

Profiles and Legacies

Farming Cells to Rebuild Skin and Melanoma

Meenhard Herlyn

Correspondence to: Meenhard Herlyn; The Wistar Institute; 36th Street at Spruce; Philadelphia, Pennsylvania 19104 USA; Tel.: 215.898.3950; Fax: 215.898.0980; Email: herlynm@wistar.org

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HISTORICAL PERSPECTIVE

The early years. The most difficult decision in my adult life was whether I should become a farmer or not. The lonely farm in the Northwestern corner of Germany next to the North Sea was filled with dairy cows, horses, pigs, chicken, cats, dogs, pigeons and rabbits, and I have wonderful memories growing up among the animals and working the fields. When I was in my second year of veterinary school in Hannover, my father became suddenly ill and I rushed home to help out. After a few months of agony, I decided to continue my studies and we sold the farm, but the dreams of being a farmer were with me for years. Back in school, I had trouble focusing on studying and instead engaged in student activities against the university authorities (it was the rebellious year of 1968). Only a transfer to the University of Vienna saved me from becoming a totally immersed student activist. I had met Dorothee early in school but then we lost contact until the final year in school when I had gone back to Hannover. In contrast to me, Dorothee had always attended classes, prepared well for exams, and knew all along that she wanted to follow in her father's footsteps and become a scientist. Just married, we worked for six months in a rural veterinary practice, but it became clear to us that this was not our life's dream. Instead, we furnished a VW van (also called a hippie van) and drove all the way to India and Nepal. Afghanistan was even wilder those days than today. Enjoying our gypsy life, we caught a sea passage to Mombasa, Kenya, and continued the adventure through Central and West Africa until we finally crossed the Sahara and returned home almost a year after departing. Dorothee got a job in physiology and immunology at the University in Munich and I started working on my dissertation in infectious diseases and immunology. Later I focused on poxviruses. My science career did not have a smooth start in those five years because we lacked good mentors and projects. I felt I was not cut out for science but wanted to give it one more try—outside of Germany.

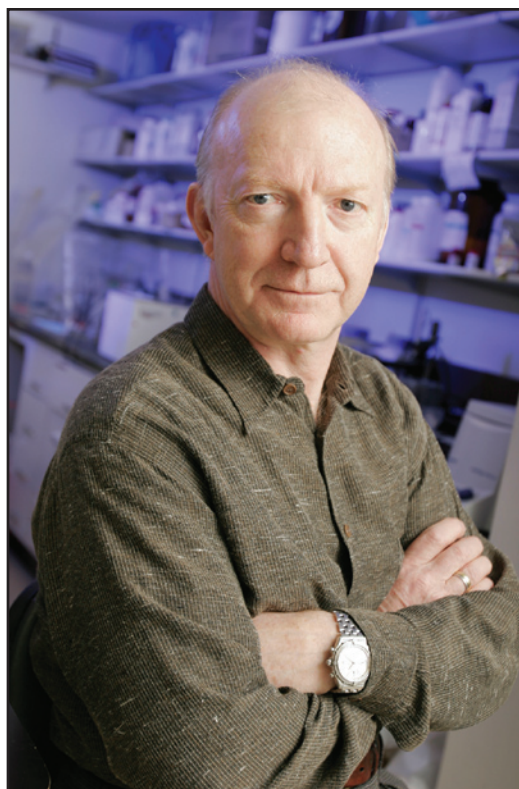
Monoclonal antibodies. Hilary Koprowski, the Director at Wistar, took us under his wings. He was a master in seizing new opportunities. Monoclonal antibodies had just come onto the scientific scene with a big splash. Within a few months we had found our niche of the next ten years. Wistar and Penn were vibrant places of research and we thrived in this rich environment, willingly following Koprowski in his search for the magic bullet of cancer therapy. We began with colorectal cancer because we had a few lines in the incubator; two melanoma cell lines served as our controls. The approach was straightforward. We injected mice with intact tumor cells, collected the spleens and fused the splenocytes with mouse myeloma cells the same way Cesar Milstein and George Kohler had published in the previous year (1975). Monoclonals were wonderful tools that told us how cancer cells differed from normal cells. We indeed thought we had found the tumor-specific bullets and that the cure was just around the corner. Those specificities much later turned out to be HLA subtypes, but they still made it exciting work. We soon established collaborations with carbohydrate biochemists, because most of the colorectal cancer antigens detected initially were glycolipids. Dorothee remained with her immunology roots and worked out the antibody-mediated cytotoxicity mechanisms using both mouse and human NK cells. The first treatments of pancreatic cancer patients were initiated at Fox Chase Cancer Center in Philadelphia. Clinically, the very first patient showed remarkable responses causing widespread excitement. But we quickly learned that the monoclonals, crudely purified from mouse ascites, induced a strong anti-mouse immunoglobulin response resulting in rapid clearance of the injected immunoglobulin from the circulation.

