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James M Grichnik MD Ph.D

Director of the Melanocytic Diseases Section
Associate Professor, Division of Dermatology, Department of Medicine
Assistant Professor, Department of Cell Biology
Duke University Medical Center, Durham, NC 27710
grich001@mc.duke.edu

My adventures on the trail to melanoma stem cell biology

Youth and Adolescence

I recall being puzzled, when handed orange, green, and black paper in 2nd grade, and told to make a pumpkin. I figured it out! I cut the orange paper in strips, looped them together at angles to make a sphere, and then rolled the green paper into a stem and assembled the final product into a fine 3D pumpkin. Imagine my surprise and dismay when I broke my concentration and noted that the other kids knew the correct way to make a pumpkin and had nice flat jack-o-lanterns with black triangle eyes and green rectangular stems. My pumpkin couldn't be displayed on the wall with the others and instead it was hung from the lights by a paper clip.

In 5th grade, I chased monarch butterflies, collected the eggs, raised the caterpillars, and calculated the total area of milkweed leaf consumed by each before forming a chrysalis. I do not remember if I was actually assigned to do a science project, but I do recall that my teacher, Mrs. Brown, seemed genuinely impressed. Prior to this, I was a mediocre student. I credit her positive reinforcement as a key turning point in my life.

Soon thereafter, I discovered chemistry, which I used to produce my own fireworks. The public library proved to be great resource for recipes; the hobby shop and drug store served as my sources for chemicals and laboratory supplies. I recall being frustrated that I was unable to purchase potassium nitrate so I learned how to produce it with fractional crystallization. My neighborhood reputation was sealed when the local sewer blew up with spectacular 10-foot flames during my 13th birthday party – a consequence of throwing a firecracker into a sewer that unknown to us had been recently supplemented with old boat fuel by one of the neighbors.

During this time, Boy Scouts also played a crucial role in my adolescence – allowing me to escape on weekends with the troop to explore the outdoors while at the same time building life and leadership skills. I ultimately achieved the rank of Eagle Scout by completing a project teaching first aid at the local fire department. My choice seemed to foretell my future career while also potentially protecting those around me from my youthful pastimes!

My upbringing was significantly influenced by my parent's difficult divorce and subsequent monetary constraints. This led to my initial jobs as dishwasher, prep cook and merchandise stocker utilizing my only source of transportation – a Sears moped. I was careful in my choice of colleges – making sure that financial aid and the option of early graduation were available – leading to my matriculation to Washington University.

Undergraduate Education

In college, my advisor was Arthur C. Wahl, the co-discoverer of plutonium, who played a critical role in the development of the atomic bomb. I was in awe of his considerable contributions and dedication. Chemistry was my primary major as I was particularly interested in polymer science at this time. However, I also found myself distracted by electronics, computing, geology, archeology, and many aspects of biology (especially fungi!). As I was exploring my future career options, I ventured into Mary-Dell Chilton's laboratory. Her work focused on Crown Gall plant tumors caused by the transfer of a fragment of DNA (T-DNA) from the bacteria Agrobacterium Tumifecians. The fact that a small coded polymer could reprogram cellular machinery was captivating! I was privileged to work under Michael Bevan, a post-doctoral candidate endeavoring to genetically sequence the T-DNA. Each day was more exciting than the previous day as the sequence fell into place. I clearly remember the day that we sequenced across the Bam HI site - a landmark we had been looking for quite some time. (*I also remember that Michael's shoes were so radioactive that they pegged the Geiger counter!).* Largely through Dr. Chilton's efforts, T-DNA became the primary vector for plant genetic engineering.

This introduction to the plant cellular transformation process sparked my interest in human cancer, as a similar problem gone awry. I was convinced that in order to be proficient at research, obtaining a PhD would be mandatory. However, I decided

that I would also need a MD to fully understand the human disease process. Thus, I decided to investigate MD PhD programs for post-graduate training. I graduated from college in three years with a double major Chemistry and Biology thanks to AP credits, a heavy workload, and a desire to avoid the expense of an additional year due to exhausted financial resources.

