Maspin

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Dedication

This book is dedicated as a loving tribute to Dr. Ruth Sager, an outstanding geneticist, and her favorite gene—Maspin, mammary serpin protease inhibitor. For more than half a century she demonstrated vision, insight and determination to develop novel scientific concepts in the face of established dogmas. Her pioneering research and original ideas continue to make contributions to biology.

With fond memories and great admiration,
Mary J.C. Hendrix
Acknowledgements

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CONTENTS

Preface ......................................................................................... x

1. Ruth Sager, Geneticist ................................................................. 1
   Arthur B. Pardee
   Personality ................................................................................... 2
   Personal History ........................................................................... 2
   Professional History ..................................................................... 3
   Cancer Biology ............................................................................. 3
   Cytoplasmic Inheritance ............................................................... 4
   Personal Legacy ............................................................................. 5
   Articles about Ruth Sager ........................................................... 5

2. Maspin in the Sager Laboratory ................................................... 8
   Ming Zhang, Shijie Sheng and Arthur B. Pardee
   Introduction and Underlying Concepts ........................................... 8
   The Biology of Human Maspin ....................................................... 9
   Mouse maspin ............................................................................... 12
   Biochemistry and Molecular Biology ............................................ 13
   Recent Developments .................................................................. 16

3. Maspin: Functional Insights from a Structural Perspective ...... 20
   Philip A. Pemberton
   Introduction ................................................................................ 20
   Biological Activities of Maspin ...................................................... 20
   Maspin is a Serpin: Implications for Function ............................... 22
   Conclusions: Future Applications—Anticipated Developments .......... 27

4. Maspin and Myoepithelial Cells ............................................... 30
   Sanford H. Barsky, Paul Kedeshian and Mary L. Alpaugh
   Introduction ................................................................................ 30
   Review of Current Research ........................................................ 31
   Future Directions .......................................................................... 53
   Abbreviations .............................................................................. 54

5. Maspin and Pericellular Plasminogen Activation  
   in Cell-Matrix Interaction ....................................................... 57
   Shijie Sheng, Hector Biliran Jr. and Richard McGowen
   Introduction ............................................................................... 57
   Maspin on Soluble Serine proteases ............................................. 57
   Maspin with Bound Plasminogen Activators ................................. 60
   Maspin in Cell-Matrix Interaction ................................................. 61
   Conclusion and Perspectives ....................................................... 64
   Acknowledgements ..................................................................... 64
6. Genetic and Epigenetic Regulation of Maspin Gene Expression in Normal and Tumor Tissue .......................................................... 68
   Frederick E. Domann and Bernard W. Futscher
   Introduction ........................................................................................ 68
   Review of Current Research ................................................................ 71
   Future Directions ................................................................................ 78
   Conclusion .......................................................................................... 80
   Acknowledgements .............................................................................. 82

7. Maspin Suppresses Breast Cancer Cell Invasiveness by Modulating Integrin Expression and Function ........................................ 84
   Richard E.B. Seftor, Valerie A. Odero, Elisabeth A. Seftor and Mary J.C. Hendrix
   Introduction ........................................................................................ 84
   Tumor Cell invasion and Metastasis .................................................... 85
   Integrins and Their Role in Tumor Cell invasion ............................... 85
   rMaspin Suppresses Breast cancer Cell Invasiveness
     In Vitro ........................................................................................... 87
   Role of MMP-2 in the Suppression of Breast Cancer Cell
     Invasiveness In Vitro by Maspin ...................................................... 90
   Maspin Re-expression Alters Cell Morphology, uPAR and α5β1
     Integrin Distribution on Human Breast cancer Cells ....................... 90
   Conclusion .......................................................................................... 91
   Acknowledgements .............................................................................. 92

8. The Role of Maspin in Tumor Progression and Normal Development ......................................................................................... 96
   Ming Zhang
   Introduction ........................................................................................ 96
   Maspin Gene Expression ..................................................................... 97
   Role of Maspin in Normal Development .......................................... 102
   Role of Maspin in Tumor Progression and Angiogenesis ............... 108
   Future Directions .............................................................................. 114

9. The Role of Maspin in Human Placental Development .......... 119
   Anuja Dokras, Lynn M.G. Gardner, Dawn A. Kirschmann, Elisabeth A. Seftor and Mary J.C. Hendrix
   Introduction ...................................................................................... 119
   Regulation of Cytotrophoblast Invasion ............................................ 119
   Tumor suppressor genes in the Human Placenta ............................... 120
   Maspin in the Human Placenta .......................................................... 121
   Discussion .......................................................................................... 122
   Acknowledgements .............................................................................. 123
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Chapter 4
This book represents the first compilation of the major research findings regarding maspin—a mammary serpin (or serine) protease inhibitor. Maspin was originally discovered through subtractive hybridization and differential display analyses, comparing the differences in gene expression in normal mammary epithelium and invasive carcinoma cells. This work originated in the laboratory of Dr. Ruth Sager, a world-renowned geneticist, at the Dana Farber Cancer Institute and Harvard Medical School. The first introduction of maspin to the research community occurred as a report in *Science* (263:526-529, 1994) and described the unique tumor-suppressing activity of this novel gene in human mammary epithelial cells.

