	4.78 (1 in 21)
Leukemia	Male	0.16 (1 in 612)	0.23 (1 in 440)	0.35 (1 in 288)	1.26 (1 in 80)	1.59 (1 in 63)
	Female	0.13 (1 in 746)	0.15 (1 in 655)	0.21 (1 in 481)	0.81 (1 in 123)	1.14 (1 in 88)
Lung & bronchus	Male	0.03 (1 in 3,552)	0.92 (1 in 109)	2.27 (1 in 44)	6.82 (1 in 15)	7.77 (1 in 13)
	Female	0.03 (1 in 3,287)	0.76 (1 in 131)	1.72 (1 in 58)	4.93 (1 in 20)	6.35 (1 in 16)
Melanoma of the skin§	Male	0.15 (1 in 691)	0.63 (1 in 160)	0.77 (1 in 130)	2.02 (1 in 50)	2.87 (1 in 35)
	Female	0.26 (1 in 391)	0.55 (1 in 181)	0.40 (1 in 248)	0.84 (1 in 120)	1.85 (1 in 54)
Non-Hodgkin lymphoma	Male	0.13 (1 in 753)	0.44 (1 in 225)	0.60 (1 in 167)	1.77 (1 in 57)	2.34 (1 in 43)
	Female	0.09 (1 in 1,147)	0.31 (1 in 322)	0.44 (1 in 229)	1.40 (1 in 72)	1.93 (1 in 52)
Prostate	Male	0.01 (1 in 7,964)	2.68 (1 in 37)	6.78 (1 in 15)	12.06 (1 in 8)	16.15 (1 in 6)
Uterine cervix	Female	0.16 (1 in 641)	0.27 (1 in 374)	0.13 (1 in 795)	0.18 (1 in 551)	0.68 (1 in 147)
Uterine corpus	Female	0.07 (1 in 1,348)	0.77 (1 in 129)	0.89 (1 in 112)	1.25 (1 in 80)	2.64 (1 in 38)

*For people free of cancer at beginning of age interval.

†All sites excludes basal cell and squamous cell skin cancers and in situ cancers except urinary bladder.

‡Includes in situ cancer cases.

§Statistics for whites only.

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Cancer is one of the top 5 causes of death for both males and females, at every age. The leading causes of death for American males under age 20 are 1) accidents, 2) homicides, 3) suicide, and 4) cancer. American females of the same age die most commonly of 1)

accidents and 2) cancer. While cancer ranks relatively high in young people, it becomes the leading cause of death in adults between the ages of 40 and 80 in both sexes. Heart disease is the most common cause of deaths in adults ages 80 and older (Siegel et al, 2013).

Table 2: The Top Causes of Death in the United States by Age and Sex

	ALL AGES		AGES 1 TO 19		AGES 20 TO 39		AGES 40 TO 59		AGES 60 TO 79		AGES ≥80	
	MALE All Causes 1,217,379	FEMALE All Causes 1,219,784	MALE All Causes 13,919	FEMALE All Causes 7,702	MALE All Causes 62,116	FEMALE All Causes 28,792	MALE All Causes 227,801	FEMALE All Causes 142,628	MALE All Causes 467,962	FEMALE All Causes 373,658	MALE All Causes 430,581	FEMALE All Causes 655,337
1	Heart diseases 307,225	Heart diseases 292,188	Accidents (unintentional injuries) 5,317	Accidents (unintentional injuries) 2,645	Accidents (unintentional injuries) 21,388	Accidents (unintentional injuries) 7,228	Cancer 54,483	Cancer 50,579	Cancer 154,168	Cancer 127,506	Heart diseases 130,332	Heart diseases 193,676
2	Cancer 296,763	Cancer 270,865	Assault (homicide) 2,031	Cancer 848	Intentional self-harm (suicide) 8,977	Cancer 4,629	Heart diseases 52,826	Heart diseases 21,353	Heart diseases 118,163	Heart diseases 74,294	Cancer 82,765	Cancer 87,264
3	Accidents (unintentional injuries) 75,022	Cerebro-vascular disease 76,769	Intentional self-harm (suicide) 1,500	Assault (homicide) 569	Assault (homicide) 7,214	Heart diseases 2,393	Accidents (unintentional injuries) 24,265	Accidents (unintentional injuries) 11,333	Chronic lower respiratory diseases 31,425	Chronic lower respiratory diseases 31,457	Chronic lower respiratory diseases 27,930	Cerebro-vascular disease 51,445
4	Chronic lower respiratory diseases 65,119	Chronic lower respiratory diseases 72,234	Cancer 1,042	Congenital anomalies 495	Heart diseases 5,256	Intentional self-harm (suicide) 2,140	Intentional self-harm (suicide) 11,858	Cerebro-vascular disease 5,283	Cerebro-vascular disease 19,751	Cerebro-vascular disease 19,317	Cerebro-vascular disease 24,649	Alzheimer disease 47,856
5	Cerebro-vascular disease 52,073	Alzheimer disease 55,103	Congenital anomalies 563	Intentional self-harm (suicide) 434	Cancer 4,256	Assault (homicide) 1,443	Chronic liver disease & cirrhosis 10,562	Chronic lower respiratory diseases 5,134	Diabetes mellitus 16,646	Diabetes mellitus 13,572	Alzheimer disease 18,689	Chronic lower respiratory diseases 35,212
6	Diabetes mellitus 35,054	Accidents (unintentional injuries) 42,999	Heart diseases 411	Heart diseases 295	HIV disease 1,295	Pregnancy, childbirth & puerperium 721	Diabetes mellitus 7,346	Chronic liver disease & cirrhosis 4,654	Accidents (unintentional injuries) 12,728	Nephritis, nephrotic syndrome & nephrosis 8,254	Influenza & pneumonia 13,134	Influenza & pneumonia 18,559

Cancer remains in the top 5 causes of death at every age in both genders. Accidents and heart diseases contribute to many of the total deaths as well.
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Cancers are named for their origins in the human body, i.e. breast cancer begins in the breast, and lung cancer begins in the lung. Cells are considered cancerous when their growth becomes abnormal due to poor or no regulation (Medline Plus). As cancer cells grow excessively they begin to form masses (tumors) that can either be benign or malignant (NCI Tumor Grade Fact Sheet). Due to physiological differences, the various cancers occur and kill at different rates in men and women. Risk factors for cancer can be

social (i.e. race), or physiological (i.e. estrogen exposure). Lung cancer results in the greatest number of deaths of all cancers in the United States, but the cancer with the highest incidence in men is prostate cancer, and in women it is breast cancer (Siegel et al, 2013.)

Estimated New Cases*

Males			Females		
Prostate	238,590	28%	Breast	232,340	29%
Lung & bronchus	118,080	14%	Lung & bronchus	110,110	14%
Colorectum	73,680	9%	Colorectum	69,140	9%
Urinary bladder	54,610	6%	Uterine corpus	49,560	6%
Melanoma of the skin	45,060	5%	Thyroid	45,310	6%
Kidney & renal pelvis	40,430	5%	Non-Hodgkin lymphoma	32,140	4%
Non-Hodgkin lymphoma	37,600	4%	Melanoma of the skin	31,630	4%
Oral cavity & pharynx	29,620	3%	Kidney & renal pelvis	24,720	3%
Leukemia	27,880	3%	Pancreas	22,480	3%
Pancreas	22,740	3%	Ovary	22,240	3%
All Sites	**854,790**	**100%**	**All Sites**	**805,500**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	87,260	28%	Lung & bronchus	72,220	26%
Prostate	29,720	10%	Breast	39,620	14%
Colorectum	26,300	9%	Colorectum	24,530	9%
Pancreas	19,480	6%	Pancreas	18,980	7%
Liver & intrahepatic bile duct	14,890	5%	Ovary	14,030	5%
Leukemia	13,660	4%	Leukemia	10,060	4%
Esophagus	12,220	4%	Non-Hodgkin lymphoma	8,430	3%
Urinary bladder	10,820	4%	Uterine corpus	8,190	3%
Non-Hodgkin lymphoma	10,590	3%	Liver & intrahepatic bile duct	6,780	2%
Kidney & renal pelvis	8,780	3%	Brain & other nervous system	6,150	2%
All Sites	**306,920**	**100%**	**All Sites**	**273,430**	**100%**

Figure 1: Estimated Incidences and Mortalities by Sex and Site
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Cancer in the United States is a multi-layered problem.

There is a social aspect to every epidemiological topic; this is clearly illustrated when considering cancer and race in the United States. While all minority populations are

generally more likely to be diagnosed at a distant stage of cancer than whites, African Americans in particular are less likely to survive for every stage of diagnosis for almost every type of cancer. Stage at diagnosis explains much of the discrepancy in survival rates between African Americans and Whites, and the extent to which other factors effect cancer survival rates remains unclear. However, some studies have shown that African Americans are less likely than Whites to receive standard cancer therapies for lung, breast, colorectal and prostate cancers, and that those who do receive similar care experience similar outcomes (Siegel et al, 2013). Additionally, these results are more pronounced in some states than others (DeSantis et al, 2011).

Table 3: Incidences and Mortalities by Site, Sex and Race in the United States (2005-2009)

	WHITE	AFRICAN AMERICAN	ASIAN AMERICAN AND PACIFIC ISLANDER	AMERICAN INDIAN AND ALASKA NATIVE*	HISPANIC/LATINO
Incidence					
All sites					
Male	543.1	619.7	327.5	423.2	418.7
Female	424.0	396.8	286.2	360.3	333.2
Breast (female)	123.3	118.0	85.9	89.1	93.0
Colorectum					
Male	52.8	65.1	41.4	50.7	46.9
Female	39.2	48.0	32.1	41.1	33.3
Kidney & renal pelvis					
Male	21.2	23.3	10.1	29.0	19.8
Female	11.2	12.1	5.1	16.6	11.4
Liver & intrahepatic bile duct					
Male	9.1	15.0	21.6	16.4	17.5
Female	3.1	4.2	8.1	7.6	6.6
Lung & bronchus					
Male	82.3	99.3	49.4	67.4	45.4
Female	57.5	51.3	28.1	49.5	26.6
Prostate	141.0	228.7	77.2	98.8	124.9
Stomach					
Male	8.4	16.3	16.1	13.0	13.5
Female	4.0	8.2	9.3	6.4	8.1
Uterine cervix	7.8	10.4	7.2	10.1	11.8
Mortality					
All sites					
Male	216.7	288.3	132.6	184.9	146.4
Female	150.8	174.6	93.2	135.9	100.6
Breast (female)	22.4	31.6	11.9	16.6	14.9
Colorectum					
Male	19.5	29.8	13.1	18.8	15.3
Female	13.6	19.8	9.6	14.6	10.2
Kidney & renal pelvis					
Male	5.9	6.0	2.9	8.8	5.0
Female	2.7	2.6	1.3	4.1	2.3
Liver & intrahepatic bile duct					
Male	7.4	11.9	14.5	11.9	11.8
Female	3.1	4.0	6.1	5.9	5.3
Lung & bronchus					
Male	65.3	82.6	35.9	48.3	30.8
Female	40.8	38.0	18.5	33.2	14.1
Prostate	21.7	53.1	10.0	19.7	17.8
Stomach					
Male	4.3	10.3	9.0	8.3	7.4
Female	2.2	4.8	5.3	3.8	4.3
Uterine cervix	2.2	4.3	2.0	3.5	3.0

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Cancer is a problem of developed as well as developing nations.