After evaluating many MD PhD programs, I was most impressed by the Baylor College of Medicine program and headed to Houston. (*Of note, Baylor was the only program that subjected me to the raw oyster test on a recruiting visit. I swallowed a few successfully and secured a position in the program.*

Post Graduate Education (PhD and MD)

I entered Baylor College of Medicine in 1982, with a great group of 168 medical students. For the first two years, we focused on the basic sciences curriculum. (*During this time, my extracurricular life was colorful – after surviving an apartment with more roaches than floor surface, I moved in with a group of students living in the Holcomb Mansion- a former Houston mayor's old house). My bedroom was the mayor's old office and my closet was the service elevator. Regrettably, we were asked to move out after the Texas Alcohol and Beverage Commission shut down one of our legendary parties. Also unfortunately, I had the opportunity to experience medical care as a patient before being a doctor. I overturned my roommate's motorcycle resulting in a significant injury to my left (dominant) hand. Surgery went well but I couldn't get a good night's rest in the hospital and worried about missing classes while occupying the hospital bed. With some pleading, I was released and given the job of my own home health care provider with a shopping bag of IV antibiotics and the required equipment to self administer the medications during the day between medical school classes.*

The basic science portion of medical school moved quickly and soon I had to choose my Ph.D program. While cancer research remained my primary focus, I found myself also particularly fascinated by neuroscience and microbiology. In the end, my best fit was in the Department of Cell Biology. I was most intrigued by the control of gene transcription. I viewed it as a primary switch that potentially could be therapeutically modulated for cancer treatment. After making the rounds, I decided to utilize a transcription reporter vector system (CAT assay) that I learned about in Jeff Rosen's laboratory to delineate the transcriptional promoting DNA elements on the chicken skeletal α-actin gene in Robert Schwartz's laboratory. Although clearly not a cancer gene, I rationalized that the information discovered would be generally applicable. Ultimately, I was successful in defining the critical DNA elements for tissue and stage restricted expression.[1] In addition, this work also revealed the promoter elements responsible for the orientation of the transcription complex.[2]

During this time, I met my wife in Houston where she was a visiting medical student. She had secured a residency position in Boston and we were evaluating the possibility of living apart for the first 3 years of our marriage. After much discussion and effort (multiple interviews, national board tests, Baylor's gracious support, NIH sanction and additional funding, and Harvard's approval), I was accepted for transfer into Harvard's MD PhD program to complete medical school. I was able to spend my final year at Baylor as a postdoctoral fellow in neuroscience and a portion of my final year at Harvard in Barbara Gilchrest's laboratory. Thus, although enrolled in the combined MD-PhD program at both schools, my PhD was awarded by Baylor and the MD was awarded by Harvard.

During the MD-PhD education process, I carefully considered the ideal career path that could promote success in a dual career path of cancer patient care and bench research. I knew that this would not be easy, the equivalent of two full time occupations, but with only part time resources. Clearly, it was important to choose a system that would be maximally synergistic between the two career arms. I sought exposure to the various clinical professions that managed cancer patients and examined the different tumor models. As the result of these efforts, I had the opportunity to participate in the pigmented lesion clinic at MGH with Thomas Fitzpatrick, Arthur Sober, and Arthur Rhodes. In contrast to the pessimism that I encountered in many of the cancer clinical practices, this clinic identified the majority of cancers early resulting in lives saved. Unfortunately, when some tumors did spread from the skin, the prognosis was dismal. This system clearly presented a great opportunity to further elucidate the diagnosis and treatment of melanoma. I was additionally impressed that dermatologists performed surgery (allowing direct access to tissue) and were trained in pathology to evaluate the microscopic characteristics (cell biology) of these lesions. Moreover, there were numerous benign melanocytic tumors (moles) for which there was also very little known. It was a very complete system ripe for investigation and I was sold!