Koprowski's remarkable science management talent brought outstanding scientists from other disciplines into the project. Radiologists attached isotopes to antibodies, pharmacologists conjugated toxins, biochemists provided knowledge on the identity of

ABOUT DR. HERLYN

Dr. Meenhard Herlyn is Professor and Chair of the Molecular and Cellular Oncogenesis Program at The Wistar Institute in Philadelphia, PA. He also holds appointments as Professor of Dermatology and Professor of Pathology and Laboratory Medicine at the University of Pennsylvania School of Medicine. Dr. Herlyn is a member of the University of Pennsylvania Comprehensive Cancer Center, and the Biology and Molecular Biology Graduate Program at the University of Pennsylvania School of Medicine. He is also chair of the Society for Melanoma Research.

Dr. Herlyn earned a D.V.M. at the Veterinary Medical School in Hannover, Germany, and a D.Sci. in Medical Microbiology at the University of Munich in Germany. In 1976 he came to The Wistar Institute with his wife, Dr. Dorothee Herlyn (also currently a Professor at Wistar), as an Associate Scientist and began his studies of skin biology and melanoma that are documented in over 380 publications and are financed by grants from the National Cancer Institute. Dr. Herlyn is on the editorial board of six major scientific journals. He is a member of a number of professional organizations, including the American Association for Cancer Research, the American Association for Investigative Pathology, the Society for Investigative Dermatology, and the American Society for Cell Biology. He has also served on several grant advisory boards and study sections for the National Institutes of Health, the National Cancer Institute, the U.S. Army, and others. Dr. Herlyn has given more than 50 invited lectures and talks in the past two years and holds several patents related to his research findings.



the proteins and glycolipids, and our colleague Alonzo Ross cloned the first melanoma antigen. However, the clinical breakthrough for monoclonal antibodies did not come, and by the mid-eighties they had lost their appeal and people gave up on them.

Between 1977 and 1985 I performed around 1000 fusion experiments and tested in excess of 100,000 monoclonals. Some of the antibodies we made in 1977 are even today being used for diagnosis of pancreas cancer. CO-19-9 detects sialylated Lewis A, which is part of a high-molecular weight mucin circulating in serum. Others like 17-1A are still being tested for therapeutic use in colon cancer. Again others (425) lingered for years until they were humanized to reduce the anti-mouse response. We had also produced antibodies against melanoma, particularly anti-ganglioside antibodies. We even tested them in patients as therapeutics, but the results were not promising and we gave up. I left the antibody field in the mid-eighties to become a melanoma biologist because I saw too many hurdles for their clinical use, whereas Dorothee stayed with them for another ten years, generating anti-idiotypic antibodies to use as vaccines. Then when I attended my first study section in 1984, each grant on monoclonals was killed by unrelenting reviewers, scaring me off for good. Today our lab has lost its expertise in hybridoma technology. We instead use the Wistar Hybridoma Core Facility for antibody production. Monoclonal antibodies in recent years have made a spectacular comeback as therapeutics thanks to new engineering technologies. Their power as research tools is unsurpassed, and in a turnaround we are currently considering entering the combinatorial antibody field.

