The money generated from Dr. Doisy’s patents on estrogens has been used for the benefit of our Department and the School of Medicine through a foundation that is today worth over $100 million. His professional accomplishments were honored with a 1944 Nobel Prize.

The history of any academic department is written by each and every faculty, staff, and trainee. However, as the Edward A. Doisy Department of Biochemistry and Molecular Biology at Saint Louis University School of Medicine enters its 90th year, it is remarkable to note that the Department has only recently recruited its fourth chairman since it began. In this essay, we offer a brief history of the department through an account of these remarkable leaders and their contributions to biochemistry and to the growth of our department.

1924-1965

In 1919, Edward Doisy (See Figure 1), a young Harvard graduate, was hired by Dr. Philip Shaffer as Assistant Professor of Biological Chemistry at Washington University in St. Louis based solely on his affiliation with Otto Folin, Shaffer’s former mentor. Four years later, Dean Hanau Loeb from Saint Louis University called Shaffer over the phone to offer Doisy a professorship, a position that Doisy would accept at Shaffer’s insistence. In January 1924, the Department of Biochemistry was founded in the Medical School at Saint Louis University with Doisy as its first chair.

While still at Washington University, Dr. Doisy had started collaboration with Edgar Allen in the Anatomy Department that culminated in the landmark localization, extraction, and partial purification of an ovarian hormone. After moving to Saint Louis University, Doisy became convinced that isolation of the hormone would require processing of large quantities of biological specimens. He commissioned from a local metal concern a large scale continuous extractor for approximately $150. When it was finished, the bill was $750 – roughly the yearly salary of a junior professor in those days. When Doisy took the bill to Dean Loeb, he expected to be fired. The Dean did blink twice but approved the purchase. Dean Loeb died in 1927 and unfortunately could not witness the extraordinary returns on the investment that he had made by paying that bill. Between 1929 and 1936, Doisy discovered and isolated estron (1929), estriol (1931), and estradiol (1936), heralding the pharmacology of female sex hormones. Dr. Doisy and his junior colleague (and first PhD student) Dr. Philip Katzman, assigned the patents from these
discoveries to Saint Louis University in 1955 in return for the generosity of Dean Loeb. The money generated from Doisy’s patents on estrogens has been used for the benefit of our Department and the School of Medicine through a foundation that is today worth over $100 million. Half of the buildings in the medical school bear the Doisy name, including the state-of-the-art Doisy Research Center, an $82 million modern construction that houses the entire Department of Biochemistry and Molecular Biology and several scientists from other basic science and clinical departments.

The discovery of estrogens did not mark the zenith of Doisy’s career. After three years of efforts using the large-scale continuous extractor that led to the isolation of estrone, Dr. Doisy succeeded in the extraction and synthesis of vitamin K from alfalfa and putrefied fish meal in 1939. When the Nobel Committee resumed its activities in 1944, after a hiatus of five years due to World War II, the 1943 Nobel Prize in Physiology or Medicine was shared by Doisy and Henryk Dam for the synthesis and discovery of vitamin K, respectively.

Dr. Doisy’s commitment to the growth and development of the Department he founded was met with challenges, of course, and perhaps none as great as the Great Depression that gripped the country in the early and mid-thirties. Dr. Harold Katzman, one of Dr. Doisy’s early faculty appointments, recalled that during this difficult time, he was summoned to Dr. Doisy’s office and told that there were no funds to pay faculty salaries. When asked (several decades later) how he reacted, Dr. Katzman responded: “I felt lucky. At least I had a job.”

Dr. Doisy remained at Saint Louis University as chair of the Department of Biochemistry until his retirement in 1965. The Department of Biochemistry was renamed the Edward A. Doisy department in his honor in 1955.

Dr. Doisy died in 1986 at the age of 92. Doisy’s extraordinary accomplishments as a scientist, educator, and member of the Saint Louis University community have shaped a legacy of excellence and dedication that continues to inspire to the present day.

1965-1982

Robert E. Olson, MD, PhD (See Figure 2), succeeded Dr. Doisy as chair of the Biochemistry Department in 1965. This represented a homecoming of sorts for Dr. Olson, as he received his PhD training in the Biochemistry Department from 1939 to 1944. He returned to his alma mater having achieved national prominence as a leader in clinical nutrition research at the University of Pittsburgh. For this research, he received the McCollum Prize from the American Society of Nutrition. In his seventeen years as chairman at Saint Louis University, Dr. Olson maintained a vigorous research program that was continuously funded by multiple NIH research grants. In addition to holding leadership positions in the Biochemistry and Nutrition Societies and serving as editor to the American Journal of Nutrition, he served (1967-77) as Director of the Anemia and Malnutrition Center in Chiang Mai University in Thailand. He obtained a multi-million dollar grant to build the Center to study protein-calorie malnutrition in northern Thai children and recruited a clinical faculty contingent to serve on its staff.

Dr. Olson also championed clinical nutrition as an important part of physician training. He initiated the AOA Medical Student Research Forum for SLU medical students to achieve local and national recognition for their research. He also initiated the student-sponsored Wendell Griffith Lectureship in the
Dr. Olson taught courses in the CMB program and many CMB students received their PhDs under the direction of Biochemistry Department faculty.

An anecdotal story illustrates Dr. Olson’s mission-oriented approach to problem-solving: Once, Dr. Olson wanted to enter one of his laboratories in the medical school but the door was locked and he did not have the key to it. He called security and an officer showed up with a large ring of keys. However, the officer found that none of them opened the door. When the officer reported this to him, Dr. Olson demanded that he shoot the lock on the door to open it. Instead, the officer went back to his office and found the key to open the door.

Dr. Olson is remembered as a charismatic leader and an excellent educator. The causes he championed were for the good of the research and teaching mission of his Department and its members. In 1982, Dr. Olson was recruited to the University of Pittsburgh. He died on August 11, 2011, in Boston, Massachusetts, at the age of 92. Dr. William Longmore served as acting chair of the department for 18 months after Dr. Olson’s departure until Dr. Sly was recruited as the next permanent chair.

1984-2010

William S. Sly, MD, (See Figure 3), succeeded Dr. Olson as chair of Biochemistry in 1984. Like Dr. Olson, Dr. Sly was a Saint Louis University graduate, receiving both his bachelor’s and medical degree at the University. Since Dr. Sly had started his independent career as a lambda bacteriophage geneticist, before moving into the genetics, biochemistry, and cell biology of human inherited disease, he brought a first-hand appreciation of the role of both basic biomedical research and translational research to his plans for the Department.
Dr. Sly arrived having already achieved international distinction from his work on lysosomal storage diseases. He identified and characterized the first patient with beta-glucuronidase deficiency, also known as mucopolysaccharidosis VII or “Sly Syndrome.” For these groundbreaking studies, he was inducted into the National Academy of Sciences in 1989.

Very few physicians have described the first defining case of a genetic disease, but Dr. Sly has accomplished this twice in his career. In 1983, Dr. Sly showed that patients with a curious syndrome of renal tubular acidosis, osteopetrosis, and cerebral calcifications suffered from a deficiency in the enzyme carbonic anhydrase II. His laboratory at Saint Louis University School of Medicine cloned and characterized many members of the carbonic anhydrase gene family, and generated mouse deficiency models. In the course of this work, Dr. Sly and his colleagues solved the first X-ray crystallographic structure of a membrane-bound carbonic anhydrase, CA XII, an enzyme implicated in tumor progression. In 2009, Dr. Sly published the first data linking mutations in carbonic anhydrase IV to an autosomal dominant form of retinitis pigmentosa. Most recently, as an extension of his interest in carbonic anhydrase, Dr. Sly is collaborating with a local biotechnology company to develop an enzyme-based carbon capture device to reduce the atmospheric emission of carbon dioxide.

In the 1990s, Dr. Sly added a third research program in hemochromatosis, the disorder of iron overload. He was the first to demonstrate that functional loss of the human hemochromatosis gene product, HFE, causes hemochromatosis by generating the knockout mouse. Research directed by Dr. Sly provided the molecular link between HFE and iron metabolism by demonstrating that HFE protein associates with the transferrin receptor. He also led the project that determined the molecular basis for the most frequent HFE gene mutation causing hemochromatosis. As an extension of this work, Dr. Sly contributed to the discovery that functional loss of HFE led to inadequate production of the iron regulatory hormone hepcidin. The recognition of this relationship between hemochromatosis and hepcidin expression has helped to bring about a fundamental rethinking of how the body regulates iron absorption and distribution.

Dr. Sly’s commitment to principles of fairness, justice, and integrity were most obviously manifested in the circumstances that brought to light a pitfall in the legal system’s willingness to rush to judgment. In 1991, a St. Louis jury convicted Patricia Stallings of first-degree murder for poisoning her infant son with antifreeze, and sentenced her to life in prison without the possibility of parole. Upon seeing the purported evidence as described in a televised episode of “Unsolved Mysteries,” Dr. Sly recognized that the blood test used by the commercial laboratory was incapable of excluding an inherited metabolic disorder. Despite skepticism from those who were otherwise convinced of the jury’s conclusion, Dr. Sly ordered his Department’s metabolic screening lab to conduct additional tests. The results pointed conclusively to a genetic disease, methylmalonate acidemia, which causes symptoms that overlap those of ethylene glycol poisoning. Dr. Sly wrote up these findings, which were forwarded to the prosecuting attorney in the case. His letter was crucial to the dismissal of the murder charges, and the innocent mother was released from incarceration.

Dr. Sly served for 20 years as co-editor for three successive editions of the definitive resource in clinical genetics, The Molecular and Metabolic Bases of Inherited Diseases. He has also served on the editorial boards of numerous
Medicine enjoyed major improvements on the campus during the past 20 years under University President Father Lawrence Biondi, with the replacement of a block of city street that ran through campus by a pedestrian mall and the return of several surface parking lots to green space. Dr. Sly’s motto throughout his administration was “What a great place to work!” He did his best to make this motto a reality for the faculty in the Department.

2010-present

Enrico Di Cera, MD (See Figure 4) assumed the chairmanship of the Edward A. Doisy Department of Biochemistry and Molecular Biology in January 2010. His arrival marked a return of this position to an internationally recognized leader in the biochemistry of coagulation. Dr. Di Cera’s work has opened important new frontiers in the control of coagulation in thrombosis and uses X-ray crystallography in the structure-guided design of novel therapies. He has greatly expanded the departmental capability in structural biology with the acquisition of a new and more powerful X-ray diffraction facility and the establishment of a proteomics core facility for large-scale production of proteins for structural biochemistry. More recently, the Department has acquired a high-performance computing infrastructure for massive parallel throughput that benefits many areas of research within the Medical School, ranging from structural and computational biology to functional genomics and proteomics.

Acknowledgments

The authors gratefully acknowledge helpful discussion and recollections of Drs. Claudette Klein, Carmine Coscia, and William Sly.
Decades of research have identified numerous elements critical to both the “seed” and “soil” components of metastasis, providing support for Paget’s 1889 hypothesis. There is hope of improved outcomes with new and innovative therapies that target these specific pathways.

“\textit{When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil.}”

~ Stephen Paget, 1889

Abstract

Most mortality from cancer is secondary to metastasis. Metastasis refers both to the process by which tumor cells establish themselves at organs distinct from where they originated and to the life-threatening lesions themselves. Metastases are often resistant to conventional therapies, highlighting a key distinction between these progeny lesions and the primary tumor from which they arose. Here, we summarize recent advances in understanding and targeting primary tumors and the mechanisms and therapeutic challenges of metastasis.