At the time, the data showed that maspin is found in normal mammary epithelial cells and is down-regulated in invasive breast carcinomas. maspin encodes a novel serine protease inhibitor (serpin) with a single 3.0kb mRNA and a protein Mr of 42,000; it contains sequencing homology with members of the serine protease inhibitor superfamily (serpins), including plasminogen activator inhibitor-1, -2 (PAI-1 and PAI-2) and α1-antitrypsin, as well as sequence homology with noninhibitor serpins such as ovalbumin. Also, maspin was shown to have tumor suppressor activity when re-expressed in aggressive cancer cells. Furthermore, treatment of breast cancer cells with recombinant maspin inhibited tumor cell motility and invasion in vitro, and this inhibitory action could be reversed with antibodies specific for the reactive loop site. Thus, this original report about maspin offered substantial promise and opportunities with respect to its development as a favorable prognostic marker in breast cancer, in addition to Maspin’s potential use in new therapeutic strategies. This publication served as the foundation from which all other maspin studies would be derived—such as the findings reported in this book.

The maspin book provides an overview of Dr. Ruth Sager—the geneticist, and presents a unique perspective of her personal and professional history, contributed by her loving husband, Dr. Arthur B. Pardee. The first chapter describes the innovation, intellect and enthusiasm that Dr. Sager dedicated to her scientific discoveries. Chapter 2 follows with a historical review of maspin research in the Sager laboratory, and describes the underlying concepts of the maspin work from its initial phase to recent developments and findings. Chapter 3 focuses on the structural aspects of maspin with respect to specific biological activities and anticipated developments. The functional insights provided in this chapter are key to our understanding of how to exploit maspin for effective therapeutic application.

Chapter 4 presents the histopathology perspectives of maspin and offers unique insight into the potential use of maspin as a reliable prognostic indicator of disease progression. Chapter 5 focuses on the biochemical assessment of maspin with respect to the complex pericellular plasminogen activation in cell-matrix interactions. An intriguing hypothetical model is presented for the intricate interactions between maspin and plasminogen activator. Chapter 6 follows with new
information regarding the genetic and epigenetic regulation of maspin gene expression in normal and tumor tissue, and addresses the mechanisms underlying maspin gene silencing—a viable target for pharmacological reactivation. Chapter 7 describes some interesting observations of the suppression of breast cancer cell invasiveness via Maspin's modulation of integrin expression, and provides additional biological targets for Maspin's activity.

Chapters 8, 9 and 10 are interrelated and complementary. Chapter 8 provides a biological role for maspin in tumor progression and normal development, and highlights a critical role for maspin in angiogenesis. Chapter 9 follows with a unique role for maspin during human placental development. Chapter 10 provides important and relevant data regarding the prognostic potential of maspin in patients with oral squamous cell carcinoma.

The contributors selected for the first maspin book are leaders in their respective fields of research. Several of the contributors were part of the original maspin research with Dr. Ruth Sager. Those of us who had the privilege of knowing and working with Ruth Sager are truly fortunate. She contributed many seminal discoveries to science, and maspin represents a major component of her legacy.

Mary J.C. Hendrix
Ruth Sager, Geneticist

Arthur B. Pardee

Ruth Sager named her favorite gene Maspin, mammary serpin protease inhibitor. Expression of this gene is lost in advanced breast cancers and inhibits tumor invasion and metastasis. This book highlights advances made in studies that developed from her laboratory’s researchers starting over a half dozen years ago. Vigorous research on maspin continues in numerous laboratories, including those of several of her past students and fellows.

Very early Ruth believed that genetics is the core of biology. She “knew she was right—and set out to prove it.” She has never ceased introducing new techniques and concepts into her field. But she found her work ignored for years—until her discoveries proved the majority wrong. “I don’t really pay a lot of attention to what other people think”.

Ruth focused her efforts on the genetics of cancer during her final 25 years. “I had really wanted to work on cancer, but it seemed like a very difficult thing to do.” Entry into the subject was by her sabbatical at the Imperial Cancer Research Fund in 1972-73. “We think that the first change in cancer is a genetic change — something acts to transform an individual cell—whether that something be a viral infection, or a chemical or radiation”.

The first question she asked was which genes cause normal cells to become cancer cells. Growth-stimulating oncogenes, recently discovered, were then being proposed as the basis of cancer. But Sager championed inactivations of tumor suppressor genes, “Nature’s own approach to cancer protection”, as being in addition deeply involved, and that they are “a vast untapped resource for anticancer therapy”. She suggested a Yin-Yang balance of these—motors and brakes of growth—for normal cellular homeostasis.

She investigated changes, between closely related normal and cancer cells, of amounts of specific mRNAs, in particular those whose expression is lost in breast cancers, as underlying the cancer phenotype. She first discovered the IL-8 related GRO gene and others including maspin, whose mRNA levels are down-regulated in tumor cells, by using subtractive hybridization. She then shifted to the then new and simpler differential display technique to discover numerous additional potential tumor suppressor genes.

Dr. Sager discovered that most of the down-regulated genes are not mutated. But rather their mRNAs are under-expressed, for example by decreased transcription rates. She introduced the concept of “Expression Genetics” now also known as Functional Genomics, the study of changed gene expressions, as compared to genetics which is based upon mutational changes in sequences of structural gene. And she began investigation of methods to reactivate their expression.

Maspin, edited by Mary J.C. Hendrix. ©2002 Eurekah.com
Personality

Ruth Sager was innovative, highly intelligent, enthusiastic, very dedicated to her science, hard working, had high standards, and expected equal dedication from her coworkers. She did not suffer fools gladly. She stated her view of science as a career. “The first thing is to be sure of your own abilities. Science is very demanding, you have to be able to think very well and also have a very good memory. You have to really love it. Science is a way of life. I think it all comes from inside. It really gets to the very core of your existence. It is much like being an artist or a dancer. It’s something that demands everything from you that you are capable of.” “I have always been intrigued by the physicists approach to scientific inquiry, particularly in the fact that the way to find out something really new is to question the basic tenet of existing theory”.