Specific aspects of cancer may vary from one region in the world to another (Ferlay et al, 2010), but cancer is the leading cause of death in the developed world and the second leading cause in the developing world. In 2008, there were about 12.7 million new cancer cases (56% in developing world) and 7.6 million deaths (64% in developing world) due to cancer. The global burden of cancer is increasing in the developing world due to both the population aging and growth; and an increasing adoption of cancer-associated behaviors, like smoking, sedentary lifestyles, and "westernized" diets (Jemal et al, 2011).

Global incidence and mortality rate rankings based on cancer site differ from American rankings, but lung, prostate and breast cancer are domestically and globally found to be among the most common and top killing cancers. It is interesting to note, though, that with the exception of brain and nervous system cancer, the lists for incidence and mortality for the developing world closely match. Members of the developing world die of their most common cancers, whereas the developed world has found ways to combat these, resulting in mortality rankings that do not closely resemble the incidence list (See figure 2).

Cancer occurs when cells cease to regulate their proliferation. The human body is composed of many specialized organs and tissues, all composed of different cells—all of these cells are capable of developing cancerous masses called tumors. Cancers all have the potential to metastasize, causing the cancer to spread to other cells through the bloodstream (MedlinePlus, 2012). When cancer cells have reached other organs in the body, the disease and the damage by disease become significantly harder to combat

(Siegel et al, 2013); likewise, treatment options become less standardized and less effective (Girotti et al, 2011).

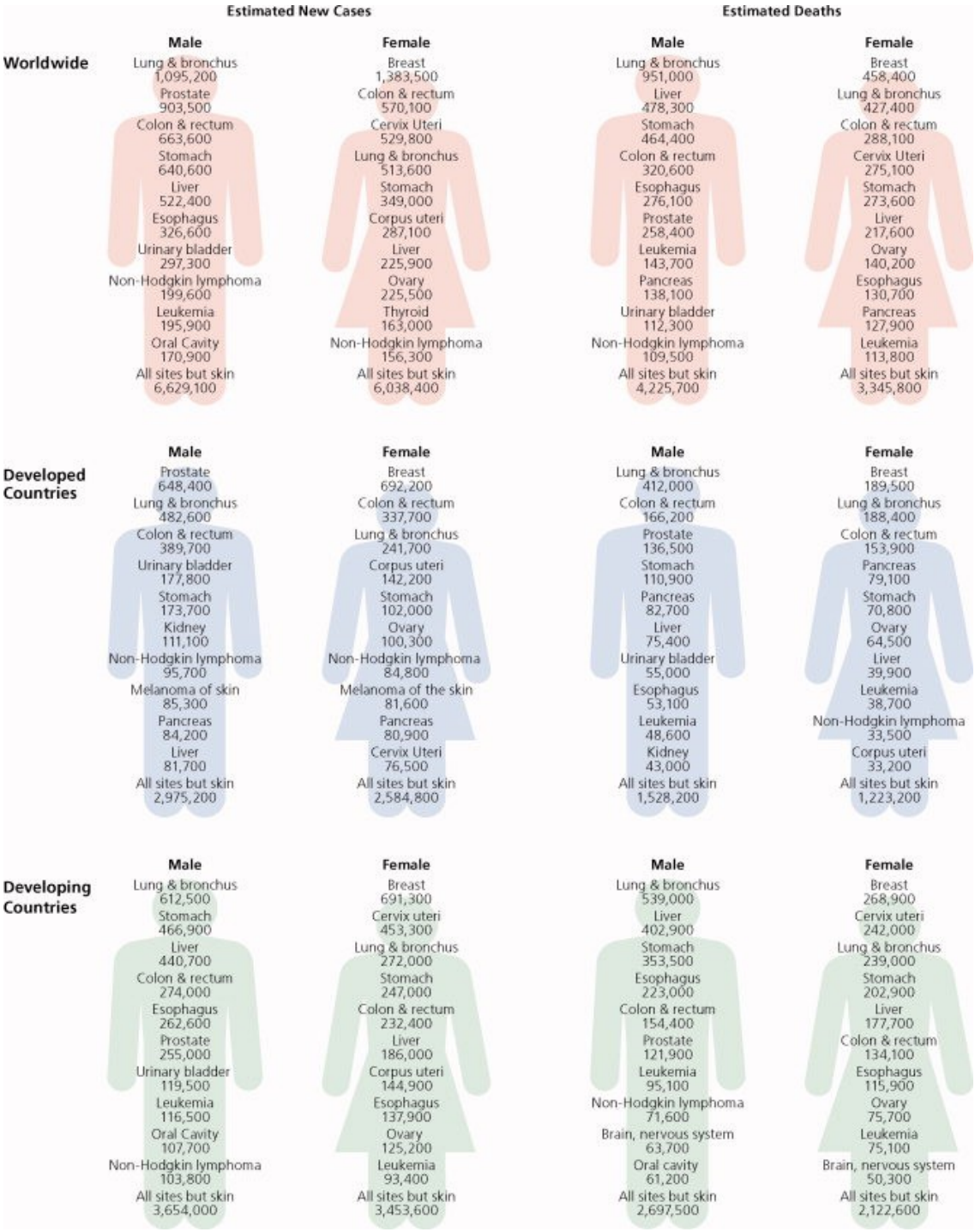

Figure 2: Estimated Incidences and Mortalities in Developing and Developed Countries
Reprinted by permission: Jemal, A., F. Bray, M. Center, J. Ferlay, E. Ward, D. Forman. "Global Cancer Statistics." CA A Cancer Journal for Clinicians (2011) **61** 69-90.

Tumor grade and cancer stage describe the progress of cancer in the human body.

Tumor grade and stage of cancer describe related but distinct aspects of cancer. Tumor grade is based on how abnormally the tumor cells have developed and can help determine whether it is benign or malignant. The stage of cancer is more descriptive of the cancer's severity and uses factors such as tumor size and lymph node involvement. Tumor grade is determined by a pathologist based on tumor cells removed in a biopsy. The grading scale used by the American Joint Commission on Cancer for all cancers:

- GX: Undetermined grade
- G1: Well-differentiated (low grade)
- G2: Moderately differentiated (intermediate grade)
- G3: Poorly differentiated (high grade)
- G4: Undifferentiated (high grade)

Grade 1 tumors are the least aggressive and grow slowly. Grade 3 and 4 tumors no longer look like normal cells and spread faster than the tumors with lower grades (NCI Fact Sheet, 2010). There are various staging systems for cancer. One of the most common is the TNM (tumor, node, metastasis) system in which cancer is described as being in Stage I, II, III, or IV. Stage IV indicates distant metastasis, or the spread of cancer beyond organs. Another system that is commonly used in cancer registries consists of 5 categories:

- In situ: cancer, or abnormal cells, are only present in the layer of cells where they developed
- Localized: the cancer remains in the organ of origin
- Regional: the cancer has spread to nearby lymph nodes or organs

- Distant: the cancer has spread to distant lymph nodes or organs
- Unknown

For most cancers in the United States, 5-year survival rates after diagnosis decrease sharply if the cancer is discovered in the distant stage; some cancers become significantly more deadly by the regional stage. Both the regional and distant stage cancers are considered to have metastasized (see Figure 3) (NCI Fact Sheet, 2010).

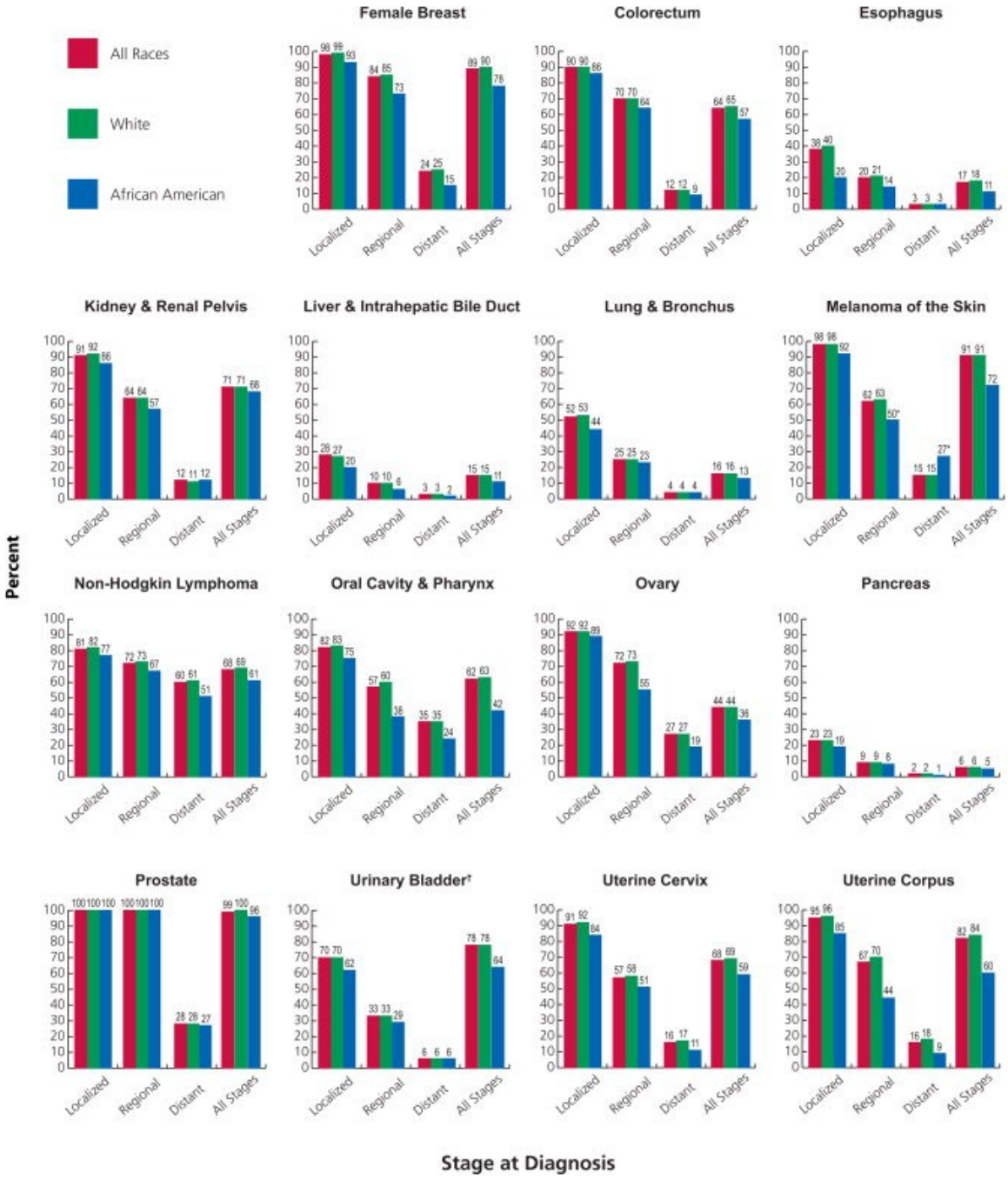

Figure 3: Five-year Survival Rates by Race and Cancer Stage
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Statement of purpose.