The English surgeon Stephen Paget proposed the “Seed and Soil” hypothesis to explain the mechanism of metastasis.\textsuperscript{1} In it, he compared the dissemination of tumor cells to the distribution of seeds: each seed carries the potential for exuberant growth, but only the seeds that fall on compatible soil will realize that potential. Analogously, among the neoplastic cells released by a primary tumor, only the few that find their way to compatible organs will establish metastases. In addition, cells that escape the primary tumor face many barriers in the process of forming clinically relevant metastatic lesions.\textsuperscript{2} The ability to target the metastatic process itself will transform cancer therapy. Here, we review the current state of our understanding of metastasis and persisting challenges uncovered by the latest research.

The Medical Toll of Metastasis

A diagnosis of cancer leads to two critical questions: (1) has the tumor metastasized and (2) will the tumor metastasize? The gravity of these questions is underlined by the fact that the leading cause of cancer-related death is disseminated metastatic disease.\textsuperscript{3} Yet most available therapies target primary tumor growth,
often showing little to no efficacy on metastatic lesions.\(^3\)

The primary metric for enduring remission is survival at five years. However, this metric fails to capture patients who relapse beyond this point because disseminated tumor cells can recur many years after seemingly successful treatment. Disseminated tumor cells can exhibit a “dormant” state, remaining quiescent for years, even decades, in tissues foreign from their origin only to re-initiate growth when they acquire adaptive mutations or a more congenial environment within that tissue.\(^2\,3\) Understanding the mechanisms of dormancy and escape from dormancy may be key to preventing relapse.

**The Cell Biology of Metastasis**

Metastasis involves tumor cell-intrinsic behaviors and systemic factors not directly related to the tumor cells, known as host-derived factors. Tumor cell-intrinsic traits include aberrant motility, invasiveness, and cellular phenotypes associated with cell survival. Host-derived factors can include endothelial cells, platelets, macrophages, regulatory T-lymphocytes, fibroblasts and bone marrow-derived cells, and secreted factors not directly derived from tumor cells.\(^2\)

In order to achieve a clinically relevant metastatic lesion, tumor cells undergo a multi-step process and often co-opt non-neoplastic cells. These steps are collectively termed the ‘invasion-metastasis cascade’ and include (1) tumor outgrowth beyond the basement membrane into the peripheral extracellular matrix, (2) recruitment of vasculature to sustain primary tumor growth, (3) invasion into this newly formed vasculature, (4) survival in the blood or lymphatic circulation, (5) arrest at the target organ, and (6) adaptation to thrive within this foreign tissue, termed metastatic colonization (Figure 1).\(^2\) Moreover, mutations resulting in neoplastic transformation and genomic instability often result in expression of tumor cell-specific antigens, which can make these cells immunogenic.\(^3\) As a result, while undergoing the invasion-metastasis cascade, metastatic cells must also evade the host immune system.\(^3\)

![Figure 1: Steps of the Invasion-Metastasis Cascade](image)

**Detachment from Primary Tumor and Traversing the Extracellular Matrix**

The first barrier to neoplastic cell dispersal is the surrounding extracellular matrix (ECM), including the basement membrane that compartmentalizes epithelial tissue. Successful neoplastic cells acquire the ability to survive detached from their substrate and secrete enzymes that dissolve the ECM, promoting dispersal from the initiating tumor. In addition to its role as a physical support for tissues and organs, ECM is also a storage site for growth factors. Thus, dissolution of the matrix can release proliferation-stimulating proteins locally in the tumor microenvironment. This release is facilitated by acquisition of a cellular phenotypic plasticity resulting from a de-differentiated state. Carcinomas, which are tumors of epithelial origin, undergo genetic reprogramming known as epithelial-to-mesenchymal transition (EMT), which is thought to endow them with motility, invasiveness, and matrix-dissolving properties that enhance their metastatic capability.\(^2\,3\) Although EMT has been consistently reported in the laboratory, observations of epithelial morphology of both primary tumors and metastatic lesions in patients have cast doubt on the relevance of EMT in human disease.\(^7\)
SCIENCE OF MEDICINE

Recent reports, however, substantiate the importance of EMT for dissemination, invasion, and arrival at the secondary site. Further, these studies reconcile clinical observations of the epithelial morphology of metastatic lesions with direct evidence for reversion of EMT by a mesenchymal-to-epithelial transition. Evidence from a mouse model suggests that, although EMT is required to enable dissemination, upon arrival to the secondary sites tumor cells that had undergone EMT must revert to an epithelial state in order to proliferate and form full-blown metastatic lesions.

Entry Into Circulation

For seeds, dispersal requires wind, water, or the digestive tracts of animals. For cells, the highways of dispersal are the lymphatic and blood circulation. Upon reaching a particular size, a tumor must recruit its own vasculature to sustain itself. Tumor-associated vasculature tends to be tortuous, dilated, and contains dysfunctional pericytes. These changes may reduce barriers to tumor cell access to the vasculature, a process called intravasation. While metastatic cells can disperse through either the lymphatic or hematogenous circulation, the blood appears to be the preferred route for most metastases. That said, sentinel node status is informative for clinical staging and management of some cancers, including melanoma, breast cancer, and prostate cancer.

Survival in Circulation

The circulatory system is a hostile environment for cells derived from solid tumors. Once in the circulation, pre-metastatic cells must acquire resistance to anoikis, a type of programmed cell death that results from loss of attachment to the ECM. The cell membranes of invading cells, unlike blood cells, are not structured to withstand the shear forces of narrow vessels. Tumor cells may bind to platelets to shield themselves from shear forces and immune cells encountered in the blood stream. The narrowest vessels, the capillaries, have internal diameters smaller than the diameter of most pre-metastatic cells, thus sieving many pre-metastatic cells from circulation. Once so arrested, tumor cells can either grow into metastatic lesions within the vascular lumen or invade organ parenchyma prior to forming a metastasis.

Exit From Circulation

Pre-metastatic cells that survive the rigors of circulation must exit by once again pushing their way out through the vessel walls. This process is the reverse of intravasation, and is thus called “extravasation.” While the most common sites for metastases are the lungs, bones, and liver, tumors with different tissue origins differ in their preferred sites of metastasis (See Table 1). The sites for extravasation may be dictated by ‘passive tropism,’ with exit simply being the nearest point to the site of intravasation that capillary sieving occurs (for example, the liver for colon cancer cells). Absence of closely apposed mural cells on the vessel walls supplying bone marrow renders them more vulnerable to pre-metastatic cell seeding. In some cases, however, tropism of pre-metastatic cells may reflect a pre-adapted state for certain organs or tissues, including the expression of surface proteins, such as growth factor receptors or cell adhesion molecules, with special affinity for those environments. Evidence suggests that circulating tumor cells are disseminated to a wide variety of organs. However, whether these disseminated cells, the “seeds,” form clinically relevant metastases depends on their ability to thrive within the “soil,” microenvironmental tissue conditions of these organs.

Adaptation of Metastatic Cells to Ectopic Sites

Many environments are hostile to seed dispersal: large bodies of water, rocky or sandy soil, and sites of excessive shade or sun exposure. Likewise, most pre-metastatic cells are poorly adapted to the environment they confront upon extravasation. Indeed, successful colonization of the metastatic site is believed to be the rate-limiting step in metastasis. Like seeds that initially

Table 1: Most Common Sites of Metastasis by Tumor

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>Main sites of metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>Lungs, liver, bones</td>
</tr>
<tr>
<td>Colon</td>
<td>Liver, peritoneum, lungs</td>
</tr>
<tr>
<td>Kidney</td>
<td>Lungs, liver, bones</td>
</tr>
<tr>
<td>Lungs</td>
<td>Adrenal gland, liver, lungs</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Lungs, skin/muscle, liver</td>
</tr>
<tr>
<td>Ovary</td>
<td>Peritoneum, liver, lungs</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Liver, lungs, peritoneum</td>
</tr>
<tr>
<td>Prostate</td>
<td>Bones, lungs, peritoneum</td>
</tr>
<tr>
<td>Rectum</td>
<td>Liver, lungs, adrenal gland</td>
</tr>
<tr>
<td>Stomach</td>
<td>Liver, peritoneum, lungs</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Lungs, liver, bones</td>
</tr>
<tr>
<td>Uterus</td>
<td>Liver, lungs, peritoneum</td>
</tr>
</tbody>
</table>

Source: http://www.cancer.gov/cancertopics/factsheet/sites-types/metastatic
find themselves in hostile soil and remain dormant until conditions favor germination, pre-metastatic cells may remain quiescent or divide only slowly for years, and at a proliferation rate that may be balanced by significant cell death. For a micrometastasis to grow beyond a diameter of 1-2 mm requires the recruitment of a microvasculature to supply nutrients to the interior of the nascent tumor, a process called 'neoangiogenesis.' Moreover, it appears that tumor-associated endothelial cells are distinct from normal endothelial cells, with distinct programs that make tumor-associated endothelia targets for novel therapeutic strategies. Additional considerations may include: (1) the ability of tumor cells at the primary site to condition the secondary site’s pre-metastatic niche congenial to tumor growth and (2) acquisition of additional mutations by the pre-metastatic cells to promote adaptation to the foreign environment of the secondary site.

**Models for Cancer Metastasis**

The steps of the invasion-metastasis cascade outlined above highlight the barriers to metastasis and behaviors tumor cells must acquire to successfully metastasize. However, they provide little insight into the genetic and molecular determinants that enable a tumor cell to disseminate and colonize secondary sites. Several models have been proposed to provide a unifying framework for the molecular genetic basis of the invasion-metastasis cascade. Each model has particular strengths and weaknesses in explaining aspects of the invasion-metastasis cascade and most are not mutually exclusive views into the mechanics of metastasis. Here, we focus on two complementary models that explain most observations on metastasis (See Figure 2).

**Clonal Selection Model**

This most widely accepted model for metastasis posits that neoplastic cells acquire metastatic capabilities through mutation and selection of the fittest clones. This hypothesis is based on the genetic instability inherent to tumor cells, which allows individual cells that acquire mutations to diverge from the parental cell followed by selection for certain mutant cells at sites foreign to the primary tumor. This model incorporates the notion that only a small subset of cells in the primary tumor ultimately acquires the necessary properties to become metastatic lesions. However, it cannot explain the observation that, in some cases, gene expression patterns within the primary tumor can predict metastatic and survival outcomes, since this small subpopulation of cells is unlikely to contribute measurably to the aggregate gene expression patterns of the whole tumor.