She was described in her fifties as “a calmly articulate and attractive woman (who looks younger by about 15 years).” Again, as “a tall, striking brunette with a ready smile and a voice that carries a merry lilt.” She described herself as “probably the happiest person I know.”

She was not at all narrowly devoted to her science, and had numerous outside interests—modern art, travel, music and theater, and a rich social life. She was a fine cook. She took up tennis late in life, and played it with great enthusiasm in spite of limited ability. She was especially fond of relaxing at Woods Hole where she had a cherished second home, where she is buried, and where her papers are stored in the Marine Biological Laboratory library.

Personal History

Ruth Sager was born in Chicago on February 7, 1918, daughter of Leon Sager, a businessman with strong intellectual interests, and Deborah Borovik Sager who died in the 1918 influenza epidemic. She and her sisters Esther and Naomi were brought up by her step-mother Hannah in an atmosphere honoring learning. She graduated at 16 from New Trier High School. She received a B.S. in mammalian physiology in 1938 at the University of Chicago, “the best thing that ever happened to me”. Her interest in science was originated by Anton J. Carlson’s lectures. “He was just a fantastic teacher.” She received a M.S. in 1944 in plant physiology at Rutgers Univ., spent the World War II years as a secretary and apple farmer. Her Ph.D. in 1948 was under Marcus M. Rhoades in maize genetics at Columbia University. Then she was a Merck Postdoctoral Fellow 1949-1951 with Sam Granick at the Rockefeller Institute, where she worked on the chloroplast. She was a staff member at the Rockefeller Institute from 1951-1955, where she chose the alga *Chlamydomonas reinhardtii* as a model organism. She was a Research Scientist 1955-1965 at Columbia University, and worked for a year in Edinburgh 1962.

For 20 years she could not obtain a faculty position, till age 48. “I guess I knew I was right, and I wasn’t terribly upset.” She was a Professor at Hunter College 1966-1975, Guggenheim Fellow at Imperial Cancer Research Fund in London 1972-1973. She was finally in 1975 appointed Professor of Cellular Genetics at Harvard Medical School, among the first women to gain Full Professorship at Harvard, and Chief Division of Cancer Genetics, Dana-Farber Cancer Institute.

She was first married to Seymour Melman in 1944, and then to Arthur B. Pardee in 1973. She had no children. She died March 29, 1997, of bladder cancer, in her
home in Brookline, Mass. at the age of 79. She worked to the end, publishing innovative articles and obtaining an NIH grant in the month before her death. She is survived by sisters Esther Altschul and Naomi Sager, and her husband.

Professional History


Cancer Biology

Among her outstanding contributions, Dr. Sager emphasized the major role of chromosome rearrangements and the accelerated evolution of cancer cells, the requirement in a cancer of more than one mutated gene, and importantly of tumor suppressor genes in addition to oncogenes. She proposed as early as 1974 that individual genetic defects could be corrected by transferring DNA into cells. “One need not be doomed by one’s genes.” She was a pioneer in the novel subject she named expression genetics, the identification by their mRNAs of genes that are functionally modified in cancers. She successfully identified numerous genes that are not mutated but whose expressions are altered in breast cancers, such as the mammary serpin Maspin.

1. She devised the first cell lines and culture medium capable of culturing and comparing normal and cancer cells. She developed a model system that allows detailed comparisons in the same culture medium between well-matched normal and tumor Chinese hamster embryo fibroblasts (CHEF cells).

2. She emphasized the multigenic basis of tumorigenicity. As with her earlier work, she was among the first to champion a then unpopular view, at a time when all attention was on mutations of growth stimulating single oncogenes such as ras. In the late 70’s she initiated investigations on negatively regulating genes—tumor suppressors. As with her research on chloroplast genetics, “ there was really no interest in tumor suppressor genes at all until about maybe....1990.” She demonstrated tumor-suppressing activity with cell hybrids and cybrids. Remarkable examples are suppressor genes such as p53 that promote programmed cell death of defective cells, and these are inactivated in tumors.

3. She provided an initial example of increased genetic instability in cancer cells. Amplification of the methotrexate resistance gene developed much faster in tumor cells than in normal cells.

4. Turning from then popular rodent cells, she decided that gene expression would best be investigated in human cells. For this purpose she created a workable human breast cancer cell culture system, in which epithelial normal and tumor cells could both grow, and at similar rates.

5. Among her final interests were effects of methylation of DNA, and its specific enzymatic cutting, and chromosome rearrangements in tumor cells.
6. The under-expressed genes, including maspin, are not mutated, unlike classical tumor-related genes. Therefore her plan was to use these under-expressed genes as markers for detection and diagnosis, and she hoped to design therapies based on restoring their functions.

Cytoplasmic Inheritance

Ruth Sager's contributions in cancer research were during her second distinguished career, a major innovator in cancer genetics in proposing, discovering, and investigating roles of tumor suppressor genes including maspin. In her first career she was at the pinnacle of research on the problem of non-nuclear or “cytoplasmic” genetics. She almost single handedly developed this subject of non-Mendelian genetics. “A vast, unexplored region of genetics was opened here today (1963).” The very existence of hereditary determinants other than nuclear genes was doubted by a large part of the scientific community, although it was proposed in 1908 from observations on higher plants. Dr. Sager gathered data and argued in support of a second genetic system in the face of great skepticism, and finally made this a respectable and exciting major area of genetics.