Pre-residency Research

Once I determined that melanoma was my area of interest, I began to explore the basic laboratory research opportunities in the field. The research of a Harvard alumna, Dr. Barbara Gilchrest, matched my interests, although she worked for Boston University and USDA Human Nutrition Research Center on Aging at Tuft's University at the time. With some convincing, the MD-PhD program allowed me to work in her laboratory, while still a Harvard student. This was a wonderful experience, and thanks to her, a huge amount of energy and excitement permeated the lab environment every day. My original project was to initiate transcription studies on the NGFR promoter in melanocytes and melanoma lines. However, unlike myoblasts

that transfected easily with calcium phosphate precipitate, the melanocytes proved troublesome with very low transfection rates and significant variability. I thus put aside my hopes of performing a deletion analysis on the NGFR promoter and focused on gaining control over the variability in the system in the brief amount of time that I was able to spend in her lab. To accomplish this, I focused on dual transfection and assay approaches to gauge one promoter's transcription strength against another. Although, transfection efficiency remained low, we showed that there were significant differences in promoter expression in the different melanoma lines. This suggested a potential method for segregating melanoma types based on active transcriptional capacity profiling. This method still offers a novel approach for the segregation of tumor types.[3] However, I was taken aback when I found out that my former lab colleagues were upset upon the publication of these findings. I had utilized control vectors with permission from my old laboratory and had acknowledged this, but I learned they had expected authorship. I am now very compulsive about making very sure all authorship roles are clearly defined before initiating any project. *The end of my medical school training was highlighted by the birth of my son, Matthew. It was wonderful being able to cross the graduation stage with him in my arms!*

Internship and Residency

I elected to do my internship training in Medicine at Boston's Beth Israel Hospital while my wife completed her residency in Anesthesia at Brigham's and Women's Hospital. One of the many wonderful attributes of Beth Israel was its advanced electronic patient data capabilities at the time. As below, this exposure influenced my decisions about how to care for patients as an attending physician. *This was also a tumultuous year of little sleep and many life changes. My son slept in a crib in the living room and was cared for during the day by a nanny from Nebraska while my wife and I alternated baby care with being on call.* For my residency training in Dermatology, we decided to look at areas of the country that would allow us to affordably raise the family in a suburban environment yet provide direct access to an outstanding academic medical center with programs in place to allow us both to advance our careers. I further desired to look at programs that I could possibly stay on staff after training to avoid future moves. It was a difficult choice but Duke offered all that we were seeking and we moved to North Carolina in 1991.

At Duke, Hilliard Siegler had established a massive surgical oncology melanoma program. However, there was not a well-defined pigmented lesion program, and thus a huge unmet need. While still in residency, I began my efforts to build a clinical pigmented lesion center and melanoma research program. As a resident, I self-funded a trip to a Keystone Conference on Melanoma to get updated on current research. I recall meeting Wally Clark, discussing KIT with Richard Spitz on a ski lift, and attending a presentation by James Mule on the use of NIH:3 mice for melanoma studies). Later in my residency, I again self-funded a trip to the NIH Consensus Conference on Early Melanoma to experience first hand the debate on "dysplastic" nevi and early melanoma. These two conferences cemented my interest in the area and provided a foundation of information upon which I could build.

I am in debt to Claude (Skip) Burton for his assistance in initiating the clinical portion of my career. In his role as my mentor, he helped me build a pigmented lesion clinic under his supervision. He also purchased a dermoscopy camera, which I used heavily and eventually acquired. (Dermoscopy is hand held surface microscopy that allows for detailed visualization of individual lesion structures). Clinically, the pivotal determinant to as to whether a lesion may be a melanoma is growth/enlargement (However, since acquired nevi have a growth phase, measurement of growth alone could not be the sole determinant). It was thus clear to me that a reliable, reproducible, and portable photographic medical record would be critical for the care of my patients. During this time, I evaluated various total body photo (TBP) image sets, output formats, and clinical approaches. The two main approaches were (1) find all moles that changed compared to TBP and then determine if they were sufficiently unusual to require biopsy or (2) first identify the unusual moles on the patient and then check TBP for change). I found the clinical approach of finding every changing mole first and traditional TBP formats (slides or prints) somewhat cumbersome and inefficient – this became a problem for me to resolve in the future, after residency.