**Cancer Stem Cell Model**

Stem cells normally replenish aged and wounded tissue. Cancer Stem Cells (CSCs) are thought to be progenitors of tumor cells with self-renewing properties that enable sustained tumor growth. In the CSC model of metastasis, CSCs are thought to be the cells that are capable of forming tumors at secondary sites because they are the only cells within a tumor that can proliferate indefinitely. The CSC model differs from the clonal selection model in that all tumors have a pre-existing subpopulation of intrinsically metastatically competent cells rather than metastatic cells arising by clonal evolution. This model could account for observations of anaplastic tumors being more malignant and of cells in de-differentiated states that are thought to induce epithelial-to-mesenchymal transition, concepts gaining prominence in metastasis biology. However, this model fails to explain differences between mutational profiles and specific gene expression in primary tumors and matched metastases.
The Molecular Genetics of Metastasis

Molecular genetic approaches to cancer over the past 35 years have uncovered two broad classes of genes that drive carcinogenesis: oncogenes and tumor suppressor genes. Oncogenes represent genes whose normal function is usually associated with cell survival and promotion of cell division. Abnormal amplification or hyperactivity of oncogenes contributes to the transformation of normal cells to neoplasms. Tumor suppressor genes, in contrast, normally act to prevent cell division and sensitize cells to signals for cell death and senescence. Their inactivation results in the loss of safeguards against uncontrolled cell proliferation. In human cells, few—if any—endogenous genes are capable of single-handedly transforming a cell. Both oncogene-activating and tumor suppressor-inactivating mutations are typically required for carcinogenesis to occur.19

Complicating the mutational profile of tumors is the intrinsic genomic instability of tumors, which results in many more mutations than are required for tumorigenesis and metastatic progression. Mutations in genes that cause tumor initiation and progression are known as “driver” mutations. However, due to the high mutational propensity of tumor cells, many “passenger” genes having no effect on tumor progression are also mutated. Resolving which mutations are drivers and which are passengers will enhance our ability to utilize targeted therapeutics. Encouragingly, it may also be possible to target certain passenger genes, as they sometimes present vulnerabilities specific to cancer cells.20

While oncogene and tumor suppressor gene mutations are drivers for tumorigenesis, these mutations contribute little to a tumor cell’s metastatic ability. New classes of metastasis-specific genes, termed metastasis suppressors and metastasis promoters, are emerging that often have little impact on primary tumor growth but significantly enhance invasive and metastatic properties of tumor cells.21

Recent research has uncovered a new class of “genes,” known as noncoding RNAs (ncRNAs), that produce RNA products that do not code for protein but enhance or suppress the expression of protein-coding genes. Various short ncRNAs (microRNAs, or miRs) and long noncoding RNAs (lncRNAs) have been implicated in carcinogenesis and metastatic progression, some acting as inhibitors and others as promoters.22,23 The discovery of these new regulators presents new opportunities for novel drugs that either mimic or antagonize the activity of ncRNAs.22 Indeed, inhibitors of microRNAs are in pre-clinical testing and molecules that can degrade messenger RNA are in early stages of clinical and pre-clinical testing.

The Tumor Microenvironment

A metastatic lesion is the culmination of complex interactions between tumor-intrinsic behaviors and host-derived factors. While much research on tumor biology has focused on behavior of the tumor cell, over the past decade the critical role of the tumor microenvironment in tumor progression and metastasis has become apparent. The tumor microenvironment is comprised of non-neoplastic cells in the immediate vicinity of tumor cells and includes myoepithelial cells, endothelial cells, lymphocytes, myeloid cells, and fibroblasts, as well as non-cellular components such as the extracellular matrix and secreted factors. It is now clear that tumor cells actively interact with their microenvironment to enhance tumor cell invasiveness and immunoevasiveness and to prime distant sites for colonization.2,3 An early indication of this was an experiment showing that ECM extract from normal cells quelled the invasiveness of tumor cells while the ECM from a tumor made tumor cells more aggressive and invasive.24

The cell-mediated immune system continuously surveys cells for intracellular antigens. Tumors evade this surveillance by, for example, turning off the expression of co-receptors that allow lymphocytes to bind tumor cells, thereby allowing them to escape detection both at the primary site and systemically. Moreover, several cell types within the tumor microenvironment, including regulatory T cells and myeloid-derived suppressor cells, suppress immune cell responses against tumor cells.5,21

Tumor cells may have interactions that reach beyond the immediate microenvironment, sending systemic signals to prime the secondary environment and fertilize its metaphorical soil prior to their arrival. In experiments that established the concept of the “premetastatic niche,” it was shown that tumor cells secrete exosomes—lipid-bound particles containing pro-metastatic proteins and RNA—into the circulation that are taken up by bone marrow derived cells (BMDC). These exosomes were shown to enhance BMDC arrival in the lung and remodeling of the lung tissue architecture to render it amenable for tumor cell colonization.13,26

Therapeutic Outlook

Depending on the type of cancer, its gene expression profile, and the characteristics of the patient, metastatic cancers can be treated with local therapy (e.g., surgery or radiotherapy), systemic therapy (e.g., chemotherapy, hormone therapy), or both.2 The standard armory of non-surgical anti-tumor therapies—radiation and drugs—targets rapidly proliferating cells. While these approaches do lead to primary tumor regression, slower-dividing
cells within the tumor, such as cancer stem cells, may survive the treatment period and seed new tumors at the primary and metastatic sites. Similarly, occult metastases that are still dormant would escape therapies targeted to dividing cells. This evasion may help explain why progress in treating metastases has been incremental.3

The good news is that the metastatic process is very inefficient so, presumably, detection and removal of the primary tumor prior to metastatic colonization may lead to complete remission. Despite this, examples exist, such as lung and pancreatic adenocarcinomas, in which primary tumors metastasize before the primary tumor is detectable with current technology. The bad news is that successful metastatic cells may differ genetically from the primary tumor from which they are derived, so targeted therapy for the primary tumor may not be effective against its metastases.14,19 In light of this difference, the ideal therapeutic would induce regression of established macroscopic metastases.

**Advances in Classification of Tumors**

Currently, two methods are used in the clinic to classify tumors for prognostication and selection of appropriate therapeutic strategies. The TNM staging method is used to predict the clinical behavior and potential prognosis of a tumor by assessing the size of the tumor (“T”), whether draining lymph nodes (“N”) contain tumor cells, and whether any distant metastases (“M”) are present. Although the TNM staging system provides clear prognosis and can indicate how aggressively a tumor should be treated in the higher stage cases (i.e., when many lymph nodes are involved or when distant metastases are detected), it is of little clinical value in cases where tumors show no signs of spread. Inevitably, some tumors showing no signs of nodal involvement or metastases will metastasize. Histological grading entails microscopic inspection of a biopsy by an experienced pathologist, provides insight into the morphological features of a tumor, and can inform therapeutic selection. Unfortunately, tumors with very similar microscopic features often have very different clinical outcomes and different oncogenic drivers.27

Enormous advances in speed and economy of DNA sequencing have resulted in systematic catalogs of tumors and corresponding normal tissue sequences. These catalogs are being assembled with the goal of identifying tumor-

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**Table 2: Molecular Markers and Indicated Therapeutics**

<table>
<thead>
<tr>
<th>Molecular marker</th>
<th>Clinical setting</th>
<th>Treatment indicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR-ABL translocation</td>
<td>Chronic myeloid leukemia; acute lymphoblastic leukemia</td>
<td>Imatinib, dasatinib, nilotinib</td>
</tr>
<tr>
<td>BCR-ABL translocation,</td>
<td>Chronic myeloid leukemia</td>
<td>Dasatinib, nilotinib</td>
</tr>
<tr>
<td>imatinib-resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PML-RAR translocation</td>
<td>Acute promyelocytic leukemia</td>
<td>All trans retinoic acid</td>
</tr>
<tr>
<td>KIT mutation</td>
<td>Gastrointestinal tumor</td>
<td>Imatinib</td>
</tr>
<tr>
<td>HER2 amplification</td>
<td>Breast cancer, gastric cancer</td>
<td>Trastuzumab, lapatinib, pertuzumab</td>
</tr>
<tr>
<td>Estrogen or progesterone</td>
<td>Breast cancer</td>
<td>Multiple hormone-based therapies (for example, aromatase inhibitors, ER/PR antagonists)</td>
</tr>
<tr>
<td>receptor-dependent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR mutation</td>
<td>Non-small-cell lung cancer</td>
<td>Erlotinib, gefitinib</td>
</tr>
<tr>
<td>ALK fusion gene</td>
<td>Non-small-cell lung cancer</td>
<td>Crizotinib</td>
</tr>
<tr>
<td>BRAF mutation</td>
<td>Melanoma</td>
<td>Vemurafenib</td>
</tr>
<tr>
<td>BRCA1/2 mutation</td>
<td>Breast, ovarian cancer</td>
<td>PARP inhibitors (e.g. olaparib)</td>
</tr>
<tr>
<td>CTLA</td>
<td>Melanoma</td>
<td>Ipilimumab</td>
</tr>
<tr>
<td>MET and VEGFR2</td>
<td>Medullary thyroid cancer</td>
<td>Cabozantinib</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Colon, lung, breast, kidney, brain cancer</td>
<td>Bevacizumab</td>
</tr>
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<td>VEGFR, PDGFR, MAP-kinase</td>
<td>Kidney, liver cancer</td>
<td>Sorafenib</td>
</tr>
<tr>
<td>pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR, PDGFR, c-KIT</td>
<td>Kidney, soft tissue sarcoma</td>
<td>Pazopanib</td>
</tr>
<tr>
<td>Non-specific Tyrosine</td>
<td>Kidney cancer, imatinib-resistant gastrointestinal stromal tumor</td>
<td>Sunitinib</td>
</tr>
<tr>
<td>Kinase inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANK/RANKL</td>
<td>Breast, prostate cancer</td>
<td>Bisphosphonates, denosumab</td>
</tr>
</tbody>
</table>

Source: Adapted from Collisson et al., 2012
Also see: http://www.cancer.gov/cancertopics/factsheet/Therapy/targeted
associated mutations that enable oncogenic transformation and metastatic progression. While the classification of tumors has for decades been based on histology, the application of genomics and transcription profiling has disclosed a more precise catalog of markers that is resulting in better therapeutic outcomes in some cases. In the context of breast cancer, mRNA expression patterns are starting to be used as prognostic tools in the clinic. Two such examples, MammaPrint and OncotypeDX, are currently being tested in clinical trials for node-negative breast cancer. Ideally, the recent advances in sequence technology, coupled with our ever-expanding understanding of mechanisms of carcinogenesis and metastasis progression, will enable us to sequence tumor RNA and DNA, not only for prognostication, but to identify deranged molecular pathways which could then be targeted to shut down oncogenic and metastatic signaling cascades.

**Advances in Targeted Therapeutics**

Over the past decade, new classes of drugs have emerged that target molecules and pathways deranged in cancer. Some of these drugs have also shown efficacy against metastases. Table 2 summarizes targeted compounds that are either in current clinical trials or have been FDA approved for the treatment of specific tumors. Unfortunately, many of these drugs show transient effects as tumor cells mutate to acquire resistance, and most patients ultimately succumb to disease. To overcome the acquisition of resistance by the tumor, the oncology field may adopt a paradigm similar to the anti-HIV therapeutic (HAART) strategy in which multiple independent pathways are simultaneously targeted. However, as the targeted therapeutics and mutational profiling in cancer are still in relative infancy, more must be learned about individual action of these drugs and their combination with classical therapeutics before the advent of trials combining multiple targeted drugs.

**Summary**

Decades of research have identified numerous elements critical to both the “seed” and “soil” components of metastasis, providing support for Paget’s 1889 hypothesis. As a result, the promise of new generations of drugs capable of disrupting both the seed and soil of metastatic progression are now within reach and, with them, the hope of improved outcomes with therapies that target these specific pathways.

**References**


**Disclosure**

None reported.
What Fruit Flies Can Tell Us About Human Birth Defects

by Dale Dorsett, PhD

Fruit flies can provide relatively rapid and inexpensive methods to understand the molecular basis diseases and to develop potential therapies, particularly for rare conditions for which standard methods for drug development are not economically feasible.