At the beginning of her research, Dr. Sager saw the advantages of studying genetics with a model microorganism that had a chloroplast, a sexual life cycle, grew rapidly, and was readily manipulated for controlling growth and mating. She chose the single cell alga \textit{Chlamydomonas}, a peerless group of organisms. .... nutritious, aesthetically pleasing, and amenable to laboratory experimentation”. With talented collaborators, especially her right hand colleague Zenta Ramanis she:

1. Developed a mating system for the organism.
2. Early investigated the genetics of the organism—both Mendelian and non-Mendelian—with clear demonstration of the maternal inheritance pattern of the latter.
3. Discovered, with Y. Tsubo, the first specific “cytoplasmic” gene mutagen, streptomycin, and identified mutants by their resistance to this drug.
4. Discovered ribosomes in the chloroplast of \textit{Chlamydomonas}, different from those in the cytoplasm, thus providing evidence that expression of genetic information as proteins is carried out by a different system.
5. Discovered, with M. R. Ishida, that unique DNA is located in isolated chloroplasts. This was the evidence that convinced most scientists that there is indeed a separate non-nuclear organelle genetic system.
6. Performed biochemical studies of the mechanism of exclusion of paternal genes.
7. Developed a system that makes genetic mapping possible, by permitting the expression of paternal as well as maternal genes.
8. Developed several mapping methods, and first published cytoplasmic linkage groups and extensive mapping of an organelle, showing that the chloroplast's DNA is circular.
9. Demonstrated with an in vitro system the basis of maternally inherited drug resistance.
10. Discovered an eukaryotic restriction enzyme.
11. Discovered that there is communication between nucleus and organelles; they send molecular signals back and forth.
12. Showed that maternal DNA is methylated and paternal DNA is not, and proposed this difference as the basis of selective destructive elimination of the paternal DNA.
**Personal Legacy**

When asked near the end of her life what she considered to be her most important contribution, she answered “Well, I don't think I’ve made it yet.” Many colleagues have carried on her research, and papers based upon her research continue to appear. She was a major constructive force in the scientific and personal lives of her many friends and students.

She was a role model for many women, being among the earliest very successful woman scientists in spite of major career obstacles. “For more than half a century Ruth Sager has been a role model for women in health-related scientific research. ..... she demonstrated vision, insight and determination to develop novel scientific concepts in the face of established dogmas. .... her pioneering research and original ideas continue to make contributions to biology”. But she was never highly active in the women’s liberation movement. When faced with the built-in prejudice of the male scientific community against women “there was nothing I could do, except to be as good (a scientist) as possible”.

She had great concerns in 1994 about politics and the future of science. “The strong influence of fashions in scientific thought continues to play an inhibitory role in scientific progress. I think science is in a rut right now. The way grants are given out just makes matters worse, because the experiment has to be so obvious and practically done already before they’ll fund it.” Her career twice demonstrated that some of the best science needs faith and support of novel ideas from the most creative minds.

**Articles about Ruth Sager**

I am indebted to these authors for many of the cited facts and quotes.

**Selected Bibliography**

Dr. Sager published two ground breaking books:
She published more than 200 articles, among them:

**Cancer Research**


Expression genetics in cancer: Shifting the focus from DNA to RNA. Proc Natl Acad Sci USA 1997; 94:952-955.


**Cytoplasmic Inheritance**


With Lane D. Molecular basis of maternal inheritance. Proc Natl Acad Sci USA 1972; 69:2410-2413.
CHAPTER 2

Maspin in the Sager Laboratory

Ming Zhang, Shijie Sheng and Arthur B. Pardee

Introduction and Underlying Concepts

Discovery of a disease-related gene marks only the beginning to a series of difficult investigations. In order to establish the functional role of the newly discovered gene, one has to obtain insights into its biological activities, genetic and epigenetic regulations, and molecular mechanisms operating under various conditions such as in cancer cells and in normal cells. Is it, as examples, involved in growth control, genetic stability, apoptosis, immortalization, differentiation, angiogenesis, invasion and metastasis, etc? What is the molecular biology of its transcription? Does it in turn control the transcription of other genes? Where is its protein product located? What are the biochemical properties of the protein? Is it an enzyme, transcription factor or a structural element, etc? Does it undergo post-translational modifications, and what effects do these have?

The following is a summarization of published work on maspin performed by researchers in and from the Ruth Sager laboratory. In order to preserve the flavor of the laboratory, the material that follows is composed of the summaries of their publications. It is intended to provide a general paradigm of how the discovery of one gene of interest can lead into a subject for intensive investigation and to novel concepts. And also it illustrates approaches that can be applied to establish functional roles of a newly discovered gene.

Tumor Suppressors

Tumor Suppressor Genes: The Puzzle and the Promise

Tumor suppressor genes are wild-type alleles of genes that play regulatory roles in cell proliferation, differentiation, and other cellular and systemic processes. It is their loss or inactivation that is oncogenic. The first evidence of tumor suppressor genes appeared in the early 1970s, but only within the past few years has a wealth of new information illuminated the central importance of these genes. Several different suppressor genes may be inactivated in the same tumors, and the same suppressors may be inactive in different tumor types (for example, lung, breast, and colon). The suppressor genes already identified are involved in cell cycle control, signal transduction, apoptosis, angiogenesis, and development, demonstrating that they contribute to a
broad array of normal and tumor-related functions. It is proposed that tumor suppressor genes provide a vast untapped resource for anticancer therapy.