As I was nearing completion of my residency, I was able to find time between clinical activities and after hours to initiate my research efforts. I felt it was important to focus my lab efforts on projects that synergized with the pigmented lesion clinic. Given the ready access to human tissue and my interest in melanoma progression, I developed a methodology for growing a small portion of a patient's melanoma as a composite graft in a larger fragment of their own normal skin on the back of an NIH:3 mouse (with IRB and animal care approvals of course). I reasoned that I would thus be able to study melanoma progression without risk to the patient. I found that, although the grafts were successful, growth of these early tumors was excruciatingly slow. Given the short life span of the mouse, they were not likely to be useful for "natural" progression studies without an exogenous stimulant. In assessing methods to accelerate or decelerate the growth process, I became particularly interested in cytokines known to be involved in melanocytic growth. After the Keystone Conference, visiting my brother in law (David Bodine) at NIH who focused on hematopoesis, and reading Nishikawa's 1991 EMBO paper, I developed a particular fascination in the SCF/KIT pathway. Serendipitously, I was the resident in the program who was asked to evaluate the rashes developing in patients receiving SCF in an oncology clinical trial (SCF was thought to be potential value in speeding recovery from chemotherapy induced bone marrow suppression). I expeditiously obtained IRB

approval to obtain sequential biopsies from these patients. This effort revealed that SCF was able to drive melanocytic proliferation and marked HMB-45 expression.[4] In some areas, the proliferation was quite marked and vaguely reminiscent of melanoma *in situ,* further driving my interest in the potential role of this pathway in melanoma.

Early Career

As I transitioned from resident to attending, the clinic became a significant responsibility. I felt I needed to put systems in place to allow me to provide high quality, efficient clinical care, while preserving the data in a manner that would allow it to be easily queried for research or quality assurance (QA) purposes. I set up an electronic medical record (EMR) system with programmer Barry Shelton and Skip Burton for medical note management. We further developed software for the display of digital CD-based high-resolution total body photos. In the era of scarce academic resources, we had to find creative way to develop these systems. Today, there exist two companies from these efforts, Malachite Incorporated (primarily for a dermatology EMR) and DigitalDerm, Inc (a service company for MoleMapCD).

In the lab, I continued to focus my efforts on the SCF/KIT pathway, due to the generous support of the Skin Cancer Foundation, the Dermatology Foundation, the American Skin Association, the KAO Corporation (Thomas B Fitzpatrick Research Award) and a NIH R29 research award. Not only could we stimulate melanocyte proliferation in normal human epidermal xenografts with SCF, but also we found that we could also drive melanocyte loss. This loss, presumably due to apoptosis was achieved using a KIT inhibitory antibody K44.2, confirming the critical homeostatic role of SCF/KIT pathway for normal human epidermal melanocytes.[5] Xenografted nevus cells were only partially sensitive to stimulation or inhibition of this pathway, but we one strongly KIT+ congenital nevus did appear to 'melt away' with KIT inhibition *(I still hold out hope of developing a "mole be gone" or "mole away" product some day)*.

Unfortunately, we not impressed with growth inhibition from the K44.2 antibody on KIT+ melanoma in culture or in xenografts, decreasing its potential utility where it was most needed. This may be explained by KIT activating mutations in some of the lines but interestingly we did note the co-expression of SCF and KIT in some KIT+ melanoma lines. The overall expression of SCF or KIT appeared to be inversely related, quite variable, and at least partially dependent on the time of feeding and culture confluence. Intracellular co-localization was noted, often without significant surface expression, suggesting the possibility of intracellular activation. We did make stable sense and anti-sense constructs in one line and did note increased pigmentation and decreased growth with the sense construct and increased growth and decrease pigmentation with the antisense construct. We also explored truncation mutants of SCF attempting to find a molecule that would bind but not activate KIT – but unfortunately both binding and activation were lost at the same point.