Abstract

Many times, when a human genetic disease is mapped to mutations in a specific gene, little is known about the biological functions of the affected gene. Development of new therapeutic methods is facilitated by understanding the gene’s biological roles. Such information can often be obtained in animal models, such as the fruit fly. Here we describe how understanding a gene’s function in fruit flies has illuminated the etiology of Cornelia de Lange syndrome.

Introduction:
Cornelia de Lange Syndrome

A few years ago, on a sweltering summer day in a park not far from Saint Louis, I met a 15-year-old boy and his mother. He only came up to his mother’s waist. As I gazed down at his shining face, the features characteristic of Cornelia de Lange Syndrome (CdLS), a rare genetic disorder, were obvious even to me, trained as a fruit fly, not a human, dysmorphologist. Talking with his mother, I was surprised to learn that he had actually just been diagnosed only the week before by his aunt, who had read about CdLS online. The boy, with verbal skills that were unusually good for someone with this syndrome, was quite excited to explain why he was different. Although CdLS can be easily suspected based on highly characteristic facial features, including arched eyebrows, thin lips, and long philtrum, there’s more to it. Individuals with this syndrome suffer a raft of more serious problems, including intellectual impairment, autism, slow growth, and limb and multiple organ abnormalities (See Table 1).1

It is not always recognized initially by physicians or other caretakers. In fact, it is not uncommon, particularly in more rural areas, for CdLS to go undiagnosed, as it was in this 15-year-old. Cornelia de Lange first described this condition, which is also called Brachmann de Lange Syndrome, in the 1930s based on two unrelated pediatric patients she saw in Amsterdam.2 Today, a CdLS-likely diagnosis is made relatively easily by a physician who has seen a few cases and recognizes the pattern of abnormalities. Early diagnosis makes a difference. It can lead to substantial improvement in management, as many of the diverse health risks can be anticipated.1

Feeding is a very common problem. On the summer day I met this patient, I also met many caring family members of other patients, including a grandfather who had used
techniques similar to those he used to handle young livestock on his farm to develop a diet that was palatable to his granddaughter. I also met a young mother who refused to accept being told by her pediatrician that poor mothering was responsible for the failure of her beautiful daughter to thrive, and who ferociously sought out other opinions until the correct diagnosis was made.

Six Degrees of Separation: Making the Connection

You might wonder why a fruit fly molecular geneticist was attending a CdLS family picnic? There is a connection! The answer goes back to the mid-1990s, when our laboratory was investigating how genes are turned on and off during the development of a fruit fly called Drosophila melanogaster. This insect is a “model organism” that offers powerful genetic and molecular tools. It has long been a tenet of the Drosophila research community that for most biological functions, ranging from metabolism and endocrinology to learning and memory, the fruit fly is equivalent to a simple small model of a human, but with a more compact genome and wings.

We were looking for genes that control limb development and discovered one that we named Nipped-B, based on the moth-eaten appearance of the mutant wings.\(^1\) Nipped-B, it turned out, controls development of several tissues.

### Table 1
Common features of Cornelia de Lange Syndrome

<table>
<thead>
<tr>
<th>Feature</th>
<th>Percentage of individuals affected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development and behavior</td>
<td></td>
</tr>
<tr>
<td>Growth retardation</td>
<td>100</td>
</tr>
<tr>
<td>Intellectual disability</td>
<td>mean IQ 53, range &lt;30 to 102</td>
</tr>
<tr>
<td>Autism spectrum disorder</td>
<td>65</td>
</tr>
<tr>
<td>Speech disorders</td>
<td>+</td>
</tr>
<tr>
<td>Self injurious behavior</td>
<td>70</td>
</tr>
<tr>
<td>Physical aggression</td>
<td>40</td>
</tr>
<tr>
<td><strong>Facies</strong></td>
<td></td>
</tr>
<tr>
<td>Microcephaly</td>
<td>93</td>
</tr>
<tr>
<td>Low posterior hairline</td>
<td>92</td>
</tr>
<tr>
<td>Bushy eyebrows, synophrys</td>
<td>98</td>
</tr>
<tr>
<td>Ocular abnormalities (ptosis, myopia, nystagmus)</td>
<td>57</td>
</tr>
<tr>
<td>Long curly eyelashes</td>
<td>99</td>
</tr>
<tr>
<td>Depressed, wide nasal bridge</td>
<td>83</td>
</tr>
<tr>
<td>Anteverted nares</td>
<td>85</td>
</tr>
<tr>
<td>Long philtrum, thin upper, downturned mouth</td>
<td>94</td>
</tr>
<tr>
<td>High arched palate</td>
<td>86</td>
</tr>
<tr>
<td>Widely spaced teeth, late eruption</td>
<td>86</td>
</tr>
<tr>
<td>Micrognathia</td>
<td>84</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
<td></td>
</tr>
<tr>
<td>Hirsutism</td>
<td>78</td>
</tr>
<tr>
<td>Cutis marmorata</td>
<td>56</td>
</tr>
<tr>
<td>Hypoplastic nipples, navel</td>
<td>50</td>
</tr>
<tr>
<td><strong>Extremities</strong></td>
<td></td>
</tr>
<tr>
<td>Micromelia</td>
<td>93</td>
</tr>
<tr>
<td>Reduction defects, oligodactyly</td>
<td>27</td>
</tr>
<tr>
<td>Clinodactyly (5th finger)</td>
<td>74</td>
</tr>
<tr>
<td>Simian crease</td>
<td>51</td>
</tr>
<tr>
<td>Proximally placed thumbs</td>
<td>72</td>
</tr>
<tr>
<td>Elbow flexion contractures</td>
<td>64</td>
</tr>
<tr>
<td>Syndactyly (toes 2 and 3)</td>
<td>86</td>
</tr>
<tr>
<td><strong>Genitalia</strong></td>
<td></td>
</tr>
<tr>
<td>Hypoplasia</td>
<td>57</td>
</tr>
<tr>
<td>Undescended testes</td>
<td>73</td>
</tr>
<tr>
<td>Hypospadias</td>
<td>33</td>
</tr>
<tr>
<td><strong>Gastrointestinal</strong></td>
<td></td>
</tr>
<tr>
<td>GE reflux</td>
<td>+</td>
</tr>
<tr>
<td>Gut duplication, malrotation</td>
<td>+</td>
</tr>
<tr>
<td>Volvulus</td>
<td>+</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>Hearing loss</td>
<td>60</td>
</tr>
<tr>
<td>Structural heart defects</td>
<td>33</td>
</tr>
<tr>
<td>Kidney, urinary tract defects</td>
<td>36</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>+</td>
</tr>
</tbody>
</table>

*a Data from references 21-25. **+** indicates reported in multiple individuals.*
It also soon became apparent, first from genetic studies with the equivalent genes in yeasts, that Nipped-B is also important in ensuring that chromosomes are dealt out equally to the two daughter cells when a cell divides. Nipped-B protein helps a protein ring called cohesin snap like a bracelet around chromosomes and hold sister chromosomes together, so they can be separated and sent in opposite directions at the moment of division (See Figure 1). It actually doesn’t take much Nipped-B or cohesin, less than 20% of normal, to ensure proper chromosome segregation. Even a small reduction on the order of 30% or less can dramatically alter growth and development. This is because many other genes that are controlled by Nipped-B and cohesin produce either too little or too much of their products when Nipped-B is reduced.

Making the Human Connection

The connection to human development, which we were sure would happen sooner or later (we could see the same gene in the DNA sequence of the human genome), came earlier than expected. In 2004, I received separate phone calls from Ian Krantz at the Children’s Hospital of Philadelphia and Tom Strachan at the University of Newcastle, who had both recently discovered that mutations in the human Nipped-B gene, which they named Nipped-B-Like (NIPBL), caused most cases of CdLS. Both were seeking any insights we might have into how NIPBL might be involved in the diverse deficits associated with CdLS. Since that time, Ian and others have found that mutations affecting portions of the cohesin ring can cause milder forms of CdLS, or a similar syndrome. Most recently, mutations in a gene encoding an enzyme that chemically modifies the cohesin ring were also found to cause CdLS.

Mapping the first CdLS gene was not for the faint of heart. The NIPBL mutations that cause CdLS are genetically dominant and it is extremely rare for the disorder to be inherited – virtually all cases are caused by sporadic mutations. As a consequence, the usual method of following family lineages could not be used. The key was that beginning in the 1970’s, Laird Jackson of Drexel University, responding to some questions from some concerned CdLS mothers, began organizing CdLS family picnics in the US, and traveling around the world to meet other CdLS families. Laird had the foresight to collect blood samples from the CdLS children, parents, and siblings, so that a large number had been collected by the time the human genome sequence became available. This collection allowed linkage exclusion analysis to narrow things down to five regions that might harbor the critical gene. Coupled with some inspired guesswork, and a balanced de novo chromosomal translocation affecting one of the five suspect regions, this analysis led to NIPBL.

Identification of NIPBL led to the first CdLS Scientific Symposium in June 2004, held in Lincolnshire, Illinois, in conjunction of the biennial family meeting organized by the CdLS Foundation that had grown out of the picnics organized by Laird many years before. I was invited to talk about our work with fruit flies, which began my association with the CdLS Foundation, as a member of the Board of Directors from 2004 to 2010, and currently as Chair of their Research Council.

One of the responsibilities that I have enjoyed is meeting CdLS families at occasional family picnics along the I-70 corridor from western Illinois to eastern Kansas. It is gratifying to reassure parents that it is nothing they did wrong that caused their child’s condition. They find it a relief to have the genetics and causes of CdLS explained to them, and also to learn that there are dedicated physicians and scientists who care about this rare condition, and are
working hard to improve the methods of diagnosis and treatment. They are excited to learn that members of the Clinical Advisory Board of the CdLS Foundation, who have diverse expertise, can be approached to address their many medical questions. They are also thrilled to learn that teams of scientists are exploring the mechanism of the disease in search for clues to treatments that could improve their children’s lives.

So, why do we think there is hope for developing effective treatments for CdLS? The problems with growth and development begin in utero, and CdLS has been diagnosed by ultrasound (e.g., reference 10). It is not beyond imagining that in the near future, CdLS, like many other rare disorders, can be diagnosed early in pregnancy with a combination of ultrasound, DNA sequencing, and molecular markers. As a rare, non-inherited genetic disorder, however, it would not become routine to check for CdLS until a safe method that can detect many genetic problems with early development is available.

If in utero diagnoses can be made, it would then be feasible to think about very early therapeutic intervention that could ameliorate many of the physical deformities. I also believe that therapeutic intervention that begins within a few weeks of birth could be very helpful. Surgery can be used to correct the occasional severe congenital heart defects, which are a leading cause of early death. We can even envision the possibility that we can improve growth and intellectual development.

Mental retardation, poor speech, and autism are major impediments that keep most individuals with CdLS reliant on constant care and from becoming independent. There is good reason to believe that brain development is highly plastic, and that early intervention may greatly improve intellectual development of children with CdLS. For instance, using a mouse model of Rett syndrome, a leading cause of mental retardation in girls, Adrian Bird’s laboratory in Edinburgh showed that turning the affected gene back on even in young adults led to substantial improvements in learning, memory, and behavior. Similarly, flies with Nipped-B and cohesin deficits show delays in axon pruning and problems with dendrite branching, two key processes in neural development, which have the potential to be corrected later in development. That’s the long-range hope.