**Expression Genetics (Subtractive Hybridization and DD)**

**Expression Genetics in Cancer: Shifting the Focus from DNA to RNA**

Expression genetics is a conceptually different approach to the identification of cancer-related genes than the search for mutations at the genome level. While mutations lie at the heart of cancer, at least in its early stages, what is recognized here are phenotypic changes that are usually many steps removed from the initiating mutation. Cancer geneticists have classically concentrated on genomic changes and have ignored the productive potential of examining downstream events based on screening for differential gene expression between tumor cells and well matched normal counterparts. Genes involved in cancer affect the normal functions of many cellular processes, not only proliferation but cell-cell and cell-matrix interactions, DNA repair, invasion and motility, angiogenesis, senescence, apoptosis, and others. However, very few cancer-related genes affecting these processes have been identified in human cancers by classical methods despite enormous efforts. I report here our success in readily isolating more than 100 candidate tumor suppressor genes from human tissue, estimated to represent roughly 20% of the total genes recoverable by this approach. Half of the genes are unknown and the other half includes representatives of most known cancer processes. Because their expression is lost during cancer progression, they may be useful tumor markers for diagnosis and prognosis. Because several of these genes are not mutated, they provide opportunities for pharmacological intervention by inducing their re-expression.

**Expression Genetics: A Different Approach to Cancer Diagnosis and Prognosis**

Expression genetics is a new approach to the identification of cancer-related genes. Instead of studying mutations at the genome level (gene mutations), expression genetics is the investigation of heredity at the RNA level. By isolating genes whose expression is up- or down-regulated in cancers, expression geneticists study their function in the context of gene regulation. A major goal of expression genetics in cancer is to correct gene expression in tumors by the application of potential therapeutic agents.

**The Biology of Human Maspin**

**Identification of Maspin**

**RNA Genetics of Breast Cancer: Maspin as a Paradigm**

Our focus is on genes that are down-regulated, but not mutated, in mammary carcinomas but not in normal mammary epithelial cells. This focus has led to the identification of a large number of candidate tumor suppressor genes, approaching
the number of known oncogenes. This research is the initial demonstration that
dysregulated genes are numerous in cancer. Implications of the fact that so many
genes are simultaneously down-regulated but not mutated in cancer cells are considered.
Expansions of such gene sets are currently under vigorous investigation, by applying
powerful methods including Differential Display, SAGE, and microarrays.

Maspin is an example of a down-regulated gene in breast cancer, discovered in
this laboratory by subtractive hybridization. Maspin encodes a novel serine protease
inhibitor (serpin), as demonstrated by sequence comparison. Maspin can be re-expressed
in tumor cells by phorbol ester treatment. It functions as a tumor suppressor gene.

Maspin: A Tumor Suppressing Serpin

Maspin, a serpin found in normal mammary epithelial cells, is down-regulated in
invasive breast carcinomas. Similar patterns of expression at the RNA and protein
levels are seen by Northern analysis with cells grown in culture and by immunostaining
of tissues. Maspin has been shown to have tumor suppressor activity. Although maspin
does not behave as a classical inhibitory serpin against any known target protease in
solution. Biological studies have shown that recombinant maspin inhibits tumor cell
motility and invasion through reconstituted basement membranes, and that its
inhibitory action is totally lost by a single cleavage at the reactive loop site. Tumor
transfectants expressing maspin are inhibited in growth and metastasis in nude mice.
The biological function of maspin is located at the cell surface.

Maspin, a Serpin with Tumor-Suppressing Activity in Human
Mammary Epithelial Cells

A gene encoding a protein related to the serpin family of protease inhibitors was
identified as a candidate tumor suppressor gene that may play a role in human breast
cancer. The gene product, called maspin, is expressed in normal mammary epithelial
cells but not in most mammary carcinoma cell lines. Transfection of MDA-MB-435
mammary carcinoma cells with the maspin gene did not alter the cells’ growth properties
in vitro, but reduced the cells’ ability to grow and metastasize in nude mice and to
invade through a basement membrane matrix in vitro. Analysis of human breast cancer
specimens revealed that loss of maspin expression occurred most frequently in advanced
cancers. These results support the hypothesis that maspin functions as a tumor
suppressor. For a review see ref. 12.

In Breast Cancer vs. Normal Cells

Production, Purification and Characterization of Recombinant
Maspin Proteins

In this paper, we report the production of recombinant glutathione S-transferase-
maspin fusion protein, expressed in the bacterium Escherichia coli, and recombinant
maspin, expressed in the insect Spodoptera frugiperda cells. The GST-fusion protein
was purified by glutathione affinity chromatography. Maspin expressed in insect cells
was purified by a combination of Bio-Rad AG1-2X anion exchange chromatography
and heparin affinity chromatography. Both recombinant proteins demonstrated strong
inhibitory effects on the invasion by two breast tumor cell lines across reconstituted basement membranes and such inhibitory effect was abolished in the presence of the polyclonal antibody made against the reactive center region of maspin. The recombinant maspin from insect cells was cleaved by trypsin specifically at the putative reactive center, as confirmed by protein sequencing. The trypsin-cleaved recombinant maspin did not inhibit invasion, indicating that intact putative reactive center of maspin is required for its biological activities. This paper provides evidence that recombinant maspin protein itself inhibits invasion and supports the role of maspin as a tumor suppressor.

Maspin Acts at the Cell Membrane to Inhibit Invasion and Motility of Mammary and Prostatic Cancer Cells

Recombinant maspin protein blocks the motility of breast carcinoma cells in culture over 12 h, as demonstrated by time-lapse video microscopy. Lamellipodia are withdrawn but ruffling continues. Both exogenous recombinant maspin and maspin expressed by tumor transfectants exhibit inhibitory effects on cell motility and cell invasion as shown in modified Boyden chamber assays. When mammary carcinoma cells were treated with recombinant maspin, the protein was shown by immunostaining to bind specifically to the cell surface, suggesting that maspin activity is membrane associated. When pretreated with antimaspin antibody, maspin loses its inhibitory effects on both invasion and motility. However, when maspin is added to these cells preceding antibody treatment, the activity of maspin is no longer inhibited by subsequent addition of the antibody. It is concluded therefore that the inhibition of invasion and motility by maspin is initially localized to the cell surface.