This thesis, titled "Metastasis and Cathepsin B," has two main purposes. 1) It is meant to summarize the literature related to cathepsin B and metastasis, and 2) it aims to

compile the evidence investigating cathepsin B as a drug target for metastasis. Metastasis, the cause of approximately 90% of cancer deaths, is best understood as a complex process mediated and facilitated by a complex network of proteases. While proteases are activated in the context of this network, they have individual potential as biomarkers and therapeutic targets. Metastasis is a large contributor to cancer mortality but also to morbidity, and the treatment process can be equally if not more draining than the disease itself. The success of targeted therapy depends on the ever-growing body of research dedicated to the relationships crucial to tumor progression and metastasis. Chapter 1, "Cancer," is an overview of cancer in the United States and in the world, and it provides context for the remainder of the thesis. Chapter 2, "Metastasis," delineates the process by which cancer cells acquire an invasive phenotype and the process they must undergo to form macrometastases in distant organs. The third chapter is titled "Proteases," and it emphasizes the relevance of the tumor microenvironment in metastasis and examines a number of key biomolecular relationships in the metastatic network. Finally, Chapter 4 is dedicated to describing cathepsin B, its inhibitors, its role in the spread of cancer, and finally, conclusions based on its potential as a drug target.

CHAPTER TWO

Metastasis

Metastasis comes from the Greek word meaning "placement" (http://www.princeton.edu/~achaney/tmve/wiki100k/docs/Metastasis.html). In the human body, metastasis is the spread of cancer to different organs in the body by growth beyond the boundaries of the primary tumor. In the first stages of cancer, surgery often provides a simple and effective solution. However, when cancer metastasizes its boundaries are no longer clearly defined or easily operable, and the solution must involve chemotherapy or targeted therapy (NCI Fact Sheet, 2012). Not surprisingly, over 90% of cancer deaths are a result of metastasis (Valastyan et al.), but metastasis is an issue of both mortality and morbidity. Metastasis is multi-step and complex, however, and without an understanding of the biomolecules and pathways involved in these processes, it would be impossible to identify biomarkers and potential therapeutic targets.

The Invasion-Metastasis Cascade is a useful and accepted way of illustrating the transition of a tumor cell from its primary tumor to metastasis.

In metastasis, tumor cells must break away from the primary tumor, enter the bloodstream, survive transport in the vessels, exit the bloodstream, and colonize distant organ sites (Pankova et al.). Carcinomas constitute about 80% of potentially fatal cancers. The process by which epithelial cell cancers (carcinomas) metastasize can illustrate metastasis in general. Collectively the seven basic steps by which an epithelial cell can extend beyond the boundaries of a primary tumor and metastasize are called the Invasion-Metastasis Cascade (Valastyan). Accordingly, each step of the process has distinctive

biomarkers and therapeutic targets, but the concentration of metastasis research is on the early stages.

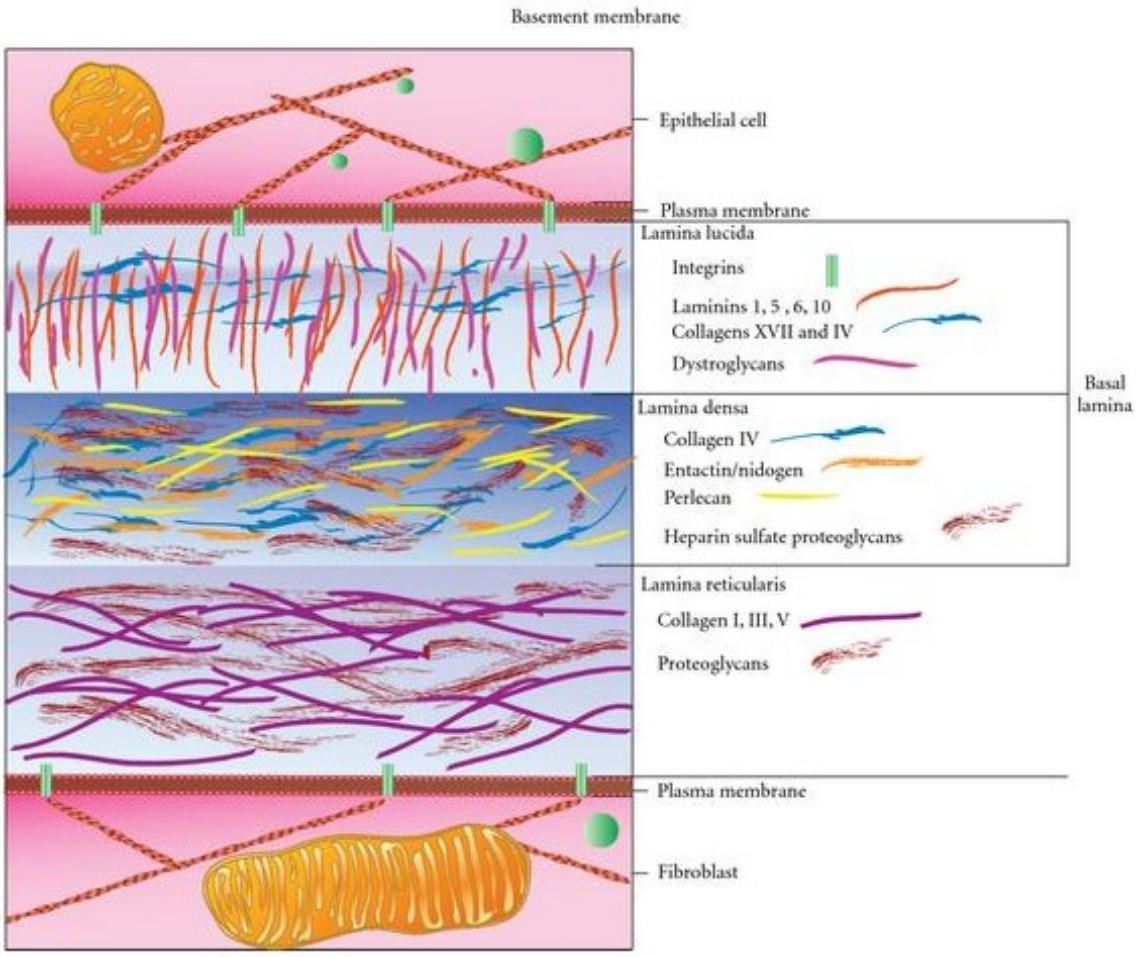

Figure 4: Components of the Basement Membrane
Reprinted by permission: David G. Menter and Raymond N. DuBois, "Prostaglandins in Cancer Cell Adhesion, Migration, and Invasion," International Journal of Cell Biology, vol. 2012, Article ID 723419, 21 pages, 2012. doi:10.1155/2012/723419

1) Cells in the "Cascade" must invade locally through the surrounding extracellular matrix and stromal cell layers. The extracellular matrix consists of the interstitial matrix and the basement membrane; collectively they provide structural support for the cells they surround. The basement membrane, a specialized extra-cellular matrix (ECM) made up of the basal lamina and the reticular lamina, separates the epithelial and stromal

compartments of epithelial tissues. It is the first structural opposition to tumor cell invasion, and it also plays an important role in signal transduction events via integrin signaling that lead to alterations in cell polarity, proliferation, invasiveness, and survival. Usually carcinomas invade in multicellular units through "collective invasion," but some individual tumor cells invade either by the "mesenchymal invasion" program or the "amoeboid invasion" program. The individual mutated epithelial cells have also shown the ability to transition from one program to the other when necessary through a process called mesenchymal-amoeboid transition (MAT), or amoeboid-mesenchymal transition (AMT) (Valastyan et al., 2011) (Pankova et al, 2010).

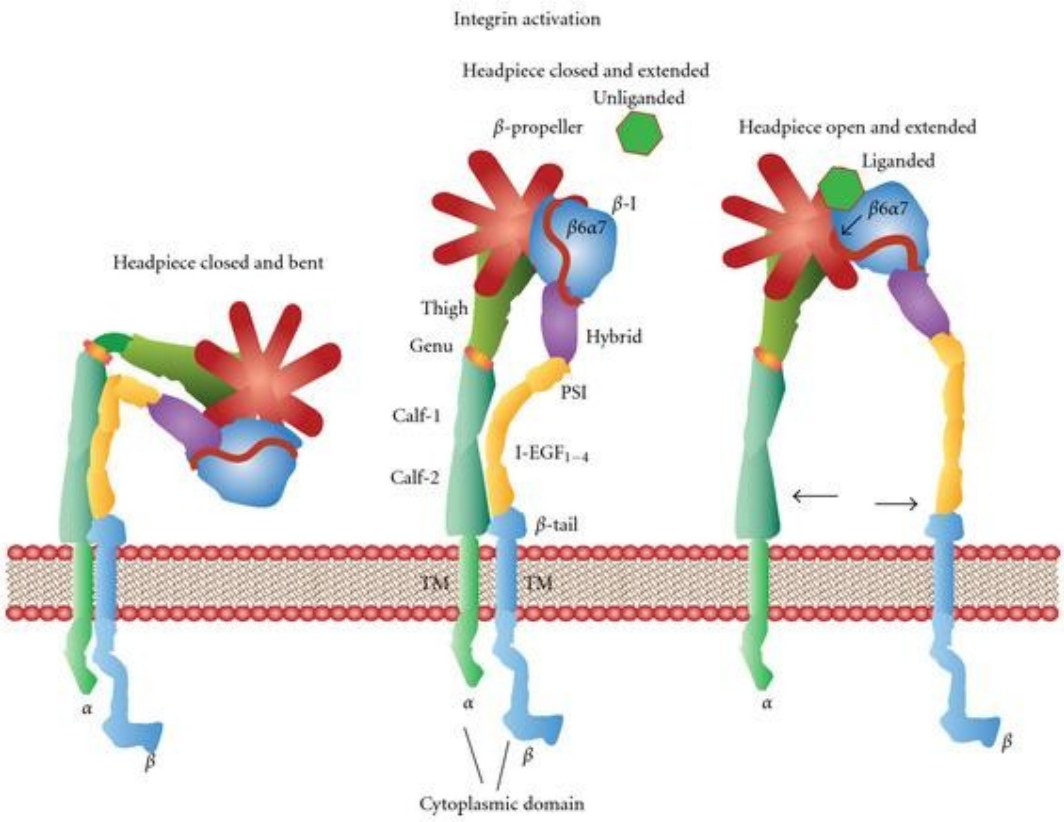

Figure 5: Integrin Activation
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2) After invasion into the stroma, cells must intravasate (enter a blood or lymph vessel). The loss of the basement membrane results in the direct invasion of the stromal compartment, where stromal cells begin to resemble inflamed tissues and are capable of further enhancing the aggressive carcinoma cell behaviors. Some stromal cells include: fibroblasts (cells that synthesize collagen), inflammatory cells (cells involved in the inflammatory response, like T-cells and macrophages), endothelial cells (cells lining the inner surfaces of organs), and adipocytes (cells that store fat). From the stroma, tumor cells are able to directly access the systemic circulation to reach distant sites through the blood or lymph (Valastyan et al., 2011).

Carcinoma cells that intravasate into the lymphatic system are important markers for disease progression, but the primary mechanism by which metastatic carcinoma cells disperse is through the blood vessels. There are a number of alterations which occur at the molecular level that promote the ability of carcinoma cells to cross the pericyte and endothelial cell barriers of the systemic circulation. For example, in breast carcinoma, the cytokine TGFβ (transforming growth factor-β) and TAMs (perivascular tumor-associated macrophages) both enhance intravasation. In addition, tumor associated blood vessels are structurally different from those normally created in the human body. This is because tumor cells stimulate neoangiogenesis, or the formation of new blood vessels within their microenvironments. The vessels formed in this process further facilitate intravasation because they are more prone to leakage and are in a continuous state of reconfiguration (Valastyan et al., 2011).

3) Upon entry to the bloodstream cancer cells become circulating tumor cells (CTCs) and must then avoid anoikis (a form of programmed cell death that usually occurs when cells leave the tissues to which they belong) and survive the rigors of transport. Once the cells are in the vasculature they are called circulating tumor cells (CTCs) and they are known as "metastatic intermediates" due to their position between the primary tumor and target organs. CTCs must survive the rigors of transport:

- Deprivation of integrin-dependent adhesion to ECM components (usually leads to anoikis)
- Damage from hemodynamic shear forces
- Predation by Natural Killer (NK) cells.