Research ‘Flies’ By

Where do we start, and what strategies might lead to therapeutic discoveries? First we need to know how NIPBL and cohesin control genes, what genes they control, and when they control them, in order delineate what is possible. Our laboratory has exploited the fruit fly to gain some of this knowledge. Using techniques that allow us to examine the entire genome, we mapped where the Nipped-B and cohesin proteins bind to chromosomes in multiple types of cells and tissues, and which genes produce too little or too much product when the amount of Nipped-B or cohesin is reduced. These studies have provided important clues.

We discovered that Nipped-B and cohesin bind selectively to hundreds of active genes that control growth and development, and that the products of many of these genes and the genes they control are often altered when there is too little Nipped-B or cohesion. Strikingly, one of the key genes that binds Nipped-B and cohesin and whose output decreases when there is too little Nipped-B or cohesion, is myc, a key growth promoter in both flies and humans. The myc gene is perhaps best known for causing cancer when it is too active, leading to unchecked growth. In Drosophila, myc is called diminutive, because the first mutation, isolated back in the 1930’s, only partially reduces gene function, giving rise to tiny flies. It turns out that the zebrafish, mouse, and human myc genes are also down-regulated when the NIPBL activity is reduced, providing one potential explanation for why CdLS was once known as “Amsterdam dwarfism.”

The overriding message of our Drosophila studies, and parallel work by other laboratories in cultured mammalian cells, zebrafish, and NIPBL mutant mice, is that NIPBL and cohesin directly control the activity of hundreds of genes important for growth and development. Many of the physical defects and other medical problems that are apparent upon birth can be handled with existing medical procedures. The diversity of the effects on gene function argue, however, that we will likely need to increase the activity of the remaining good NIPBL gene if we are to improve future development. It is easier to control a river at its source than at the delta.

The reality is that drug companies would go out of business if they poured buckets of money into developing treatments for disorders that occur on the order of once per ten thousand births. Different strategies have to be developed for identifying and developing effective drugs for orphan diseases. A model that we are testing for CdLS is to use fruit flies to screen libraries of chemical compounds that have already been approved for human use for the ability to enhance expression of Nipped-B. The idea is that this is a rich source of bioactive compounds, and it is not nearly as expensive to repurpose a drug as it is to develop a new one.

In collaboration with the laboratory of Justin Fay at Washington University, we screened a library of FDA-
approved drugs for those that improve the growth of yeasts that have modest defects in their NIPBL and cohesin genes, and then tested the dozen or so that had the desired effect for their ability to improve some of the mutant phenotypes displayed by Nipped-B mutant flies. This analysis identified a few chemically-related compounds that were able to normalize the levels of NIPBL RNA in cell lines derived from individuals with CdLS. One of these compounds is used to treat other pediatric conditions. That’s hopeful! We are now seeking funding to test these compounds in a mouse model of CdLS, and consulting with physicians, allied health professionals, and CdLS families to decide what biomarkers can be used to conduct a meaningful clinical study in CdLS patients.

Conclusion

My take-home lesson is fruit flies and other non-vertebrate organisms can prove to be useful models for human maladies. They can provide relatively rapid and inexpensive methods, both to understanding the molecular basis of the disease and to developing potential therapies, particularly for rare conditions for which standard methods for drug development are not economically feasible.

Acknowledgments

The author is grateful to the CdLS families for their willingness to share their experiences, the CdLS Foundation, and Ian Krantz, Matt Deardorff, Laird Jackson, and Antonio Musio for many illuminating discussions. Work in the Dorsett laboratory is supported by grants from the National Institutes of Health (R01 GM055683, P01 HD052860).

References


Disclosure

NIH GM055683, HD052860 - not a commercial organization - government.
The Two Faces of DNA Repair: Disease and Therapy

by Alessandro Vindigni, PhD & Susana Gonzalo, PhD

A major breakthrough in the treatment of breast cancers with the poorest prognosis, BRCA1-deficient and triple-negative breast cancers, was the finding that these tumors are exquisitely sensitive to PARP inhibitors.

Abstract

Our genome is the blueprint for our bodies. A number of sophisticated mechanisms help protect our genome from life-threatening cellular mistakes and environmental insults. Much current research focuses on understanding these mechanisms, how they prevent disease, and whether they can be targeted for therapeutic purposes. Here, we review the main mechanisms maintaining genome integrity, how their malfunctioning results in disease, and the exciting progress toward targeting these mechanisms for cancer treatments.

Genome Instability

Maintaining the stability and the correct sequence composition of the three million bases that form our genome is critical for a correct transmission of genomic information. Unfortunately, our genome is under constant attack by agents that arise from either normal metabolism or exposures to natural or artificial products in the environment.\(^1\) As many as \(10^5\) lesions in DNA can occur per cell per day. DNA damage can result from side products of our normal metabolic activities, such as free radicals and reactive oxygen and nitrogen species, as well as from environmental factors such as UV radiation, X-rays, and chemical compounds.\(^2\) Our cells have evolved elegant mechanisms to cope with all these threats.\(^1\) Improper or inefficient repair of DNA damage causes mutations, abnormal chromosome structures, or loss of genetic information that could ultimately lead to a large number of human syndromes, including premature aging, various cancer predispositions, and genetic abnormalities.\(^1,3\) A large body of evidence has also implicated alterations in nuclear architecture and chromosome structure (so-called “epigenetic changes”) in genomic instability. Furthermore, defects in the structure of chromosome ends and in the integrity of the energy factories of our cells, the mitochondria, profoundly impact the stability of our genome. The degree of genomic instability caused by alterations in any of these mechanisms will determine whether the cell survives, undergoes a permanent growth arrest known as senescence, or dies.


The DNA Damage Response

The cell has evolved a complex pathway, termed the DNA damage response, to sense, signal, and ultimately repair DNA lesions arising from endogenous or exogenous insults. A variety of DNA repair mechanisms, with specificity towards different categories of DNA damage, have been identified. These include mechanisms devoted to repair single-stranded DNA lesions, such as those caused by UV light and certain chemicals, and mechanisms responsible for the repair of DNA double-strand breaks, such as those caused by X-rays and anti-tumor agents like cisplatin and mitomycin C. Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two main pathways required to repair double-strand breaks. These two pathways have been extensively characterized due to the fact that these lesions are particularly dangerous to the cell. While NHEJ is an error-prone mechanism for DNA double-strand break repair, HR repairs the damage with great fidelity by utilizing sister chromatids as templates for recombination (See Figures 1 and 2).

DNA damage response is a complex pathway that mobilizes and recruits a variety of nuclear proteins to sites of DNA damage. Activation of this pathway can halt cell-cycle progression until the damage is restored, or initiate mechanisms of growth arrest or cell death if the damage is beyond repair. A growing list of factors has been clearly implicated in DNA damage response. These factors include sensors of the lesions that recruit and activate the ATM and ATR kinases at sites of damage. ATM is a master regulator that activates a variety of factors involved in DNA damage response and in cell cycle control. This process also facilitates changes in chromosome regions surrounding the break. These changes in turn permit the recruitment of the machinery required to repair DNA double-strand breaks. Two effectors have recently been in the spotlight due to their key roles in regulating the balance between the main pathways of double-strand break repair: the breast cancer type 1 protein (BRCA1), which promotes HR, and the tumor suppressor p53-binding protein 1 (53BP1), which facilitates NHEJ.

Diseases Associated With Genomic Instability

Almost invariably, mutations in DNA repair factors cause syndromes linked to genomic instability. For example, loss of ATM function causes the genome instability syndrome Ataxia-telangiectasia (A-T). A-T is a devastating disorder characterized by progressive neuromotor dysfunction, thymic atrophy and immunodeficiency, predisposition to lymphoid malignancies, and high sensitivity to DNA damage-inducing agents such as X-rays. Three other diseases,
Xeroderma pigmentosa (XP), Cockayne syndrome (CS), and Trichothiodystrophy, are associated with defects in a specific single-strand break repair pathway. In addition, alterations in other factors with key roles in DNA replication stress response are linked to genomic instability syndromes, such as Fanconi anemia and Schimke immunoosseous dysplasia. RecQ helicases are an important family of DNA unwinding enzymes that play a key role in the maintenance of genome stability. Mutations in the genes of three human RecQ helicases are linked to defined genetic disorders associated with genomic instability, cancer predisposition, and features of premature aging; namely, Bloom syndrome (BLM gene mutations), Werner syndrome (WRN gene mutations), as well as Rothmund–Thomson, Rapadilino, and Baller–Gerold syndromes (all caused by mutation of RecQL4).

In recent years, the generation of mouse models deficient in different DNA damage response pathways has provided important insight into the requirements for DNA repair pathways. In many instances, mice lacking different DNA repair factors die during embryonic development, and conditionally deficient mouse models have been developed to test the effects later in life. Mouse models with DNA repair deficiencies often develop premature aging phenotypes and cancer. One of the best-characterized models is the histone H2AX deficient mouse. H2AX is a key coordinator of DNA damage response and mice that lack this protein exhibit radiation sensitivity, growth retardation, immunodeficiency, and male

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**Figure 2**

A) Main double-strand break repair pathways. NHEJ directly seals the broken DNA ends and occurs primarily during the G1 phase of the cell cycle. HR is a high-fidelity repair mechanism that occurs primarily during the S and G2 phases of the cell cycle and requires the information contained in the sister chromatid. The figure on the right schematically shows a replication fork with a double-strand break on one of the strands.

B) Schematic model for the HR mechanism.
1) A double-strand break is present on the DNA template.
2) HR is initiated by resection of a double-strand break to provide 3’ single-stranded DNA overhangs.
3) Strand invasion by these 3’ single-stranded DNA overhangs into a homologous sequence is followed by DNA synthesis at the invading end.
4) The second double-strand break end is captured to form an intermediate with two Holliday junctions (HJs). The structure is resolved at the HJs in non-crossover or crossover products by specific HJ resolvases (black and grey arrow heads).
infertility. These defects are associated with an impaired ability to assemble DNA repair factors at damaged sites. Similarly, mice that lack 53BP1 show growth retardation, chromosomal instability, increased radiosensitivity, immunodeficiency, and cancer susceptibility. Mice deficient in BRCA1 are embryonic lethal, whereas conditional BRCA1-deficient mouse models have consistently increased risk of developing cancer, especially when combined with mutations in other tumor suppressor proteins such as p53.

Genomic Instability and Cancer

Genomic instability has been most extensively studied in the context of cancer. Hereditary or acquired mutations targeting DNA repair genes are often associated with aberrant DNA replication and increased genomic instability, leading to a neoplastic phenotype. In particular, mutations or chromosome rearrangements can contribute to the loss of tumor suppressor functions, or abnormal activation of oncogenes, which in turn result in uncontrolled cell growth, the hallmark of cancer. BRCA1 is one of the best examples illustrating a direct link between DNA repair activities and tumor suppression: women born with a mutation in BRCA1 have a high risk of developing breast and ovarian cancer. Like BRCA1, loss of other DNA repair factors increase the risk of tumorigenesis, as well as the sensitivity to DNA damaging strategies such as radiation or chemotherapy. In addition, deregulation of mechanisms of chromosome segregation leads to an abnormal dosage of chromosomes or aneuploidy, often triggering growth arrest or cell death, or providing the cells with a proliferative advantage.