During this period, the Duke pigmented lesion clinic continued to expand (*as did my family with the birth of my daughter Sarah in 1994*). As anticipated, there was (and is) a great unmeet need for this group of patients. Thanks to Skip Burton with his initial dermoscopy camera, and my good friend Harold Rabinovitz, I established clinical expertise in dermoscopy. Through networking about the optimal uses for dermoscopy, I had the opportunity to build many academic relationships, join part of growing international community, and witness the founding of the International Dermoscopy Society. This is a group of intensely committed and opened minded individuals with a common goal of enhancing and optimizing early melanoma detection. This group helped me to crystallize my approach to melanoma detection. Although clinicians all have slightly different practice habits, I believe that the most efficient method for melanoma detection is to first complete a head to toe to clinical examination to identify lesions that are distinct from that patient's average mole pattern. Then I evaluate the structure of these lesions with dermoscopy, and compare these lesions with previously acquired digital TBPs for change. If a melanocytic lesion is **g**rowing and **u**nusual, especially if **n**on-uniform, it should be excised.[6] For teaching purposes I use "smoking **G.U.N.** of melanoma detection." Personally, I believe that the **G.U.N.** approach is better than the traditional ABCDEs for early melanoma detection, especially in patients with numerous dysplastic nevi.

Current Career

The beauty of clinical practice is that you get a first hand exposure to the biologic process. It is free of dogma, it is what it is. For example, in the clinic it is clear that most melanomas develop in normal skin. However, the literature on melanoma progression might lead one to believe that melanoma always starts as a mole, then becomes a dysplastic nevus, and then turns into a melanoma. In the clinic, it is clear that almost all dysplastic nevi are benign, yet the popular press would have one believe that they are all pre-cancers and it is just a matter of time before the melanoma develops. In the clinic, it is clear that the melanocytic system is dynamic, and is capable of replenishing itself (as we see in the recovery from vitiligo) from a protected, less differentiated precursor population. However, the literature tends to portray this epidermal cell population as static and homogenous.

These clinical findings led me to question whether the differentiated melanocyte was really the cellular origin of melanoma. In graduate school, I worked with immature muscle cells, myoblasts, and studied the transcriptional activation of genes as cells differentiated into mature muscle tissue. Given the findings in vitiligo, it seemed obvious that immature melanocytic

cells must exist in the skin in order to replenish the melanocytes. My first evidence of a less differentiated melanocytic cell came from our studies on the KIT receptor; these experiments clearly delineated a KIT+ epidermal dendritic cell population that had not yet activated TRP-1.[7] KIT also identified cells in the dermis but the majority of these cells were clearly mast cells as they expressed tryptase. The proof of the existence of at least one state of a less differentiated precursor of melanocytic cells, led me to hypothesize that melanocytic neoplasias resulted from mutations in a melanocytic stem cell population; however, proving this would prove to be quite a challenge.

A Stem Cell Model

I rationalized, given stem cell biology, that I might be able to identify immature tumor stem cells in melanoma. I was able to identify a melanoma line that clearly gave rise to a secondary population of more differentiated cells (suggesting a transition from a precursor to a more mature state) and attempted to seek collaborations to help obtain the preliminary data required for an R01 submission. Interest in the idea that cell of origin for a melanoma was a melanocytic stem cell increased; however, in the end my efforts to obtain R01 grant funding failed. Despite this lack of funding, I felt the idea was too important to give up. I was able to obtain benefactor and dermatology division support and with the help of a technician continued to pursue this idea. After much effort, we were able to demonstrate that metastatic melanoma lines included a subpopulation of small, slow cycling cells with the capacity to give rise to larger more melanized cells that appeared to eventually fully differentiate.[8] The very existence of these cells supports that concept of melanoma derivation from a stem cell and not dedifferentiation from a (fully differentiated) melanocyte.