There are a number of possible reasons that tumor cells are able to avoid anoikis. For example, the expression of tyrosine kinase TrkB, a suppressor of anoikis, is of interest, because it must be expressed for metastatic progression in some cancers. Another reason that CTCs might avoid anoikis is that many of them are likely to become physically trapped in capillary bloods during their first round of circulation, meaning that they arrive at their new microenvironments within minutes of intravasation and leave the circulation long before anoikis would occur. Finally, to protect against these factors and avoid immune detection, CTCs can form large emboli through interactions with blood platelets in a process mediated by the expression of tissue factor and/or L- and P-selectins by the carcinoma cells (Valastyan et al., 2011).

4) Theoretically CTCs could stop at a wide variety of places in the body but in practice they tend to settle in a limited number of locations. What is uncertain is whether this "selection" is passive or active. It is possible that CTCs simply stop in capillary beds due to the layout of the vasculature and size restrictions of vessels, but it is also a possibility

that the cells actively home to specific organs due to genetically-templated specific ligand-receptor interactions between the cells and the microvasculature (Valastyan et al., 2011).

5) Once the CTCs arrive in a distant organ's microvasculature, they can take one of two routes: 1) form a microcolony that ruptures the walls of surrounding vessels and or 2) penetrate the endothelial cell and pericyte layers that separate vessel lumina from the stromal environment (extravasate). In addition the cells secrete factors that perturb the distant microenvironment to facilitate extravasation. For example, in order to facilitate extravasation of breat carcinoma cells into the lungs, angiopoietin-like-4 (Angptl4), EREG, COX-2, MMP (matrix metallo-proteinase)-1, and MMP-2, disrupt pulmonary vascular endothelial cell-cell junctions (Valastyan et al., 2011).

6) CTCs must initially survive in the foreign microenvironments in order to form micro-metastases. Since a CTC is at first poorly adapted to survive its new microenvironment, primary tumors must release systemic signals that induce organ-specific upregulation of fibronectin from resident tissue fibroblast so that hematopoietic progenitor cells can modify the local microenvironments at the organs to make them more hospitable. One of the others ways disseminated cancer cells adapt is by utilizing cell-autonomous programs, like Src tyrosine kinase signaling (Valastyan et al., 2011).

7) CTCs must reinitiate their proliferative programs at metastatic sites to generate macroscopic, clinically detectable neoplastic growths.

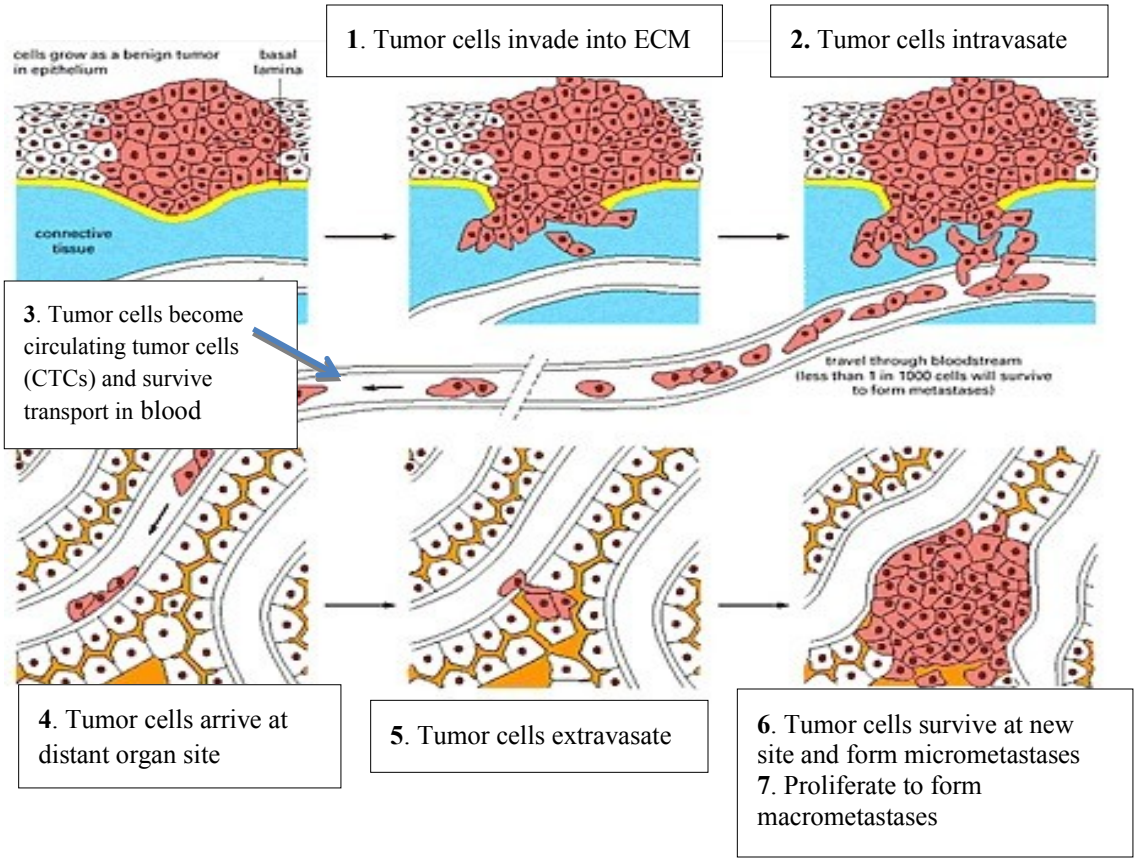

Figure 6: The 7-step Invasion-Metastasis Cascade
Reprinted by permission and modified: Alberts, B. (Bruce Alberts) et al. Molecular Biology of the Cell ,4th ed., New York: Garland, 2002, p. 1325

The Invasion-Metastasis Cascade is unidirectional (Mason et al.), which means that stopping cancer cells in early stages keeps them from progressing to further in the cascade. Given this logic, abundant research has been conducted to delineate the first step, local invasion, as well as its associated biomarkers. As mentioned earlier, researchers have postulated two mechanisms by which individual cancer cells invade, and the cells in these programs may switch between the single-cell migration strategies (Pankova et al, 2010).

Individual tumor cells can proceed toward metastasis via the epithelial-mesenchymal transition, amoeboid migration, or both.

Mesenchymal cells have a unique spindle-like shape and exhibit fibroblast-like motility (Pankova et al., 2010). The origins of the mesenchymal cells involved in pathological processes like tumor invasiveness and metastasis have long evaded researchers, but recently, evidence is emerging that EMTs (epithelial-mesenchymal transitions) are a source of many of these cells (Kalluri and Weinberg, 2009). These transitions show that epithelial cells are plastic, which allows them to acquire a mesenchymal phenotype (Zeisberg and Nielson, 2009). The process called epithelial-to-mesenchymal transition is characterized by loss of cell-cell adhesion molecule E-cadherin and gain of mesenchymal markers and promigratory signals (Yilmaz and Christofori, 2010). When epithelial cells undergo EMT they have enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis and greatly increased production of ECM components mesenchymal cells. There are three types of EMT that are distinguishable based on their functions. Type 1 EMT is involved in implantation, embryogenesis, and organ development. Type 2 EMTs are related to tissue regeneration and organ fibrosis. Finally, Type 3 EMT is associated with cancer progression and metastasis (Kalluri and Weinberg, 2009). The same basic molecular processes are required in order for an epithelial cell to become a mesenchymal cell in any of the three types of EMT:

1. Activation of transcription factors
2. Expression of specific cell-surface proteins
3. Reorganization and expression of cytoskeletal proteins
4. Production of ECM-degrading enzymes, and

5. Changes in the expression of specific microRNAs

(Kalluri and Weinberg, 2009).

These steps are orchestrated by transcription factors Slug, Snail, Twist, ZEB1, and ZEB2, which suppress expression of epithelial markers and induce expression of other markers associated with the mesenchymal state. EMT has a reverse process called mesenchymal-epithelial transition, or MET, that is driven by genes such as paired box 2 (Pax2), bone morphogenetic protein 7 (Bmp7), and Wilms tumor 1 (Wt1) (Kalluri and Weinberg, 2009).

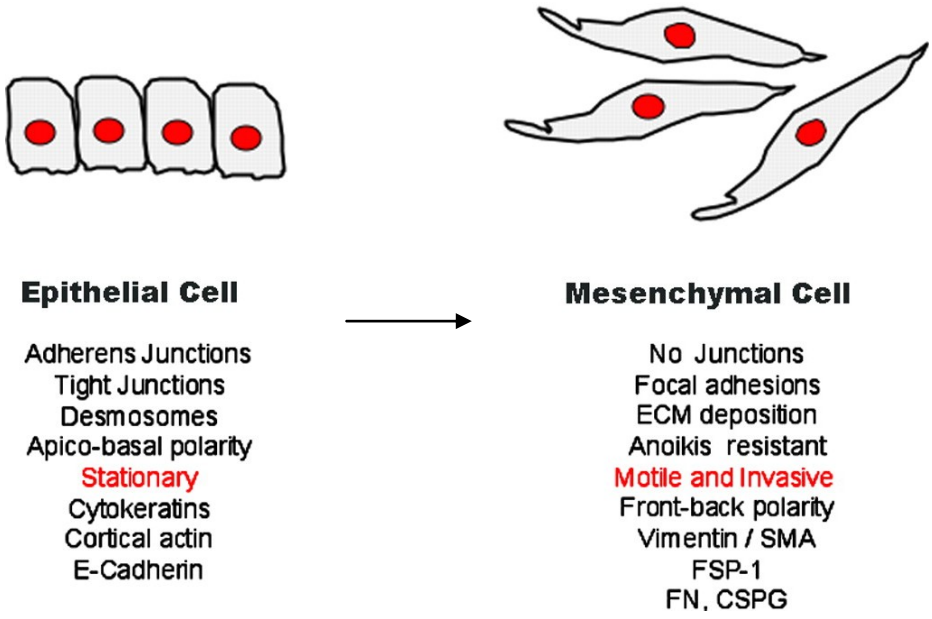

Figure 7: Epithelial-Mesenchymal transition—adapted from
©Lee J M et al. J Cell Biol 2006;172:973-981, doi: 10.1083/jcb.200601018

Mesenchymal invasion is not the only method by which carcinoma cells begin metastasis. Amoeboid migration is named after the motility displayed by amoebas, characterized by the expansion and contraction cycles of the cell body mediated by actin and myosin (Pankova et al, 2010). The amoeboid metastatic invasion program is

dependent on the Rho/ROCK/MLC pathway but is independent of proteases, stress-fibers, and integrins (Micuda et al., 2010). This pathway is responsible for the enhanced contractility found in amoeboid-like invasive strategies that allow tumor cells to squeeze through gaps in ECM fibers or deform the ECM (Pankova et al. 2010). Fast generation of contractile forces is mostly governed by the Ca^{2+}/Calmodulin stimulated myosin light chain kinase (MLCK), but on a longer time frame, contractile force generation are regulated by kinases of the Rho-family GTPases (Micuda et al., 2010).