Genomic Instability and Cancer Treatment

The presence of defects in specific DNA repair pathways in most cancers has been exploited for the design of commonly used chemotherapy and radiotherapy regimens in the attempt to selectively target actively proliferating cancer cells. For example, platinum salts (carboplatin or cisplatin) are frequently given in combination with taxane paclitaxel to patients with advanced ovarian cancer. Platinum salts cause DNA...
inter- and intrastrand crosslinks that are repaired by a combination of mechanisms, including HR. These agents are particularly effective in patients with ovarian cancer because these tumors are commonly characterized by defects in key HR genes.15

An alternative approach based on inhibitors that directly target DNA damage response at the level of cell cycle regulation or DNA repair mechanisms has recently emerged as a new paradigm in cancer therapy.16 These inhibitors often enhance the effectiveness of radiotherapy and chemotherapy in cells, and are thus currently being tested in preclinical and clinical trials. Understanding the mechanism of action of these drugs is of high clinical relevance, and has allowed the identification of genetic defects in DNA metabolism pathways that can either confer resistance or sensitivity to certain chemotherapeutic agents. Knowing which DNA repair pathway is defective in a tumor will facilitate the design of the appropriate therapeutic regimen. However, inactivation of a specific DNA repair pathway is often compensated by the activation of an alternative pathway, which becomes essential for tumor cell survival. This dependence on an alternative mode of repair can be exploited therapeutically by “synthetic lethality” approaches. Synthetic lethality is a genetic concept that refers to the combined inactivation of two pathways to induce cell death when inactivation of each individual pathway is not effective.17 These new approaches have shown efficacy and reduced toxicity in preclinical studies, and hold great promise for cancer treatment as long as the defective pathways in a specific tumor can be identified. Thus, it is envisioned that these synthetic lethality approaches will need to be complemented by the discovery of robust biomarkers that allow the stratification of patients based on the type of DNA repair defects, so that the proper combination therapy can be delivered to the specific patient. These types of discoveries will bring us closer to individualized medicine.

New Frontiers for Cancer Treatment

Current research in our Department aims to understand the mechanisms of action of DNA replication and repair inhibitors with the ultimate goal of developing new drug combinations for cancer therapy. DNA topoisomerase I inhibitors, such as irinotecan and topotecan, could be regarded as the first generation of anticancer agents that target DNA replication. DNA topoisomerase I inhibitors are widely used for chemotherapy, but they are also highly toxic to many normal cell types. The mechanism of tumor response to DNA topoisomerase I inhibitors and the combination of DNA topoisomerase I inhibitors with other drugs for more effective tumor treatments is an area of vibrant investigation. Their cytotoxicity has been associated with their ability to generate single-strand breaks, which are eventually converted to double-strand breaks when the replication forks collide with the single-strand break.18 Recent studies provided new insight into the molecular basis of DNA topoisomerase I inhibitor cytotoxicity by showing that clinically relevant doses of DNA topoisomerase I poisons trigger a process called “fork reversal” where the replication forks back up, thus preventing the collision of the fork with the single-strand break generated by the DNA topoisomerase I inhibitor (See Figure 3).19 Following this discovery, our studies provided the first mechanistic insight into how this process occurs by showing that two DNA repair factors, the poly(ADP-ribose) polymerase 1 (PARP1) and the human RECQ1 helicase, play central roles in the accumulation and restart of reversed forks after DNA topoisomerase I inhibition.20 These studies provide a new rationale for the development of novel targeted inhibitors to sensitize cancer cells to lower DNA topoisomerase I inhibitor dosages that are not toxic to normal cells.20 Inducing fork reversal (DNA topoisomerase I inhibitors) and targeting the factors required for proper processing and restart of the reversed forks (PARP1 and RECQ1) should in principle synergize, thus increasing the DNA topoisomerase I inhibitor-sensitivity of cancer cells.

PARP inhibitors (olaparib and veliparib are the most extensively studied) have also received much attention as a promising therapeutic strategy for tumors that are deficient in the HR mechanism of DNA repair.21 A major breakthrough in the treatment of breast cancers with the poorest prognosis, BRCA1-deficient and triple-negative breast cancers, was the finding that these tumors are exquisitely sensitive to PARP inhibitors.21 Phase II studies with PARP inhibitors have shown a significant response rate in women carrying BRCA1 mutations with tolerable side effects.21 Furthermore, PARP inhibitors potentiate the antitumor activity of current therapeutic strategies, such as genotoxic agents and ionizing radiation. A significant fraction of advanced BRCA1-related cancers
acquire resistance to PARP inhibitors. Landmark studies have demonstrated that loss of 53BP1 is “synthetically viable” with the loss of BRCA1 and induces resistance of BRCA1-deficient cells to PARP inhibitors.24 Targeting the degradation of 53BP1 represents a new strategy for breast cancer therapy. Interestingly, our recent studies identified a mechanism contributing to the loss of 53BP1 in breast cancers. In particular, activation of the protease cathepsin L was found to contribute to 53BP1 degradation in breast cancer cells.25 These findings provide a novel paradigm for the treatment of breast tumors that are resistant to PARP inhibitors due to the loss of 53BP1, such as the case of triple-negative and BRCA1 related cancers. In particular, inhibition of cathepsin L activity via treatment with vitamin D or specific cathepsin L inhibitors is expected to increase the levels of 53BP1, reduce proliferation, and restore the sensitivity of these tumors to PARP inhibitors and other DNA damaging strategies. These novel therapeutic strategies hold great promise for diagnosis, prognosis, and customization of breast cancer treatment.

Acknowledgments

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References


Disclosure

None reported.

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The Double Life of Antibiotics

by Mee-Ngan F. Yap, PhD

Ultimately, it is through research and understanding of the microbes and the biological effects of each molecule at the metabolic level within the cell and within the microbial communities that will lead to new insights into antibiotic design.

“...The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily under-dose himself and by exposing his microbes to nonlethal quantities of the drug make them resistant.” ~Alexander Fleming

Abstract

Antibiotic resistance is a persistent health care problem worldwide. Evidence for the negative consequences of subtherapeutic feeding in livestock production has been mounting while the antibiotic pipeline is drying up. In recent years, there has been a paradigm shift in our perception of antibiotics. Apart from its roles in self-defense, antibiotics also serve as inter-microbial signaling molecules, regulators of gene expression, microbial food sources, and as mediators of host immune response.

The Growing Threat of Antibiotic Resistance

In 1945, the Scottish bacteriologist Alexander Fleming, who received the Nobel Prize for the discovery of penicillin, delivered the above cautionary statement in his Nobel lecture. His prophecy has since been fulfilled. Following the development of penicillin and sulfonamide (by the 1939 Nobel Laureate Gerhard Domagk), the serendipitous discoveries of antibiotics in the 1940s had inspired intense research into new human therapeutics. Fourteen classes of antibiotics representing six distinct mechanisms of action were introduced to clinical and veterinary uses before 1970 (See Figure 1). They became the miracle drugs of the twentieth century and enabled common life-threatening infections to be treated for the first time. Yet after decades of extensive use (misuse, overuse, and habitual use), the medical community is facing an unprecedented challenge in combating antibiotic resistance. At the same time, bottlenecks have slowed drug discovery and development.

Every year at least two million people fall ill to hospital-acquired infections and approximately 100,000 of the cases are fatal, the majority due to resistant bacterial and fungal strains. The empty pipeline for antibiotics and the rapid erosion of drug efficacy pose
serious health risks in modern medicine. As antibiotics become ineffective, cases of hospital-acquired pneumonia will rise, life-threatening septicemia will become a greater concern in abdominal surgery, and the risks of opportunistic infections will be the limiting factor for patients undergoing cancer treatments and transplant surgery. According to World Health Organization estimates, new antibiotic-resistant strains have claimed more lives than HIV, influenza, and traffic accidents combined in the past decade. The infections are caused by members of gram-positive bacteria, including methicillin- and vancomycin-resistant Staphylococcus aureus (MRSA and VRSA), multidrug-resistant and extremely drug-resistant Mycobacterium tuberculosis (MDR-TB and XDR-TB), and Clostridium difficile. Most recently, the emergence of the gram-negative enterobacteria and vibrio species carrying the New Delhi metallo-β-lactamase-1 (NDM-1) gene not only left infected patients untreatable by the last line of drug, carbapenem, but also raised worldwide concern over the spread of the resistant gene through the transmissible plasmid.

Falling Behind the Bugs

Antibiotics are generally prescribed in restricted quantity and for a short period, so the profits associated with new antibiotic discovery are considered much less lucrative than those associated with long-term treatments for chronic diseases such as cardiovascular diseases and diabetes. However, that is not to say that the pharmaceutical industry lacks incentive solely due to financial burden and lengthy regulatory hurdles. In reality, the development of novel antimicrobial drugs is scientifically daunting. Almost all new drugs approved after 1990 were either complete synthetic (oxazolidinones) or semi-synthetic derivatives of existing scaffolds originated from soil-dwelling actinomycetes (See Figure 1). The discovery of new classes of antibiotics with distinct scaffolds and novel modes of action has been constrained by the lack of chemical diversity in current combinatorial libraries, the narrow scope of structural- and target-based screening approaches, and off-target signals in whole-cell screening, and poor success in developing effective screens to validate potential targets that were identified through comparative genomic analysis.

Mechanisms of Antibiotic Resistance and Tolerance

There are hundreds of antibiotics on the market, but they are very similar in that they each interfere with microbial cell growth by inhibiting only a handful of drug targets. These sites are usually macromolecules of essential biosynthesis pathways, such as the ribosome (protein synthesis), topoisomerase, DNA and RNA polymerase (nucleic acid synthesis), cell wall and membrane (peptidoglycan and lipid synthesis), and dihydrofolate reductase (folate metabolism) (See Figure 2). Not surprisingly, antibiotic resistance often evolves by reprogramming and camouflaging these targets through at least four general mechanisms: immunity, efflux, target modification, and drug inactivation. Some bacteria are immune to a drug simply because they have an impermeable barrier or produce protective proteins (TetM and FusB) that mask the drug binding sites. Active efflux involves multidrug resistance transporters that eject antibiotics directly to the outside of the cells or secrete them into the periplasm. Degradation and covalent modification can disarm the antibiotics. Furthermore, modifications of the target entail mutations in the target proteins or enzymatically modifying the targets that disable
drug binding, for example, by methylation of the ribosomal RNA (See Figure 2).³,⁵,⁶

Research in our lab focuses on studying the molecular mechanism of resistance mediated by rRNA methylation that is induced by subinhibitory concentrations of antibiotic. In staphylococci and streptococci, the erm genes encode for the methyltransferase enzymes that modify the drug target in 23S rRNA of the bacterial ribosome and reduce antibiotic binding. The expression of erm is regulated at the translational level by at least one upstream leader peptide via an unusual ribosome stalling mechanism. A threshold concentration of antibiotic, such as erythromycin, binds to a ribosome translating the leader peptide and causes the ribosome to stall. The stalling denatures an inhibitory mRNA hairpin in the intergenic region that masks the translation initiation site of erm and thereby facilitates the synthesis of Erm enzyme.⁷ Clinical studies have shown that Erm leader peptides are highly diverse and the antibiotic inducibility is drug type-specific, but the underlying mechanism is poorly understood. Our major goal is to understand the selective induction of antibiotic resistance in different erm homologous systems, specifically to compare the molecular interactions of the regulatory leader peptide with the ribosome in the presence and absence of antibiotic ligands. The work could potentially lead to rational drug design for non-inducer antibiotics.