Maspin Suppresses the Invasive Phenotype of Human Breast Carcinoma

The exploitation of maspin as a potential diagnostic and/or therapeutic tool has remained limited due to the lack of knowledge concerning its molecular and biological mechanism(s) of action. The work reported here demonstrates that treatment of MDA-MB-435 cells with recombinant maspin enhances the selective cell adhesion to a fibronectin matrix and induces the conversion from a fibroblastic to a more epithelial-like phenotype. In addition, a blocking antibody to integrins is shown to abrogate the inhibitory activity of maspin on tumor cell invasion through a fibronectin matrix-containing barrier in vitro. Further molecular analyses revealed that recombinant maspin induces higher cell surface levels of some integrins and reduced levels of others in the metastatic human breast carcinoma cell line MDA-MB-435, concomitant with its ability to inhibit the invasive process in vitro. Taken together, these data address the hypothesis that maspin reduces the invasive phenotype of MDA-MB-435 cells by altering their integrin profile, which in turn converts these cells to a more benign epithelial phenotype, with a reduced invasive potential. These data provide new insights into the biological significance of this tumor suppressor gene found in normal mammary epithelium and may form the basis of novel therapeutic strategies in the management of breast carcinoma.
In Prostate Cancer

Maspin Acts at the Cell Membrane to Inhibit Invasion and Motility of Mammary and Prostatic Cancer Cells

Since breast and prostate are both glandular epithelial tissues, prostate cancer and breast cancers have numerous common pathological features. We showed that recombinant maspin exerts a potent inhibitory effect on three prostatic cancer cell lines in both invasion and motility assays. An oligopeptide-derived polyclonal antibody against the reactive site loop sequence of maspin neutralized the inhibitory activity of maspin in both assays. This data suggests that maspin may block the progression of breast cancer and prostate cancer by a similar mechanism. (Additional results with prostate cancer are presented below.)

Mouse Maspin

In Mouse Breast Cancer

mMaspin: The Mouse Homolog of a Human Tumor Suppressor gene Inhibits Mammary Tumor Invasion and Motility

In order to examine the role of maspin in an intact mammal, we cloned mouse maspin cDNA by screening a mouse mammary gland cDNA library with the human maspin cDNA probe. The deduced protein sequence of mouse maspin (mMaspin) is 89% homologous with human maspin. Like its human homolog, mMaspin is expressed in normal mouse mammary epithelial cells and down-regulated in mouse breast tumor cell lines. The expression is altered at different developmental stages in the mammary gland.

The recombinant mouse maspin was produced as a GST-fusion protein in *E. coli* and was purified by glutathione affinity chromatography. Addition of the recombinant mMaspin protein to mouse tumor cells was shown to inhibit invasion in a dose-dependent manner. As with the human protein, recombinant mMaspin protein also inhibited mouse mammary tumor motility. Deletion in the putative mMaspin reactive site loop (RSL) region resulted in the loss of its inhibitory functions. Taken together, these data suggest that the homologous proteins play similar physiological roles in vivo.

Reduced Mammary Tumor Progression in WAP-TAg/WAP-Maspin Bitransgenic Mice

Maspin is a unique serpin involved in the suppression of tumor growth and metastasis. To investigate whether increased levels of maspin protect against tumor progression in vivo, we established a transgenic model in which maspin is targeted to mammary epithelial cells by the whey acidic protein (WAP) promoter for over expression. We crossed these maspin transgenic mice with the WAP-TAg mouse model of tumor progression. Maspin over expression increased the rate of apoptosis of both preneoplastic and carcinomatous mammary epithelial cells. Maspin reduced tumor growth through a combination of reduced angiogenesis and increased apoptosis. The
number of pulmonary metastases was reduced with maspin over expression. These data demonstrate that targeted over expression of maspin can inhibit tumor progression in vivo, likely through a combination of increased apoptosis, decreased angiogenesis, and inhibition of tumor cell migration.

**In Normal Breast Development**

**Maspin Plays an Important Role in Mammary Gland Development**

Maspin, a unique member of the serpin family, functions as a class II tumor suppressor gene. Despite its known activity against tumor invasion and motility, little is known about maspin's functions in normal mammary gland development. In this paper, we show that maspin does not act as a tissue plasminogen activator inhibitor in the mammary gland. However, targeted expression of maspin by the whey acidic protein gene promoter inhibits the development of lobular-alveolar structures during pregnancy and disrupts mammary gland differentiation. Apoptosis was increased in alveolar cells from transgenic mammary glands at midpregnancy. However, the rate of proliferation was increased in early lactating glands to compensate for the retarded development during pregnancy. These findings demonstrate that maspin plays an important role in mammary development and that its effect is stage dependent.