The myosin II regulatory light chain (MLC) phosphorylation activates the myosin II motor protein complex, which interacts with actin to activate the myosin ATPase and finally result in contraction. The myosin light chain phosphatase (MLCP) and MLCK are responsible for phosphorylation of MLC. MLCP is composed of 3 subunits: 1) A catalytic subunit PP1, 2) a myosin-binding subunit MYPT-1, and 3) a small non-catalytic subunit. When occurring over long time frames, myosin activity is regulated by small monomeric GTPases RhoA and RhoC, which act through their downstream effector, Rho-associated protein kinase (ROCK). ROCK either phosphorylates MLC directly or through MLCP (Micuda et al., 2010).

Amoeboid cell motility in promoted by an upregulation of ROCK has also been shown to correlate with increased metastatic potential. Additionally, ROCK inhibition has been shown to reduce the invasive behavior of tumor cells (Micuda et al., 2010).

Mesenchymal	Amoeboid
Morphology	
Elongated	Rounded
Attachment to the ECM	
Via integrin clusters creating focal contacts and adhesions	Weak, short-term, integrins diffused in the membrane
Migration in the ECM	
ECM degradation, remodeling	Proteolysis-independent pushing through the ECM
Organization of actin cytoskeleton	
Actin meshwork (leading edge), stress fibers (traversing the cell)	Contractile actin cortex
Velocity of the locomotion	
Low	High
Cell membrane extensions	
Filopodia and lamellipodia	Intensive blebbing

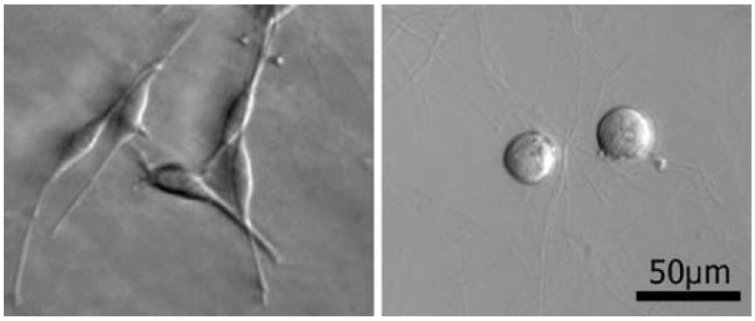

Table 4: Differences in Mesenchymal and Amoeboid Phenotypes
Reprinted by permission: Pankova, K., D. Rosel, M. Novotny, J. Brabek. "The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. Cellular and Molecular Life Sciences (2010) **67** 63-71.

Mesenchymal-Amoeboid Transition/Amoeboid-Mesenchymal Transition (MAT/AMT):

Suppression or enhancement of the activity of specific molecular pathways that determines whether a cell invades through the mesenchymal or amoeboid program can cause the cell to switch to the other type of invasiveness, in a process known as MAT/AMT (Pankova et al. 2010).

Most tumor cells invade in groups.

Individually invading tumor cells are rarely observed in metastasis, however, which has led to an unresolved debate as to whether or not EMT is required for invasion.

In addition, the cells found in the distant metastases of a number of epithelial cancers (breast, prostate, and colon), show little evidence of undergoing EMT. Either the cells in these processes simply do not undergo EMT, or the single circulating tumor cells are hard to detect. One theory is that the "leading" cells in a group of invading cells undergo EMT briefly and revert back to their epithelial phenotypes upon reaching their distant destinations (Friedl et al., 2012).

The biomolecules in a tumor microenvironment that provide the necessary support for tumor cells to proceed toward metastasis is ultimately more important to the development of metastasis drugs than how the cells change along the way. Understanding the biomolecules in the tumor surroundings may help researchers determine drug targets.

CHAPTER THREE

Proteases

Whether or not tumor cells metastasize depends greatly on the tumor microenvironment and its constituents.

Proteases are protein-degrading enzymes (Shah and Bano, 2009); as a group, they make up 2% of all proteins (Antalis et al.). There are 570 known human proteases, making them one of the largest groups of enzymes in the human body (Frohlich, 2010). When the human body is functioning as it should, under the regulation of over 200 endogenous inhibitors (Reiser et al, 2010), extracellular proteases help maintain tissue homeostasis (Kessenbrock et al., 2010). But they also are known to assist in migration, invasion, angiogenesis, modulation of signaling pathways and metastasis (Mason and Joyce, 2010). Proteases are classified by their location, where they cleave on the amino acid chain, and their catalytic mechanism. Exopeptidase proteases cleave at the amino terminus or the carboxy terminus of a peptide substrate, while endopeptidase proteases cleave peptide bonds in the inner region of polypeptide chains (Frohlich, 2010). Additionally proteases are categorized by catalytic mechanism, and five of these classes are known to be associated with human cancer: serine, cysteine, aspartic, metalloproteinases, and threonine (Rothberg et al.).

Cutaneous malignant melanoma (CMM) is the most aggressive skin cancer; based on a worldwide incidence of 3-7% per year it is also the most rapidly spreading cancer. The difficulties in treating CMM lie in the differential diagnosis to other melanocytic lesions, lack of prognostic markers, and no efficient treatment of advanced melanoma. To

say that there are no prognostic markers is not completely accurate—for years the medical community has relied on the multi-step model by Clark and Elder. According to this model melanoma develop in a continuum from nevi (moles). Melanocytic nevi become melanocytic atypia, radial growth melanoma, vertical growth melanoma, and finally metastatic melanoma. All of these progressions lead to an increase in the thickness of the lesion, which, based on the Breslow index, is the most predictive measure of prognosis and survival of the patient. The correlation is intuitive—the thicker the lesion, the more likely the cancer would metastasize, leading to poorer prognoses (Frohlich, 2010).

However, most melanomas appear in healthy skin without nevi as precursors, which the Clark and Elder model cannot explain. And, even a short list of similar melanocytic lesions helps illustrate the difficulty in diagnosis: ancient nevi, balloon nevi, blue nevi, Clark's nevi, Spitz nevi, Halo nevi, recurrent nevi, and genital nevi (Frohlich, 2010). Since a visual examination of lesions cannot reliably detect malignancy and the differences in prognoses and treatment are so drastic, protease expression levels are of great interest. The detection or marked increase in the expression of a given protease, like cathepsin B, could be used as a biomarker for malignancy, thereby reducing the guesswork and misdiagnoses (Frohlich, 2010). One *in vitro* and *in vivo* study on metastatic melanoma showed that while cathepsin B is not expressed at high levels in primary melanoma cell lines, it is expressed at high levels in metastatic cell lines, thereby showing cathepsin B's potential as a prognostic marker (Mataresse et al, 2010).

In addition, the protease network provides a useful framework for the study of individual proteases that participate in metastasis. While cancer is caused by genetic

mutations that cause poor cell regulation, it is maintained by the local environment (Taddei et al, 2013). Paget's "seed and soil" theory states that tumor cells, or the "seeds", are supported in their primary tissue environment, or "soil" (Spano et al.). Fibroblasts, inflammatory cells, endothelial cells, and adipocytes encourage the tumor cells by increasing proteolytic activity and basal inhibitor levels, ultimately promoting tumor formation. The protease production supported by stromal cells is involved in the epithelial mesenchymal transition of tumor cells. While the basal inhibitor level increase may seem odd at first, it can be explained as preventing excessive degradation of the ECM, as proteases cannot work effectively without substrate (Frohlich, 2010).

Tumor-producing proteases function as part of an extensive, multidirectional network of proteolytic interaction, because proteases are synthesized as either inactive or marginally active zymogens and remain this way until they either autocatalyze or become cleaved by other proteases. In addition, protease networks involve various other components of the tumor microenvironment, and these networks interact with other important signaling pathways in tumor biology (chemokines, cytokines, and kinases). Given the interconnected nature of protease function, studying them individually and without regard for their place and roles in the network would be impractical. Rather we should study pathways and the ways in which they are amplified and suppressed as a more contextual approach (Mason and Joyce, 2010).

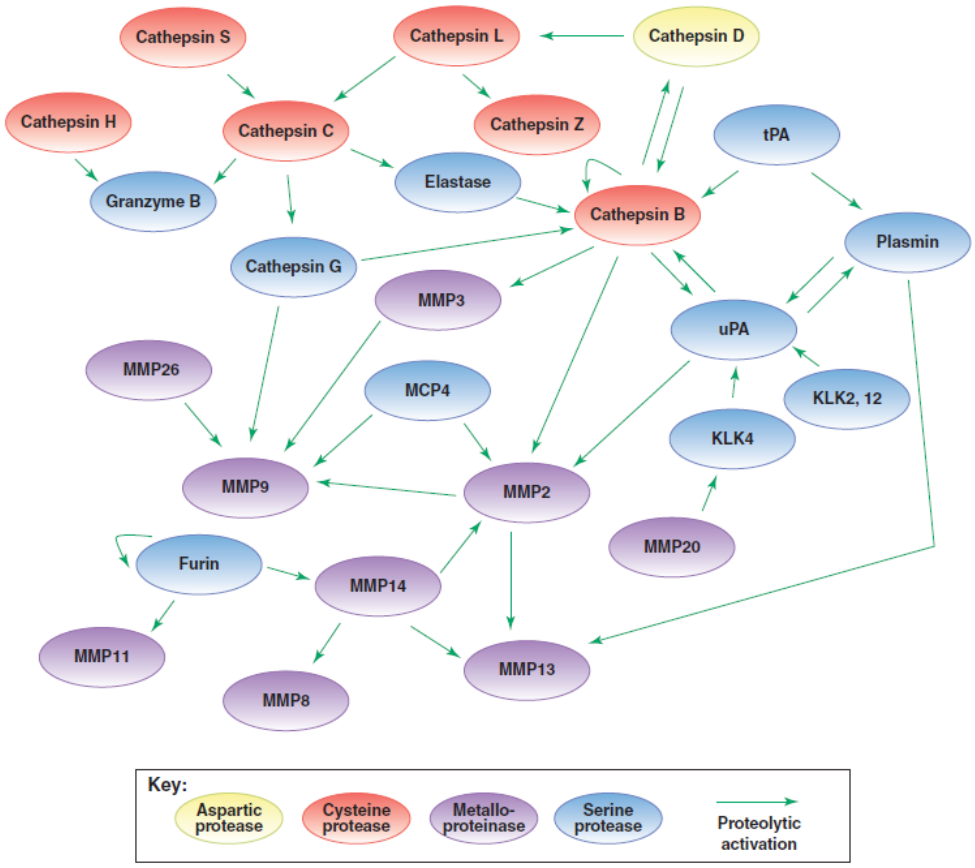

Figure 8: Protease Activation Network in Metastasis
Reprinted by permission: Mason, S., Joyce, J. "Proteolytic networks in cancer." Trends in Cell Biology (2011) **21** 228-237.

In order for invasion to occur, the ECM must be degraded. First, the glycoproteins that protect collagen against proteolytic attack must be removed, so that the collagen can subsequently be degraded. This work is done by the serine proteases and metalloproteinases found on the plasma membranes of tumor cells as well as cathepsin B (catB), which is found both intra- and extra-cellularly. All the while, the proteases are activating one another. Cathepsin D (catD) activates both catB and cathepsinL (catL), which activate urokinase plasminogen activator. Then, plasmin and catB activate metalloproteinases (MMP-2, MMP-3) that activate other metalloproteinases (MMP-9, MMP-13). Proteases in the stromal cell cancers (90% of breast, pancreas, and gastric

cancer) assist in extravasation by degrading collagen type IV of the blood vessels (Frohlich, 2010), (Mason and Joyce, 2010).

MMPs and cathepsins are the two most important protease families in the tumor microenvironment.