In some cases, antibiotic tolerance arises when the microbes are able to withstand the drug killing effect by an altered metabolic pathway, such as an attenuation of hydroxyl radical production. This observation has provided an explanation on how seemingly unrelated genetic mutations away from a primary target can result in increased drug tolerance.⁵,⁹ Because most antibiotics are derived from bacterial natural products, the producers possess self-defense mechanisms that contribute to resistance. Recent studies have shown that horizontal gene transfer is extensive between environmental, clinical, and commensal bacteria by means of plasmids, transposons, integrons, and bacteriophages. Widespread antibiotic use is another complication, which exerts strong selective pressure in bacterial population and consequently induces mutagenesis in hypermutator strains. This evolution encourages the formation of persister cells that usually survive in biofilms (bacterial aggregates) (See Figure 2)⁶,¹⁰ and has hampered the complete eradication of pathogens during chronic infections.

**Reservoirs of Antibiotic Resistance**

According to a recent survey from the FDA, approximately 13.1 million kg (~80%) of antimicrobial drugs in the United States are sold for animal husbandry and fisheries annually. In contrast, only 3.3 million kg are administered for human use.¹¹ Nontherapeutic and subtherapeutic uses of antibiotics in agribusiness began in the 1950s for inclusion in feed and water for poultry and livestock to prevent disease and to promote growth. The application fattens the animal faster and helps to mitigate the impact of disease spread because animals are regularly raised in crowded, unsanitary, and stressful conditions. The result is reduced cost of time and feed. Recognized scientific studies have repeatedly shown that low-dose antibiotics not only propagate resistant bacteria, but also disseminate...
these strains into nearby communities as well as the food supply. During the last few years, the new MRSA strain CC398 and a multidrug resistant strain of Salmonella enterica serovar Heidelberg have been found in high abundance in US retail turkey products, farm animals, and even humans who have had no direct contact with the animals. Resistant determinants are also widespread in soil, sewage, and water systems, at high prevalence compared with background resistance in nature. The example of bidirectional zoonotic exchange is an alarming sign of inadvertent creation of resistant strains that are too powerful for current medicine, given that the seven classes of antibiotics used in livestock production are also commonly used in treating human infections. To help preserve long-term effectiveness of medically important antimicrobials, FDA officials in April 2012 called for a voluntary halt to the routine use of several performance-enhancing antibiotics except for circumstances where prescription of such drugs is needed to treat and control animal illness. While farmers and stakeholders have argued tenaciously that eliminating antibiotics would result in larger economic loss and have little or no impact on reducing human infections, alternatives to antibiotics in agriculture should be explored in the United States. Approaches such as improving livestock hygienic practices and reducing overcrowding have had substantial success in Europe in the decades following the bans of antibiotics in animal production.

**Multiple Roles of Antibiotics in Non-Clinical Settings**

The popular press tends to paint microbes as disease-causing agents that should be eliminated using antibiotics, and that the role of antibiotics is to inhibit the cohabitating competitors. In contrast to these conventional views, a growing body of evidence has demonstrated that antibiotics serve a role far beyond that of a direct weapon. It has been shown that all antibiotics, regardless of their target and mode of action, exhibit the phenomenon of “hormesis” that is manifested by biphasic response of high-dose inhibition and low-dose stimulation. At subinhibitory doses, the concentration likely to be found in the environment, antibiotics act as signaling molecules to modulate gene expression and intercellular signaling (See Figure 2). For instance, aminoglycoside (AG) antibiotics such as tobramycin could interact with the AG response regulator and trigger secondary messenger cyclic diguanylate, which in turn leads to biofilm formation. In another example, subtherapeutic antibiotic treatment of young mice increased the metabolic activity of their gut microbiome by altering the expression of genes involved in the conversion of carbohydrates to short-chain fatty acids. Furthermore, bacterial cells exposed to subinhibitory concentrations of different antibiotics displayed distinct transcription profiles that are associated with pleiotropic effects in metabolic processes. Of note, it was found that the production of hydroxyl radicals induced by low antibiotic treatment could generate subpopulations of mutant strains that are resistant to other antibiotics but not the one applied. The isolation of naturally occurring bacteria that can subsist on both natural and synthetic antibiotics as a sole carbon source (See Figure 2) further expands the unrecognized role of antibiotics within bacterial communities. These discoveries have ignited the idea of removing antibiotic “pollution” from the environment by using the bacteria’s ability to devour antibiotics. Finally, quorum-sensing molecules that are important for mediating intra- and interspecies communication could exhibit antimicrobial activity at high concentrations, which further illustrates the common dual role of naturally occurring small molecules and suggests a potential resource for antimicrobial discovery.

**Immunomodulatory Benefits of Macrolide Antibiotics**

Relatively little is known about the beneficial effects of antibiotics on eukaryotic cell responses, except for the adverse effects usually associated with drug toxicity or compromised innate immunity. Interestingly, long-term macrolide prophylaxes are common pulmonary practices for the treatment of patients with chronic airway diseases, such as chronic obstructive pulmonary disease (COPD) and diffuse panbronchiolitis (DPB). Macrolides belong to the polyketide class of antibiotics that exert their bacteriostatic efficacy by inhibiting protein synthesis. Although the reduction of airway inflammation can in part be attributed to the antimicrobial activity of macrolide, the effects are believed to be negligible because the treatment dosage is too low to fight infection. The biological effects of macrolides are multifaceted but the exact mechanism is not well understood. They have been found to modulate (attenuate) the production of pro-inflammatory cytokines, to reduce epithelial expression of adhesion molecule, and to enhance phagocytosis and...
inhibition.\textsuperscript{25,26} Development of a macrolide-based anti-inflammatory drug devoid of the antimicrobial properties is currently of potential clinical interest.

The Future is an Arms Race: Bugging Out

Preventing and controlling infectious diseases relies on widespread collaboration in drug discovery, the judicious use of available antibiotics, improved hygiene, and well-developed methodologies for disease diagnosis. At the top of the list is drug discovery, which is essential to the long-term success of combating disease and requires a reinvigorated search in underexplored microbial niches. Robust screens designed to avoid rediscovering known scaffolds, leveraging existing libraries of synthetic molecules, and exploring the metagenomics approach to access the secondary metabolites from unculturable bacteria are all equally important components in drug discovery in need of further refinement.

In the short-term, antibiotic combination therapies that generate synergistic effects may slow the rise of resistance. To conserve drug effectiveness, tighter regulation on misuse and abuse of antibiotics must be imposed. More efficient and precise diagnostic tools tailored for individual patients will help guide more appropriate drug therapies based on the identified pathogen. Ultimately, it is through research and understanding of the microbes and the biological effects of each molecule at the metabolic level within the cell and within the microbial communities that will lead to new insights into antibiotic design. With an ever-greater range of new genomic and chemical biology tools, the race against antibiotic resistance is just the beginning of a new era for modern medicine.

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Disclosure

None reported.
MIF inhibitors analysis will allow us to identify pathophysiological signaling events and involved molecules, which have not been detected by traditional methods.

Abstract

The Cho and Baldan labs focus their efforts on novel pathways that control atherogenesis. MIF (Macrophage migration inhibitory factor) recruits macrophages to atherosclerotic lesions and activates the production of matrix proteinases, which in turn destabilize atherosclerotic plaques. On the other hand, miR-33 coordinates the expression of several sterol transporters essential for high-density lipoprotein metabolism and bile secretion. Thus, both MIF and miR-33 are promising therapeutic targets to manage patients at risk of developing atherosclerosis.

Macrophage Migration Inhibitory Factor in Atherosclerosis

A proinflammatory cytokine called macrophage migration inhibitory factor (MIF) consists of 115 amino acids and exists as a trimer in physiological conditions (See Figure 1). From the historical experiments by John David in 1964, MIF was discovered as the first lymphokine attracting immune cells such as macrophage, T, and B cells. Since then, protective roles of MIF in the immune system have been reported, including the fact that MIF deficient mice are more susceptible to the infection of Leishmania major than the wild-type. In contrast, high levels of MIF were observed in major inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and atherosclerosis. MIF activates the expression of various proinflammatory cytokines and chemokines. This activation implies that MIF is a master regulator of inflammatory responses in the human body. Intriguingly, MIF has a catalytic activity, missing in canonical cytokines and chemokines, and converts a C-O double bond into a single bond and vice versa. It is not clear whether this catalytic activity is physiologically relevant. This catalytic activity has been used as a molecular trap to identify small molecules binding to MIF.

Migration of mononuclear leukocytes is one of the key features of atherosclerosis. MIF recruits macrophages to the site of atherosclerosis and activates them to produce matrix metalloproteinases, which in turn degrade collagen fibrils, providing mechanical strength to atherosclerotic plaque. As the instability of the plaque is directly related to the progression of the disease, MIF inhibitors could be...
potential therapeutics for atherosclerosis in combination with drugs targeting other disease factors, including cholesterol, which is under investigation by the Baldán lab. To achieve this goal, understanding the molecular mechanism of MIF–mediated cell migration is essential.

**MIF-receptor Signaling**

MIF binds to an invariant chain of MHC class II CD74, canonical chemokine receptors CXCR2 and CXCR4. Since CD74 is lacking a signaling domain, it forms a hetero-dimer with CD44, which then interacts with intracellular signal transducers (e.g., src-kinases) and activates downstream signaling pathways (e.g., MAPK, ERK, and AKT). CD74 can form a heterodimer with CXCR2 and CXCR4 to mediate various MIF signaling in any given environment. In general, chemokine-receptor interaction is complicated due to their promiscuous binding patterns. For instance, MIF receptor CXCR2 and HIV co-receptor CCR5 can recognize seven chemokines, and they have been implicated in atherosclerosis. Furthermore, chemokine receptors, belonging to the type A G protein coupled receptor (GPCR) family, can bind to different G proteins (e.g., G, G, G, G, etc.) depending on ligands and co-factors. Very little is known about the molecular mechanism of MIF-receptor interaction and their cellular outcomes. We are currently investigating these by employing multidisciplinary means, including structural biology, chemical biology, and label-free cell-based assay.

**Small Molecule MIF Modulators**

Some of the prototypic catalytic MIF inhibitors exhibited inhibitory effects against the immunological functions of MIF. Inspired by this, we identified two dozen structurally and chemically diverse catalytic MIF inhibitors through high-throughput screening (HTS) of small molecule libraries. Several inhibitors were crystallized in complex with MIF and revealed covalent and non-covalent binding to the first amino acid, proline. Interestingly, some of them exhibited a non-competitive inhibition pattern, indicating that they might not bind to the catalytic site but bind to another part of MIF. In our recent study, a novel allosteric binding site was discovered from the complex crystal structures of MIF and ibudilast, which had been used as an asthma drug for two decades in Japan. We also tested whether the inhibitors modulate MIF-mediated cell responses. Indeed, different inhibitors altered the MIF-mediated cell responses in distinctive ways. We are investigating which receptors and signaling pathways were affected and using these small molecule MIF modulators as molecular probes for further mechanistic studies.