**Biochemistry and Molecular Biology**

**Transcription Control**

**Transactivation through ets and Ap1 Transcription Sites Determines the Expression of the Tumor-Suppressing Gene Maspin**

Tumor invasion and metastasis are processes poorly understood at the molecular level. Maspin is a serine protease inhibitor (serpin) with tumor-suppressing function in the mammary gland. Maspin gene expression is decreased with malignancy and is lost in metastatic cells. We show in this report that differential expression of maspin in normal and carcinoma-derived mammary epithelial cells is regulated at the transcriptional level. We have identified the ets and Ap1 sites in the maspin promoter that are active in regulating maspin expression in normal mammary epithelial cells but inactive in tumor cells. The ets site alone is sufficient to activate transcription in a heterologous promoter, whereas the Ap1 site cooperates with ets in activation. The enhancing function by ets and Ap1 elements is decreased in primary tumor cells (21NT) and is abolished in invasive tumor cells (MDA-MB-231). Thus, loss of maspin expression during tumor progression results at least in part from the absence of transactivation through the ets and Ap1 sites.
**Expression of Maspin in Prostate Cells is Regulated by a Positive ets Element and a Negative Hormonal Responsive Element Site Recognized by Androgen Receptor**

Prostate cancer is the most common cancer in men. The molecular mechanisms leading to its development are poorly understood. Maspin is a tumor-suppressing serpin expressed in normal breast and prostate epithelium. We have found that expression of maspin in normal and carcinoma-derived prostate epithelial cells is differentially regulated at the transcriptional level. We have identified two different kinds of cis elements, ets and hormonal responsive element (HRE), in the maspin promoter. The ets element is active in regulating maspin expression in normal prostate epithelial cells but inactive in tumor cells. The HRE site is a negative element that is active in both cell types. This negative DNA sequence can repress a heterologous promoter recognized by the androgen receptor. We conclude that expression of maspin is under the influence of both a positive ets and a negative HRE element. Loss of maspin expression during tumor progression apparently results from both the absence of transactivation through the ets element and the presence of transcription repression through the negative HRE element recognized by androgen receptor.

**p53 Regulates the Expression of the Tumor Suppressor Gene Maspin**

Maspin has been shown to inhibit tumor cell invasion and metastasis in breast tumor cells. Maspin expression was detected in normal breast and prostate epithelial cells, whereas tumor cells exhibited reduced or no expression. However, the regulatory mechanism of maspin expression remains unknown. We report here a rapid and robust induction of maspin expression in prostate cancer cells (LNCaP, DU145 and PC3) and breast tumor cells (MCF7) following wild type p53 expression from an adenovirus p53 expression vector (AdWTp53). p53 activates the maspin promoter by binding directly to the p53 consensus-binding site present in the maspin promoter. DNA-damaging agents and cytotoxic drugs induced endogenous maspin expression in cells containing the wild type p53. Maspin expression was refractory to the DNA-damaging agents in cells containing mutant p53. These results, combined with recent studies of the tumor metastasis suppressor gene KAI1 and plasminogen activator inhibitor 1 (PAI1), define a new category of molecular targets of p53 that have the potential to negatively regulate tumor invasion and/or metastasis.

**Protease Inhibition-Structure and Kinetics**

Maspin is a protein of 42 kilodalton with extensive sequence homology to members of the serpin family. Serpins are divided into two classes (inhibitory and non-inhibitory) and both play very important roles in vivo. The following is evidence that supports maspin’s role as a protease substrate or inhibitor.
The Tumor Suppressor Maspin Does Not Undergo the Stressed to Relaxed Transition or Inhibits Trypsin-Like Serine Proteases: Evidence that Maspin Is Not a Protease Inhibitory Serpin

The role of tumor suppressor proteins in the development of malignancy has made the understanding of their molecular mechanisms of action of great importance. Maspin is a tumor suppressor produced by a number of cell types of epithelial origin. Exogenous recombinant maspin has been shown to block the growth, motility, and invasiveness of breast tumor cell lines in vitro and in vivo. Although it belongs to the serine proteinase inhibitor (serpin) superfamily of proteins, the molecular mechanism of maspin is currently unknown. Here we show that the reactive site loop of maspin exists in an exposed conformation. The reactive site loop of maspin, however, does not act as an inhibitor of proteinases such as chymotrypsin, elastase, plasmin, thrombin, and trypsin but rather as a substrate. Maspin is also unable to inhibit tissue and urokinase type plasminogen activators. Stability studies show that maspin cannot undergo the stressed-relaxed transition typical of proteinase-inhibitory serpins, and the protein is capable of spontaneous polymerization induced by changes in pH. It is likely, therefore, that maspin is structurally more closely related to ovalbumin and angiotensinogen, and its tumor suppressor activity is independent of a latent or intrinsic trypsin-like serine proteinase-inhibitory activity.

Tissue-Type Plasminogen Activator is a Target of the Tumor Suppressor Gene Maspin

Maspin is structurally a member of the serpin (serine protease inhibitors) superfamily but deviates somewhat from classical serpins. We find that single-chain tissue plasminogen activator (sctPA) specifically interacts with the maspin reactive site loop peptide and forms a stable complex with recombinant maspin. Major effects of maspin are observed on plasminogen activation by sctPA. First, maspin activates free sctPA. Second, it inhibits sctPA preactivated by poly-D-lysine. Third, maspin exerts a biphasic effect on the activity of sctPA preactivated by fibrinogen/gelatin, acting as a competitive inhibitor at low concentrations (< 0.5 micromolar) and as a stimulator at higher concentrations. Fourth, 38-kDa C-terminal truncated maspin further stimulates fibrinogen/gelatin-associated sctPA. Maspin acts specifically; it does not inhibit urokinase-type plasminogen activator, plasmin, chymotrypsin, trypsin, or elastase in solution. Our kinetic data are quantitatively consistent with a model in which two segregated domains of maspin interact with the catalytic and activating domains of sctPA. These complex interactions between maspin and sctPA in vitro suggest a mechanism by which maspin regulates plasminogen activation by sctPA bound to the epithelial cell surface.