MMPs were first described in 1962 as a family of zinc-dependent endopeptidases (Kessenbrock et al. 2010). They were originally believed to exist in the matrix—hence the name—but they have also recently been found in the cytosol and nucleus, although their functions there are unclear. They are believed to have both degrading and regulatory functions, although the regulatory functions are less understood (Mannello and Medda, 2012). There are 23 matrix metalloproteinases (MMPs) in humans. They are named for their architectural features, but all of them use a metal ion in their catalytic mechanism (Frohlich et al.), and most of them have 3 common domains: a pro-peptide domain, a catalytic domain, and a zinc ion in the active site. Like many proteases, MMPs are initially enzymatically inactive. This is because of an interaction between a cysteine residue of the pro-domain with the zinc ion of the catalytic site, and can MMPs can only be made active when this interaction is disturbed (Kessenbrock et al. 2010).

MMPs have been studied extensively, because "increased activities of MMPs have been detected in all cancer lesions so far studied" (Frohlich, 2010). However, the MMP "family" label can be misleading; the 23 human MMPs are diverse in activation requirements and functions. For example, other than autoactivation and activation by oxidation, MMP-9 can be activated when plasmin activates MMP-3, which in turn activates MMP-9. Cathepsin G, a serine protease, can also activate MMP-9. A broad spectrum inhibition approach was likely ineffective in clinical trials for treating

pancreatic, brain, lung, or renal cancer because the approach assumed similarity in function among MMPs (Mason and Joyce, 2010; Kruger et al., 2012).

In addition, most MMPs have 2 roles. MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-11 can promote or inhibit growth, invasion, angiogenesis, or host defense. MMP-12, MMP-13, and MMP-20 help protect against cancer, but MMP-14, MMP-15, MMP-16, MMP-24, and MMP-26 promote many tumors. ADAM (A disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) cut off extracellular portions of transmembrane proteins. Specifically ADAM-12 assists in tumor adhesion; and ADAM-10, ADAM-15, ADAM-17 and ADAMTS-4 are involved in angiogenesis (Frohlich, 2010).

MMPs are regulated by TIMPs (tissue inhibitors of metalloproteinases). But TIMPs are complicated in that they have both inhibiting and activating effects, as well as functions independent of metalloproteinase inhibition. For example, while MMPs are overexpressed in cancer, TIMP-1, though an inhibitor of MMPs, actually had negative effects in cancer patients when highly expressed because of its anti-apoptotic effects (Frohlich, 2010). Because of the poor performance of MMP inhibitors in clinical trials (Kruger et al., 2010), it might be beneficial to focus attention on other potential drug targets. A number of cathepsin inhibitors have been shown to limit metastasis *in vivo,* indicating their potential to be effective in clinical trials in the future.

The first cathepsins were discovered in the stomach; the word “cathepsin” is derived from the Greek word “kathepsein,” which means to digest or boil down (Reiser et al, 2010). While they are normally found in lysosomes, cathepsins can also play a large role in tumor protease networks (Mason and Joyce, 2010). Eleven of the fifteen known

cathepsins are cysteine proteases (Reiser et al, 2010), which are named for their cleavage of peptide bonds using a reactive cysteine residue at the catalytic site. The cathepsins that are not cysteine proteases are aspartic proteases cathepsin D and cathepsin E, and serine proteases cathepsin A and cathepsin G. Aspartic proteases contain 2 aspartic acid residues in the active center, and serine proteases are characterized by the catalytic triad: serine, aspartic acid, and histidine. In most cases, cathepsins are found within lysosomes as they are activated in the low ph found within, but in instances of metastasis they are also found extracellularly, where they degrade the extracellular membrane to facilitate invasion. Cathepsins are known to be effective at both intra- (in lysosomes) and extra-cellular degradation of ECM components (Frohlich, 2010).

A few cathepsins play key roles in the proteolytic network. For example, cathepsin K is considered to be one of the most important in collagen I degradation in bone (Garnero et al, 1998), and cathepsins L and B have the highest expression levels in melanoma (Girotti et al., 2012). Cathepsin L is a unique case in cathepsins because it is the only one to be upregulated in only malignant cells (Lankelma et al., 2010). These cysteine cathepsins are found in lysosomes of all living cells (Katanuma, 2011). Some researchers would argue that of the proteases, cathepsin B "appears to be the most important of all cathepsins in regards to tumor progression" (Mason et al.). While it is impossible to discuss cathepsin B's individual role in metastasis without regard to the proteases and other extracellular components with which it interacts, it does possess unique traits and takes part in its own pathways (Mason et al.). The focus of chapter 4 will be Cathepsin B status as a metastasis biomarker and therapeutic target.

CHAPTER FOUR

Cathepsin B

Current treatments of metastasis, a health concern of both mortality and morbidity, have limited efficacy. One solution would be to target its specific, fundamental enzymes.

One of the most common areas for metastasis in breast cancer is the bones (Withana et al, 2012). Symptoms of bone metastases include: bone pain; pathological fractures; urinary incontinence; bowel incontinence; weakness in the legs; epidural spinal cord compression; and nausea, vomiting, and confusion caused by hypercalcemia (high levels of calcium in the blood) (Mayo Clinic, 2012). The survival rate for breast cancer patients with distant metastases is about 23%, compared to 85% for breast cancer patients who are diagnosed at stage I (Withana et al, 2012). While a variety of treatment options are available to cancer patients, they become significantly less effective as cancer progresses. Surgery provides effective treatment in the beginning stages of cancer, as well as radiation therapy, which kills cancer cells (but also surrounding cells) by damaging their DNA—normal cells can recover from this, while cancer cells cannot regenerate themselves. Most chemotherapy indiscriminately eliminates rapidly growing cells of the body, but targeted therapy can selectively target cancer cells. These are used either alone or in combination with another, or even both; as implicated by the mechanisms by which radiation and chemotherapy treat cancer, the side effects of the current treatments of metastasis can be tremendous (NCI FactSheet, 2012).

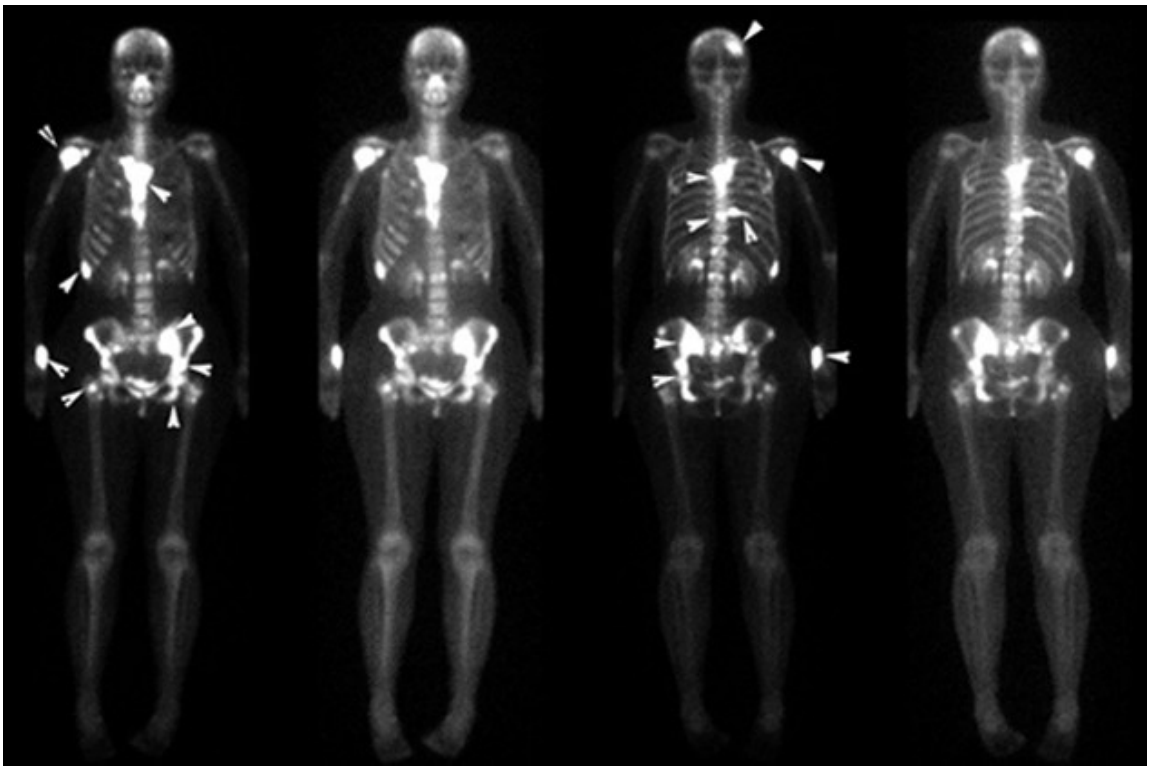

Figure 9: Multiple Bone Metastases from Breast Cancer
http://www.meddean.luc.edu/Lumen/MedEd/Radio/curriculum/Surgery/Met_bone_list1.htm

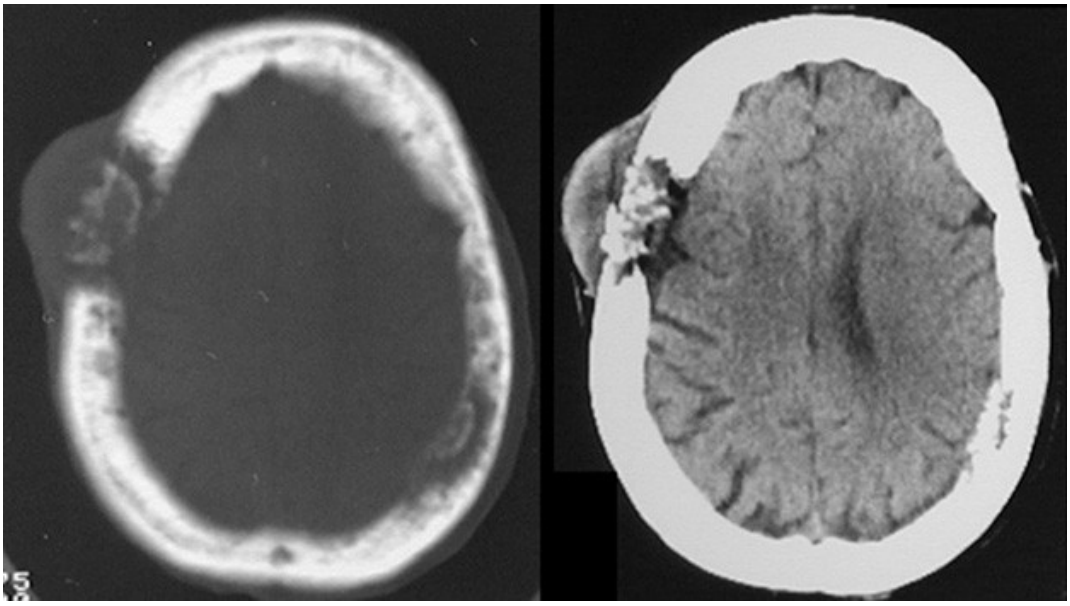

Figure 10: Bone Metastasis
http://www.meddean.luc.edu/Lumen/MedEd/Radio/curriculum/Surgery/Met_bone_list1.htm

For this reason, targeted therapy, although relatively new, is an attractive approach. Targeted therapy has the ability to stop the very proteins that drive metastasis at the biochemical level; thus it can provide the specificity that radiation and chemotherapy lack as well as reach areas of the body that surgery cannot.