**Innovation in MIF-receptor Interaction Study**

We are currently identifying MIF residues critical for receptor binding to ultimately link this information to the resulting intracellular signals in leukocytes and lymphocytes. We employ X-ray crystallography to obtain atomic resolution of the molecular structures of MIF in complex...
with its inhibitors. Among the MIF receptors, CXCR2 and CXCR4 are 7-transmembrane proteins, and it is difficult to work with them in vitro. One of the classical ways of studying GPCR is artificially replacing a downstream target gene with a reporter gene to monitor the activation of the receptor of interest. With this reporter-based assay, one can monitor limited cellular events at only a single time point. To overcome this limitation, we have established a label-free technology that measures holistic cellular response in real-time (See Figure 2A). We successfully measured dynamic mass redistribution (DMR; relocation of intracellular molecules upon the ligand-induced activation of receptor) from MIF-treated cell lines. Three different immune cell lines, THP-1 (monocyte), Jurkat (T cell), and Ramos (B cell), were activated with MIF and generated distinctive DMR patterns (See Figure 2B). B cells reacted at the earliest time points, T cells the second, and monocytes the latest. In addition, T cells responded in a negative mode at earlier time points and shifted to a positive mode. Opposite DMR modes and the amplitudes of the responses imply the activation of distinctive signaling molecules and pathways in each cell line.

To identify the signaling molecules and pathways involved, we perform the label-free assay in the presence of signaling molecule/pathway-specific inhibitors. Evaluation of small molecule agonists and antagonists in physiologically relevant conditions is one of the advantages to using this technology. Different catalytic MIF inhibitors altered the DMR responses of different cell lines in distinctive ways (See Figure 2C). We are planning to use this highly sensitive label-free technology to work with primary cells from disease patients. This analysis will allow us to identify pathophysiological signaling events and involved molecules, which have not been detected by traditional methods.12

**Future Direction of MIF Research in Atherosclerosis**

One of our research interests is the molecular mechanism of MIF-receptor interaction in chronic inflammatory conditions, including atherosclerosis. Since

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**Figure 2**

Label-free technology for monitoring cellular events. (A) MIF (red) binds to receptors on the cell surface and activates the intracellular signaling events represented as dynamic mass redistribution (DMR; black indicates movement of signaling molecules). Cells are plated on a fibronectin-coated biosensor. (B) DMR is represented as the peak wavelength value difference (delta PWV) before and after the addition of MIF. (C) One of the HTS MIF inhibitors alters the MIF-mediated DMR responses compared to those of MIF alone in panel B.
Atherosclerosis is a progressive disease of the artery wall where large numbers of monocytes infiltrate the sub-endothelial space and subsequently differentiate into macrophages and become lipid-loaded. At later stages, more advanced plaques develop, containing inflammatory cells, a necrotic core, and a fibrous cap. When unstable plaques rupture, a thrombotic event is initiated that ultimately results in heart attack, stroke, or peripheral artery disease. According to the American Heart Association, atherosclerosis-related cardiovascular disease accounts for > 30% of all deaths in the U.S. The exact cellular and molecular events that lead to the early development and progression of atherosclerotic lesions are not yet completely understood, but several risk factors have been identified in large population studies. For example, the Framingham Heart Study recognized elevated cholesterol in blood as a risk factor for heart attack in 1961. Since then, great progress has been made to understand the regulation of both intracellular cholesterol homeostasis and lipoprotein metabolism.

Our laboratory is interested in the regulation of sterol and triglyceride homeostasis by microRNAs (miRNAs), which are small, non-coding 20-24 nt RNAs that promote the silencing of their target genes by binding to specific, partially complementary regions in the 3’-UTR (untranslated regions) of the target mRNA. This binding results in RNA interference and/or translational repression of the target gene. miRNAs can be expressed from their own promoter or can be encoded within the introns of other genes (so they are expressed when the “hosting” mRNA is transcribed). Regardless, the original miRNA transcript (Pri-miRNA) is sequentially cleaved by the exonucleases, Drosha (nuclear) and Dicer (cytoplasmatic), to generate the Pre-miRNA (100-150 nt) and the mature miRNA (20-24 nt) molecules, respectively. It is the mature miRNA that is finally incorporated into the RNA-Induced Silencing Complex (RISC). Other, “non-canonical” pathways that are independent of Drosha or Dicer have also been described. Nevertheless, the expression of a particular miRNA can be tissue, developmental, and even disease-specific, revolutionizing our understanding of epitranscriptional regulatory networks. Multiple studies have shown that miRNAs function as key mediators in multiple normal and disease-related biological processes. Consequently, novel therapeutic approaches that exploit miRNA-dependent gene silencing offer a promising future for the management of multiple diseases.

### HDL-cholesterol and miR-33

In 2010, five different laboratories, including ours, reported on miR-33. This miRNA is expressed from within an intron of SREBP-2, a transcription factor that functions as a master regulator of intracellular cholesterol homeostasis promoting the expression of cholesterogenic genes (including HMGCR, the rate-limiting enzyme of the cholesterol biosynthetic pathway), the LDL-Receptor, PCSK9, and other cholesterol-related genes. The clinical importance of the SREBP-2 pathway is revealed in patients taking statins to decrease plasma LDL-cholesterol levels. We and others showed that the expression of miR-33 is physiologically regulated by the levels of intracellular cholesterol (induced by sterol depletion and suppressed by sterol loading) and stimulated by treatment with statin drugs, both in vitro and in vivo. These initial studies also showed that miR-33 functions to repress the expression of the cholesterol transporter ABCA1, and identified the sequences in the 3’UTR of ABCA1 that bind to miR-33.

ABCA1 plays a critical role during the early steps of high-density lipoprotein (HDL) biogenesis by mobilizing intracellular cholesterol towards ApoA-1 in circulation, and patients with functional mutations in this transporter develop hypoalphalipoproteinemia (low HDL-c levels) or, in the most severe cases, Tangier disease (< 5% normal HDL-c levels). Consequently, mice in which we overexpressed miR-33 (via adenoviral transduction) showed decreased hepatic ABCA1 levels and reduced circulating HDL-c levels, compared to control animals. More importantly, the opposite was also true: silencing hepatic miR-33 (by using antisense oligonucleotides) led to elevated...
hepatic ABCA1 mRNA and protein levels and elevated HDL-c in blood, compared to control mice.18-20 These results suggested that sustained silencing of hepatic miR-33 might be used therapeutically in hypercholesterolemic patients to raise the levels of anti-atherogenic HDL-c and thus reduce the risk of cardiovascular disease.

Bile Secretion and miR-33

We reported that miR-33 also controls the expression of sterol transporters involved in bile secretion, namely ATP8B1 and ABCB11.24 Severe functional mutations in these transporters result in Progressive Familial Intrahepatic Cholestasis (PFIC) type-1 and -2, respectively.25 Other, less severe mutations lead to Benign Recurrent Intrahepatic Cholestasis (BRIC) type-1 and -2, respectively.25 We characterized the miR-33 response elements in the 3’UTR of these genes and showed that manipulation of hepatic miR-33 levels in mice (by using adenoviral vectors or antisense oligonucleotides) results in changes in bile secretion rates and overall bile collection from the gallbladder.24 Additionally, we showed that treating mice with both a statin and a Pagen diet (a high fat, high cholesterol, sodium cholate diet) resulted in a cholestatic phenotype, compared to mice that were fed the diet but did not receive the drug. Importantly, this liver phenotype could be rescued by pre-treatment with anti-miR-33 oligonucleotides,24 suggesting that miR-33 could mediate some of the effects of statins in vivo.

Reverse Cholesterol Transport and miR-33

Collectively, the data discussed above suggest that miR-33 is physiologically induced by sterol depletion, prevents the loss of intracellular sterols, and controls the expression of sterol transporters via both the basolateral membrane (via ABCA1 towards HDL lipoproteins) and the apical membrane (via ATP8B1 and ABCB11 towards the bile). Both HDL and bile metabolism are essential components of the Reverse Cholesterol Transport (RCT) pathway, which mobilizes extra-hepatic cholesterol towards the liver for subsequent biliary secretion and ultimately loss through the feces.26 While this pathway is a very inefficient way of removing sterols (> 95% bile acids are reabsorbed in the intestine and shuttled back to the liver), it is the main mechanism we have to remove excess cholesterol (the other main pathway is known as Trans-Intestinal Cholesterol Excretion-TICE).

The RCT is predicted to be atheroprotective, by facilitating the removal of sterols from macrophages in atheromata.26 We24 and others27 showed that an acute (two to four week) treatment of either wild-type or atherosclerosis-prone Ldlr−/− mice with anti-miR-33 oligonucleotides resulted in accelerated RCT in vivo, compared to mice treated with control oligonucleotides. In these experiments, mice receive an intraperitoneal injection of macrophages loaded with radiolabeled cholesterol, and the RCT is estimated by the recovery of radiolabeled sterols in blood, liver, gallbladder, and feces. Collectively, these data provided further support to the hypothesis that silencing miR-33 in hypercholesterolemic patients might prevent cardiovascular events.

Challenging the miR-33 Hypothesis: When All that Glitters Is Not Gold

Studies in non-human primates showed that a 16-week treatment with anti-miR-33 oligonucleotides increased plasma HDL-c, while at the same time reduced VLDL-triglycerides.28 These latter authors also reported that a 4-week antisense treatment following 16-weeks of a high fat, high cholesterol feeding accelerated the regression of atheromata in Ldlr−/− mice.27 A recent report showed that miR-33/ApoE double knock-out mice had decreased atherosclerosis, compared to atherosclerosis-prone ApoE−/− mice.29 In contrast, we recently showed that long-term (12-week) treatment of Ldlr−/− mice with anti-miR-33 oligonucleotides did not change the size or composition of atheromata, compared to mice receiving control oligonucleotides or saline.30 These latter results are paradoxical, especially when considering the fact that previously described miR-33 targets (e.g., ABCA1, ATP8B1, ABCB11, and others) were induced in the livers of mice receiving the antisense,30 as expected. Also intriguing is the fact that by the end of the three-month experiment, the plasma levels of HDL-c were not significantly different among the different treatments, and that VLDL-triglycerides were indeed increased in mice receiving anti-miR-33 treatment.30 The different outcome between the four-week regression study27 and our twelve-week progression study30 need to be elucidated. Differences in the progression of vascular lesions following whole-body miR-33 deficiency29 or treatment with anti-miR-33 oligonucleotides30 are also intriguing, and might reflect the impact of miR-33 expression in several cell types (hepatocytes, macrophages, endothelial cells). It is conceivable that anti-miR-33 therapy could be effective
in some, but not all, cell types where miR-33 is normally expressed. It will also be important to establish whether different chemistries alter the bioavailability and potency of anti-miR-33 oligonucleotides in either liver or lesions.

What We Missed in Mice

It is important to note that primates, but not rodents, express a second miR-33 gene (miR-33b) from an intron of SREBP-1. Hormones, dietary challenges, and even statin treatment differentially regulate SREBP-1 and -2, and this control may be critical to fully understanding the role of miR-33a/b in lipid homeostasis, since the expression of miR-33a and miR-33b is expected to parallel that of their hosting gene. In marked contrast to our atherosclerosis study, a longitudinal study in non-human primates did show a sustained elevation of HDL-c, as well as a strong decrease in triglycerides. Whether the differences in cholesterol and triglyceride metabolism between mice and primates in response to anti-miR-33 treatment are due to miR-33b remains to be established, but anti-miR-33 oligonucleotides should block the action of both miR-33a and miR-33b.

The discrepancies between rodent and primate models thus raise an important concern regarding the direct translation of data from rodent models to human physiology and metabolic disorders. Using a combination of in vitro experiments and cell culture techniques, we are currently exploring other metabolic pathways that respond to miR-33a/b overexpression or silencing that might explain the specificities between mice and primates.

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