The Surface of Prostate Carcinoma DU145 Cells Mediates the Inhibition of Urokinase-Type Plasminogen Activator by Maspin

Maspin is a novel serine protease inhibitor (serpin) with tumor suppressive potential in breast and prostate cancer, acting at the level of tumor invasion and metastasis. It was subsequently demonstrated that maspin inhibits tumor invasion, at least in part, by inhibiting cell motility. Interestingly, in cell-free solutions, maspin does not
inhibit several serine proteases including tissue-type plasminogen activator and uroki-
nase-type plasminogen activator (uPA). Despite the recent biochemical evidence that
maspin specifically inhibits tissue-type plasminogen activator that is associated with
fibrinogen or poly-lysine, the molecular mechanism underlying the tumor-suppres-
sive effect of maspin remains elusive. The goal of this study was to investigate the
effect of maspin on cell surface-associated uPA. In our experimental system, we chose
prostate carcinoma DU145 cells because these cells mediate plasminogen activation
primarily by uPA, as shown by two different colorimetric enzyme activity assays. Pu-
rified recombinant maspin produced in baculovirus-infected Spodoptera frugiperda Sf9
insect cells binds specifically to the surface of DU145 cells, inhibits the DU145 cell
surface-bound uPA, and forms a stable complex with the uPA in DU145 cell lysate.
The inhibitory effect of maspin on cell surface-bound uPA was similar to that of an
uPA-neutralizing antibody and was reversed by a polyclonal antibody against the reactive
site loop sequence of maspin. The Ki value for maspin in cell surface-mediated
plasminogen activation was 20 nM, which was comparable to the Ki values for
plasminogen activator inhibitor 1 and plasminogen activator inhibitor 2, respectively.
Furthermore, the proteolytic inhibitory effect of maspin was quantitatively consistent
with its inhibitory effect on the motility of DU145 cells in vitro. Our data demon-
strate an important role for the prostate carcinoma cell surface in mediating the in-
hibitory interaction between maspin and uPA. Thus, future maspin-based therapeutic
strategies may prove useful in blocking the invasion and metastasis of uPA-positive
prostate carcinoma.

Recent Developments

Cancer Marker Detection

Linking Gene Expression Patterns to Therapeutic Groups
in Breast Cancer

A major objective of current cancer research is to develop a detailed molecular
characterization of tumor cells and tissues that is linked to clinical information. Toward
this end, we have identified approximately one-quarter of all genes that were aberrantly
expressed in a breast cancer cell line using differential display. The cancer cells lost the
expression of many genes involved in cell adhesion, communication, and maintenance
of cell shape, while they gained the expression of many synthetic and metabolic enzymes
important for cell proliferation. High-density, membrane-based hybridization arrays
were used to study mRNA expression patterns of these genes in cultured cells and
archived tumor tissue. Cluster analysis was then used to identify groups of genes, the
expression patterns of which correlated with clinical information. Two clusters of genes,
represented by p53 and maspin, had expression patterns that strongly associated with
estrogen receptor status. A third cluster that included HSP-90 tended to be associated
with clinical tumor stage, whereas a forth cluster that included keratin 14 tended to be
associated with tumor size. Expression levels of these clinically relevant gene clusters
allowed breast tumors to be grouped into distinct categories. Gene expression
fingerprints that include these four gene clusters have the potential to improve prog-
nostic accuracy and therapeutic outcomes for breast cancer patients.
Detection in Blood

High-Sensitivity Array Analysis of Gene Expression For the Early Detection of Disseminated Breast Tumor Cells in Peripheral Blood

Early detection is an effective means of reducing cancer mortality. Here, we describe a highly sensitive high-throughput screen that can identify panels of markers for the early detection of solid tumor cells disseminated in peripheral blood. The method is a two-step combination of differential display and high-sensitivity cDNA arrays. In a primary screen, differential display identified 170 candidate marker genes differentially expressed between breast tumor cells and normal breast epithelial cells. In a secondary screen, high-sensitivity arrays assessed expression levels of these genes in 48 blood samples, 22 from healthy volunteers and 26 from breast cancer patients. Cluster analysis identified a group of 12 genes that were elevated in the blood of cancer patients. Permutation analysis of individual genes defined five core genes (P < 0.05, permix test). As a group, the 12 genes generally distinguished accurately between healthy volunteers and patients with breast cancer. Maspin was an excellent marker in this set. Mean expression levels of the 12 genes were elevated in 77% (10 of 13) untreated invasive cancer patients, whereas cluster analysis correctly classified volunteers and patients (P = 0.0022, Fisher’s exact test). Quantitative real-time PCR confirmed array results and indicated that the sensitivity of the assay (1:2 x 10(8) transcripts) was sufficient to detect disseminated solid tumor cells in blood. Expression-based blood assays developed with the screening approach described here have the potential to detect and classify solid tumor cells originating from virtually any primary site in the body.

Therapy

Maspin Is an Angiogenesis Inhibitor

Maspin, a unique member of the serpin family, is a secreted protein encoded by a class II tumor suppressor gene whose downregulation is associated with the development of breast and prostate cancers. Overexpression of maspin in breast tumor cells limits their growth and metastases in vivo. In this report we demonstrate that maspin is an effective inhibitor of angiogenesis. In vitro, it acted directly on cultured endothelial cells to stop their migration towards basic fibroblast growth factor and vascular endothelial and to limit mitogenesis and tube formation. In vivo, it blocked neovascularization in the rat cornea pocket model. Maspin derivatives mutated in the serpin reactive site lost their ability to inhibit the migration of fibroblasts, keratinocytes, and breast cancer cells but were still able to block angiogenesis in vitro and in vivo. When maspin was delivered locally to human prostate tumor cells in a xenograft mouse model, it blocked tumor growth and dramatically reduced the density of tumor-associated microvessels. These data suggest that the tumor suppressor activity of maspin may depend in large part on its ability to inhibit angiogenesis and raise the possibility that maspin and similar serpins may be excellent leads for the development of drugs that modulate angiogenesis.