One of the most successful stories of targeted therapy is that of the tyrosine kinase inhibitor Imatinib (Gleevec) in chronic myeloid leukemia (CML). CML is a disease of the white blood cells that results in an accumulation of immature white blood cells in the blood and bone marrow, and the only effective treatment before Gleevec was a bone marrow transplant. Most CML patients were not young or healthy enough to undergo such a procedure, reaffirming the need for alternative treatments (NCI Gleevec Q & A, 2001). Gleevec emerged from highly successful clinical trials in 1999 for approval from the Food and Drug Administration in 2001 to treat CML. All 31 patients in the clinical trial had their blood counts return to normal, and 9 of 20 were found to be completely clean of metastasis 5 months later. Very few side effects were reported (NCI Gleevec Q & A, 2001). In a 2011 study, the survival rates in the patient group receiving Imatinib did not statistically differ from the general population; this result illustrates the transformative nature of Imatinib on CML patients, whose life expectancies previously ranged from 4-6 years. Additionally, the incidence of secondary malignancies in Imatinib patients did not statistically differ from that of the general population, which further supports the case that targeted therapy can have fewer side effects than chemotherapy (Smith, 2011).

The effectiveness of Gleevec can be attributed to its specificity—specificity requires finding the exact proteins or agents responsible for specific cancers. In 95% of

CML patients, the chromosome 9 and 22 fusion known as “Philadelphia translocation” creates the oncogene “BCR-Abl” that codes for the BCR-Abl tyrosine kinase that seems to be responsible for CML. The tyrosine kinase inhibitor Imatinib binds the phosphate-binding site, forcing the kinase into a “closed” or inactive conformation (NCI Gleevec Q & A, 2001).

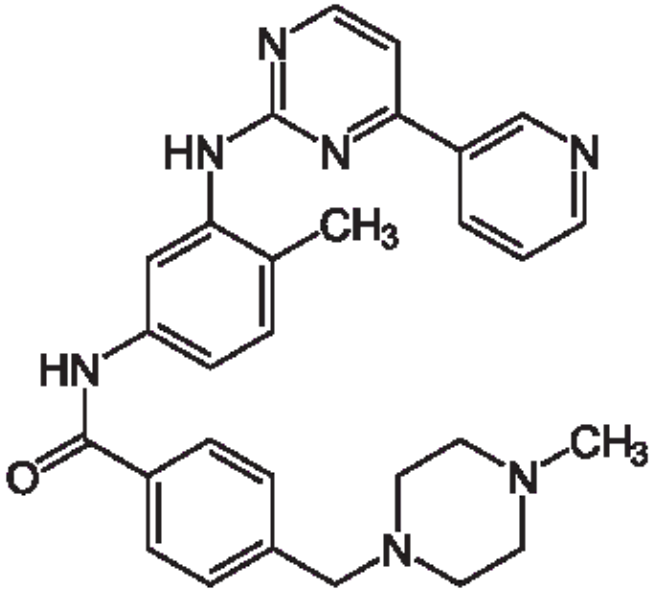

Figure 11: Structure of Imantinib (Gleevec)

Because of the success of Gleevec in combatting CML (and potentially gastrointestinal stromal tumor, glioma, and soft tissue sarcoma), targeted therapy principles might be applied to other cancers. Cathepsin B, a cysteine protease implicated in many number of cancers (breast, melanoma, glioblastoma, among others), is involved in numerous pathways involved in tumor progession and metastasis and could prove to be a useful drug target.

Cysteine proteases, when first discovered, were thought to be exclusively lysosomal proteases, involved merely in waste degradation and endosomal processing.

Slowly they have been observed to play roles in other organelles as well as extracellularly (Turk et al. 2011). Cathepsin B is one of the most abundant lysosomal cysteine proteases (Rozman-Pungercar et al, 2008). It is one of 11 papain-like cysteine proteases (Rozman-Pungercar et al, 2003), which means that its binds similarly to papain, the first cysteine protease to have its structure identified (Worthington Manual, 2013). Papain was named for its discovery in the latex of papaya; its active site contains 7 subsites (S1-S4, S1'-S3'), one for each of 7 possible substrate-binding residues (P1-P4, P1'-P3') (Worthington Manual, 2013). Cathepsin B has the same binding sites, but it differs from other papain-like cysteine proteases in that it contains one heavy and one light chain, as well as an "occluding loop" of 20 residues located near its active site. Cathepsin B catalyzes the hydrolysis in a mechanism involving a cysteine residue in its active site.

Cathepsin B is able, because of the "occluding loop," to exhibit exopeptidase (carboxy dipeptidase) activity in addition to endopeptidase cleavage (Illy et al., 1997). Specifically, when the surrounding pH is below 5.5, Cathepsin B exhibits more dipeptidyl carboxypeptidase activity than endopeptidase activity (Stachowiak et al., 2004). An additional property of the occluding loop is its location above cathepsin B's active site and thus has the ability to restrict inhibition. Without the central 12 residues of cathepsin B's occluding loop, cathepsin B has no exopeptidase activity, and its binding affinity for the inhibitor cystatin C increases 40-fold and its affinity for procathepsin B 50-fold (Illy et al., 1997).

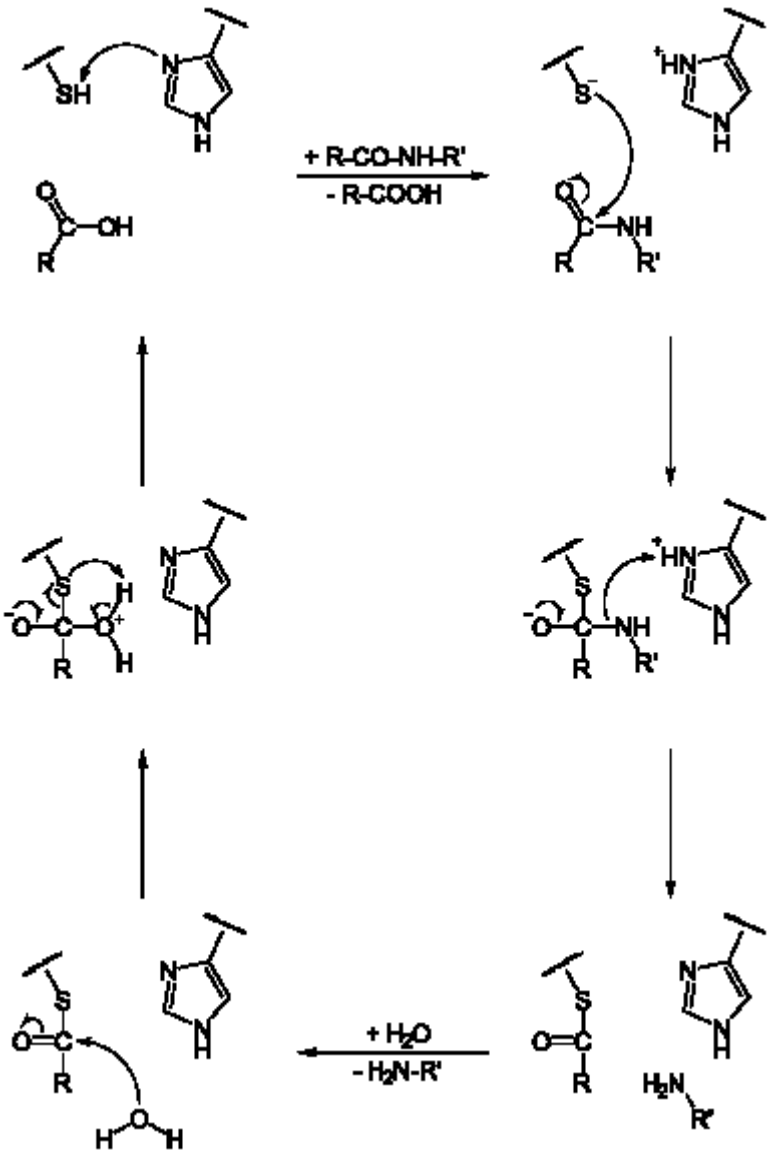

Figure 13: Mechanism for Cysteine Protease Cleavage

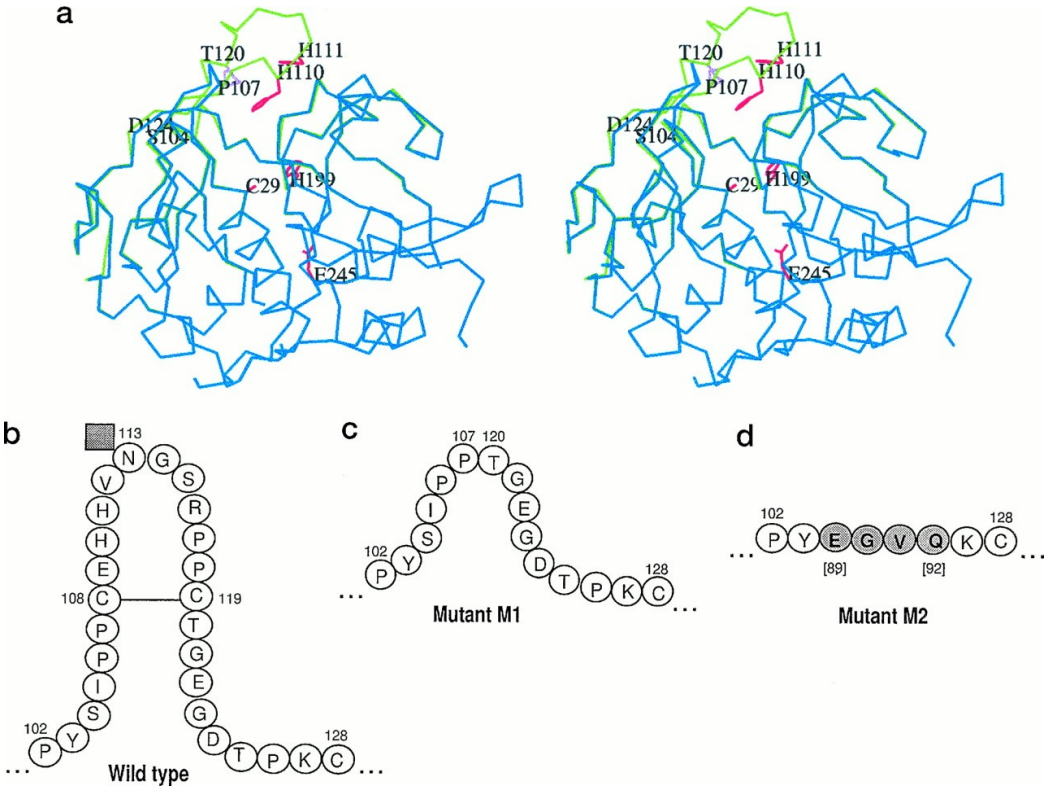

Figure 14: Occluding Loop of Cathepsin B
Illy C et al. J. Biol. Chem. 1997;272:1197-1202

The human body usually regulates the action of endogenous cysteine proteases with cystatins. Cystatins are found primarily in body fluids and tissues and provide a strong regulatory system for leaking cysteine proteases from dead and dying cells. In this way cystatins protect cells undergoing both endogenous and exogenous proteolysis. Cystatins are grouped into 3 "families" (Shah and Bano, 2009). Family 1 (stefins) cystatins are mostly intracellular. These members contain about 100 amino acid residues and lack disulfide bonds; the group includes human stefin A, human cystatin A and human cystatin B. Family 2 (cystatins) inhibitors are primarily extracellular. Each member is made up of about 120 amino acid residues and 2 intrachain disulfide bonds. Cystatin C belongs to this group. Finally, family 3 (plasma kininogens) contains 3 further groups: low molecular weight kininogens (LWWK), high molecular weight (HMWK)

and T-kininogens. These kininogens are single chain glycoproteins with additional disulfide bonds (Shah and Bano, 2009).