The hypothesis that a stem cell serves as the cell of origin for melanoma (and nevi) fits well with the clinical presentation. Mutations from ultraviolet light exposure (or other mutagenic event) could accumulate in the "dormant" stem cell. When called upon to give rise to melanocytes, a normal stem cell would generate melanocytes, a stem cell with a set of benign mutations would generate a benign mole, whereas a stem cell with a complement of malignant mutations would result in a melanoma. (Certainly, secondary mutations could develop in benign nevus cells leading to a secondary melanoma, but this would not be a requirement for all melanoma formation.) More differentiated tumor cells would be susceptible to immunologic destruction while the tumor stem cells would be protected. Our *in vitro* studies demonstrated that the tumor stem cells are small, weakly adherent, and viable even when floating in culture. These cells would be readily released into the lymph system from the primary tumor, and while the more differentiated cells may adhere to the lymph node, the tumor stem cells may pass on through and enter the circulatory system. The tumor stem cells may persist for prolonged periods in the body and only give rise to tumors when the appropriate conditions are present. This model accounts for latency and tumor dormancy, death from melanoma despite a strong immunologic response, the development of the majority of melanomas in skin without pre-existing nevi, and explains why sentinel lymph node biopsies (and lymph node dissections) fail to improve patient survival. It suggests that to cure melanoma, we will have to eliminate the tumor stem cells.

The stem cell model allows one to think of melanoma as an aberrant developmental system, one attempting to create melanocytic tissue from a primitive embryonic cell. This conceptual framework allows us to consider that it is the underlying mutations, not the stem cell type, that create cancer subtypes (i.e. certain mutations will give rise to melanoma, while different mutations could give rise to other types of cancers). The stem cell model also allows us to evaluate other theories. I propose that genomic instability is primarily due to aberrant expression of meiosis machinery (from aberrant differentiation towards germ cells) during mitosis in tumor stem cells (not telomere crisis or faulty DNA repair).[9] The collision of meiotic and mitotic pathways would create a fine milieu for shredding genetic material.

The stem cell model has also changed the way I look at moles in the clinic. Dermoscopy reveals a vast number of distinct mole growth patterns, patterns that have yet to be sufficiently categorized. Like coat colors of mice, these different patterns are certainly a reflection of the underlying mutation and the tissue environment in which the neoplasm develops; however, the specific mutations leading to specific patterns are unknown. In the clinic, we have the opportunity to follow the growth of these lesions, and I view them as a rich resource for the study of various mutations on stem cell melanocytic developmental biology.

Future Directions

Without current research funding, the pigmented lesion clinic is now absorbing most of my time. My patients are wonderful and I enjoy spending working with them but I must admit the melanoma detection process has now become routine. I am sure technology can be developed to take over the clinical screening process. Along this line, I hold a patent (through Duke and Digitalderm, Inc) for the use of growth parameters for melanoma detection in such potential devices. I look forward to the day when we can reliably transform a series of high-resolution 3D surface images on top of one another to resolve individual mole growth and change. Lesions not following established parameters for normal acquired nevi would be concerning for melanoma. Not only would this be an excellent research tool to clarify the dynamics of mole growth and other skin conditions, it would also create time for other research pursuits.

What does the future hold for me? I am eager to investigate tumor and nevus markers (I believe a large number of benign lesions are being over interpreted as early melanoma – possibly due to "pagetoid" features which may be normal in early nevus growth, further some melanomas are being missed), genomic instability (no question – it's meiosis!), and differentiation treatment options (probably the best way to kill these resistant stem cells is to differentiated them into a more responsive form). However, I have not been successful in obtaining the R01 grants required to sustain a full laboratory operation while also being significantly involved in patient care. This is in part due to the increasing competitiveness of the grants but it is also due to the ever-increasing clinical productivity measures set for clinicians. I suspect many other MD PhDs are also facing a similar dilemma. I believe NIH needs to consider a separate granting procedure for MD-PhD clinician-scientists if this group is to survive. My current goal is to significantly reduce my clinical workload in order to regain time to spend on research activities. I may have to think of creative ways to accomplish this goal, some that may take me out of academic medicine and possibly out of melanoma research, but wherever this leads me, I plan to have fun and continue to challenge dogma whenever possible. I am proud of the strength of pigment cell and melanoma research nationally and internationally and I am sure that the future will be bright.

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