Unfortunately, while cathepsin B is upregulated in metastasis, the body's protective cystatins exhibit impaired inhibitory abilities. For example, stefin A taken from sarcoma was less able to inhibit a number of papain-like proteases, such as Cathepsin B, than stefin A from liver (Shah and Bano, 2009). This is not to say that they are ineffective; breast cancer metastases where cathepsin B and stefin A are simultaneously expressed were correlated with good prognoses (Withana et al., 2012.) However, cathepsin B is often abnormally expressed with pathological effects, and synthesized inhibitors have been useful in elucidating the actors involved in activating cathepsin B-dependent tumor progression.

Structure-based exogenous inhibitors of cathepsin B have been useful in determining pathologies stemming from abnormal expression of cathepsin B.

Using X-ray crystallography images of cathepsin B, researchers have been able to synthesize inhibitors that have been used *in vitro* and *in vivo.* A broad-spectrum inhibitor, E64 (epoxysuccinate) was used as a frame compound for specific cathepsin B inhibitors, CA-030 and CA-074.

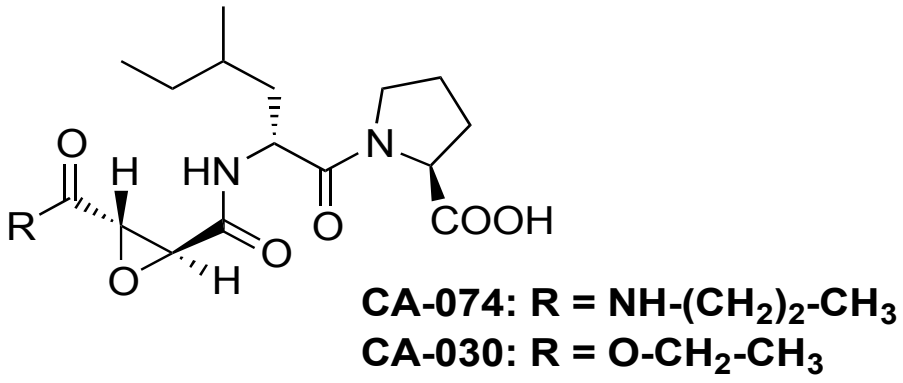

Figure 15: Structures of cathepsin B Inhibitors CA-074, CA-030

Both specific inhibitors were designed to have a proline carboxylate (negative charge) at the C-terminus to bind with the strong positive charges of the adjacent histidines in the occluding loop of cathepsin B. Binding an isoleucine epoxysuccinyl group next to a proline allows the epoxysuccinyl group to easily approach Gln-23 on the surface of the substrate binding pocket of cathepsin B to make an oxyanion hole. This way, the active site of cys-29 of cathepsin B easily binds with that of epoxysuccinate. They inhibit cathepsin B at 10^-7 M. In addition to their potential as therapeutic targets, synthesized cathepsin B inhibitors can and have helped elucidate a number of the enzyme's pathological pathways (Katanuma, 2011).

A number of cancers implicate cathepsin B-dependent pathways in tumor progression and metastasis. In a number of in vivo *studies, inhibiting cathepsin B was shown to limit metastasis.*

Using synthetic inhibitors like the ones described above, Cathepsin B has been linked in a number of *in vitro* and *in vivo* studies as being integral in the metastatic process, particularly in facilitating invasion. While cathepsin B is typically found in lysosomes and endosomes, in cancer it is secreted into the cytoplasm and pericellular space (Frohlich, 2010). The extracellular placing is critical in metastasis, because

cathepsin B degrades matrix proteins like elastin and collagen (Diez et al., 2010) surrounding tumors to facilitate invasion (Girotti et al., 2011). One example of this was shown in glioblastoma, where cathepsin B was translocated to cell surfaces under the direction of netrin-1, an axon-directing protein (Shimizu et al., 2013).

Cathepsins B and L are the most expressed cathepsins in melanoma (Frohlich, 2010). Cathepsin B is not found in primary melanoma cell lines, like cathepsins D and L, but it was found to be highly expressed at the surface of metastatic cell lines. Cathepsin B inhibitor CA-074, as well as specific antibodies against cathepsin B, were found to limit invasion and metastatic melanoma *in vitro*, whereas specific inhibitors for cathepsins D and L were ineffective (Materrese et al., 2010). The cathepsin B-dependent pathway involved in melanoma tumor progression appears to be regulated by SPARC (secreted protein acidic and rich in cysteine), mediated by collagen I and α2β1 integrins (Girotti et al., 2011).

In addition to melanoma, cathepsin B has been shown to play a role in the progression of glioblastomas, which are notoriously invasive tumors of glial cells. Netrins are axon guidance molecules that play key roles in brain development by guiding axon growth and neural cell migration in the nervous system cathepsin B has also been shown to play a role in the invasion and angiogenesis of glioblastoma in a pathway promoted by netrin-1. The netrin-1 pathway was completely inhibited by a leupeptin, a cysteine protease inhibitor. Specific inhibitors of cathepsins B, S, D, and L were tested, and; while the inhibitors of cathepsins D and L had no effect, both the cathepsin B inhibitor CA-074Me and cathepsin S inhibitor (to a lesser degree than CA-074Me) significantly reduced netrin-1 induced invasion (Shimizu et al, 2013).

In numerous experiments, cathepsin B has been shown to increase the invasiveness of breast cancer cells *in vivo;* in addition, inhibitors of cathepsin B have been successful in reducing breast cancer-related bone metastases *in vivo*. One transgenic experiment of human cathepsin B in mice showed that cathepsin B expression had no effect on breast tumor onset. However, the cysteine protease did contribute to increased tumor weight and accelerated tumor growth, both in the primary tumor and the lung metastases (Sevenich et al., 2011 Recycle Bin). More recently, cathepsin B-deficient mice were crossed with mice with a predisposition for developing mammary carcinoma. The proliferation of primary tumors and lung metastases was reduced, showing that cathepsin B does play a role in non-transformed and premalignant human breast epithelial cells. In addition, cathepsin B-and-L-specific inhibitor CA-070 caused a reduction in structure size and proliferation as well as an increase in apoptosis in the tumor (Mullins et al., 2013). Finally, a third *in vivo* study simultaneously affirmed the above findings and reported that cathepsin B inhibitor CA-074, unlike a broad spectrum cysteine cathepsin inhibitor, was able to reduce bone metastasis in breast tumor-bearing mice (Withana et al., 2012). Broad spectrum inhibitors, like JMP-OEt and E64, were shown to work better in inhibit cathepsin L more effectively than cathepsin B (Withana et al., 2012; Mullins et al., 2013). This might be explained by the fact that targeting only cathepsin L, while effective in reducing invasion and metastasis, induces compensatory activity by other cathepsins (Lankelma et al., 2010).

Discussion and conclusions.

Although the treatment options for patients with metastasis currently seem bleak, cancer death rates have declined about 20% overall since 1991 (the peak of cancer

deaths). But there is still room for improvement, as cancer deaths are estimated to comprise a quarter of American deaths in 2013. In addition, the purpose of metastasis research also encompasses the morbidity associated with late stage cancer, such as that associated with lung and bone metastases. Because of the burden of mortality and morbidity of metastasis and the accompanying lack of efficacious treatment, targeted therapy presents an attractive alternative option.

Metastasis research requires attention to specificity, as myriad enzymes contribute to the biochemical processes involved in tumor progression and metastasis. Some enzymes contribute more actively to the metastatic network than others; matrix metallo-proteinases and cathepsins are particularly prominent. While the focus of this thesis was cathepsin B as a potential drug target in metastasis, any specific treatment therapy development must take into account the activation and compensatory processes. Cathepsin B has been implicated in pathways leading to increased invasiveness and often angiogenesis in breast cancer metastasis, cutaneous malignant melanoma, glioblastoma, and others. One of its inhibitors, CA-074, has been tested to successfully limit invasion and the formation of metastases in the above-mentioned cancers, both *in vitro* and in mice.

Other proteases, like MMP-9 and cathepsin L, also act influentially in the metastatic protease network—so why target cathepsin B? Metastasis is clearly not the result of any single actor or pathological pathway but the culmination of their effects. Many of these enzymes are often scrutinized simultaneously in studies attempting to discern the processes underlying specific cancers, and inhibiting cathepsin B was shown to be more effective in reducing the invasiveness and metastases of breast cancer,

glioblastoma, and melanoma, compared with inhibiting cathepsins L, D, and K. The success of targeted therapy is conditional on a practical understanding of the workings of metastasis, but ultimately, experimental results should be most heavily weighted in deciding on drug therapy targets.

Perhaps more compelling than the fact that inhibiting cathepsin B has been shown to effectively limit tumor progression in certain cancers, the types of cancer in which cathepsin B plays a role are among the most common. Breast cancer is the most common cancer as well as the top-killing cancer in women worldwide. While the survival rates in the United States are high for women who are diagnosed relatively early with breast cancer (85%), the rates drop precipitously when the cancer metastasized to distant sites (23%); not to mention, bone and lung metastases would lower the quality of life in these patients significantly. With toxic chemotherapy being the only viable option for these women, many fall into depression and most do not survive (Siegel et al, 2013). Cathepsin B inhibition showed promising results in limiting breast cancer metastasis in mice, and might be effective in clinical trials.

Certainly, it is possible that cathepsin B inhibitors will fail to provide the desired results in clinical trials (lowering metastasis). This was the case in clinical trials for MMP inhibitors (Kruger et al., 2012); however the inhibitors used in these trials were broad-spectrum inhibitors that assumed uniformity of function in MMPs. The effects of broad-spectrum cathepsin inhibitors have shown analogous results—specific cathepsin B inhibitors have been shown to be more efficacious. In the end, the treatment for metastasis may be comprised of not one but many specific protease inhibitors, due to the tendency of cathepsins to compensate for one another in cancer networks. The drug

cocktail might inhibit cathepsin B, cathepsin L and certain MMPs—the possibilities are endless. With an accumulation of knowledge of specific proteases involved in metastasis, we might be able to discern the best treatment options. For now, cathepsin B has at least shown potential as a biomarker and therapeutic target *in vitro* and *in vivo.*

APPENDIX

APPENDIX

Literature Searches

Databases: Scifinder, Scopus, Science Direct, PubMed:

"Cathepsin B and Metastasis,"—English, Reviews only, 2009-
"Cathepsin B"—English, Reviews only, 2009-
"Metastasis"—English, Reviews only, 2009-
"Tumor Metastasis"—English, Reviews only, 2009-
"Cystatins and Metastasis"—English, Reviews only, 2009-
"Mesenchymal Invasion Process"—English, Reviews only, 2009-
"Amoeboid Invasion" –English Reviews only, 2009-
"Cathepsin B and Metastasis,"—English Journals only 2009-
"Cathepsin B"—English Journals only 2009-
"Metastasis"
"Serpins"
"Cystatins"
"Cure for cancer,"—English, Reviews only, 2009-
"Cathepsin B Inhibitor"—English, Journals and Reviews, 2009-

Google Scholar:
"Cancer" articles (including patents) 2009-
"Cancer statistics, 2013" articles (including patents) 2009-
"Cancer quality of life" articles (including patents) 2009-
"Cathepsin B inhibitor" articles (including patents) 2009-

Google Search:
"Cancer"
"Metastasis diagram" (images)
"Metastasis lymph node"
"Occluding loop"

Science Direct:
"Matrix Metalloproteases" 2009-, journals only
"Matrix Metalloproteases and metastasis" 2009-, journals only

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