Melanoma progression. In 1978 Koprowski introduced us to Wallace H. Clark, Jr., a charismatic pathologist, who may be called the founder of modern melanoma pathology. His sessions were remarkable excursions into the life of an abnormal melanocyte. From simple H&E stained sections of a formalin-fixed tumor, he

made the cells become alive. He saw the tumor as a dynamic organ, in which the malignant cells control stromal fibroblasts, vessels, and inflammatory and immune cells. The concerted imbalance of normal homeostasis was dissected and interpreted by Clark and his colleague David Elder to develop new diagnostic and prognostic models that have become, despite initial opposition among their colleague pathologists, the standard for melanoma clinical and pathological assessment. Clark's views were fascinating and he captivated many including my long-term clinical colleagues David Elder, DuPont Guerry, and Lynn Schuchter. He taught us that tumor progression is a dynamic process of cellular changes. Cells within a melanocytic lesion are in a continuous flux resulting in persistence (survival) of the pre-malignant and malignant cells despite an otherwise hostile environment. He encouraged me to use the monoclonal antibodies to translate his imagination into real, quantitative data to show the dynamics of tumor-associated protein expression.

To better understand the underlying cellular processes in melanoma progression, we needed a model that could duplicate the disease process and that would allow manipulating progression. Wallace Clark enthusiastically endorsed the guinea pig melanoma model, then the Sinclair swine model, followed by the Xiphophorus fish model. Each model was dismissed because of either difficulties in reagents, high costs, or dissimilarities with the human disease. Only in the last few years have new mouse genetic models emerged that begin to reflect better the human disease. This was achieved using gene knockout techniques for p16(INK4A), p53 or PTEN, or overexpression of genes including mutated RAS, BRAF, CDK4 or others. Our focus is not only on classical oncogenes and tumor suppressor genes but also on the tumor microenvironment that drives transformation and progression. Carola Berking had shown that overexpression of growth factors in the human dermis can induce melanoma-like lesions. We are now expecting that aberrations in one or two genes with appropriate

stimulation of the microenvironment will transform melanocytes to melanoma and John Lee is leading this search for the best way.

Modeling human melanoma. Starting in the late seventies we had begun culturing human cells, first from melanoma metastases. In comparison to other human malignancies, melanoma cells from metastases can be cultured at a high success rate that exceeds 60% if the laboratory personnel have appropriate experience. We established over 350 cell lines, but those from advanced primary lesions were easier to obtain than those from biologically early radial growth phase primary melanomas, with dysplastic nevi being most difficult. The reasons are not entirely clear but the poor growth could reflect the poor growth characteristics of these cells in situ suggesting that many lesions, if not resected may not have progressed to aggressive primary melanomas but rather senesced. Today we have better culture techniques and media. However, access to primary melanomas has become more difficult because the pathologists became more restrictive of what they could spare (not so much for scientific but for legal reasons). We needed to grow normal melanocytes to have a good comparative cell for characterizing tumor antigens on the melanoma cells, and in 1982, Magdalena Eisinger's laboratory showed the field that the phorbol ester TPA is an excellent mitogen for melanocytes (but not for melanoma cells or keratinocytes). Tom Maciag followed a few years later by using growth factors and pituitary extracts instead of the phorbol ester. The melanocytes grew (relatively) rapidly but they posed a problem for us because the cells expressed in culture melanoma-associated antigens that they did not express in normal skin. The explanation for this came in the late eighties, when Istvan Valyi-Nagy showed that co-culture of melanocytes with keratinocytes down-modulated the expression of tumor antigens within three days. Keratinocytes dominate the melanocytes not just for the expression of molecules but they control growth, migration, and invasion. We still do not know the molecular mechanisms behind this phenomenon. We learned more how they do it. Ie-Ming Shih showed that we need cell-cell contact and Mei-Yu Hsu identified E-cadherin as most critical. Most recently Mizuho Fukunaga identified a matrix-cellular protein, CCN3, as critical for attachment to the basement membrane (without being a ligand itself). Much of this work was possible because Satya (Kappettu Satyamoorthy) and Mark Nesbit had established a strong infrastructure in the lab on adenoviral vectors for overexpression of genes. Later, for gene knock down we used lentiviral vectors, and Zhao-Jun Liu, Trish Brafford and Steve Kazianis were instrumental in developing the technology.

The strong influence of the tissue context for the phenotype of melanocytes stimulated us to develop additional models, including those for the normal human esophagus, colon, and breast and we learned how fibroblasts induce differentiation of endothelial cells to form microcapillary networks. The three-dimensional structures for normal skin, esophagus and breast have become staples in the lab to investigate the role of the microenvironment on normal tissue homeostasis and tumor progression.

Stem cells. Approximately in 2000, two years after Jamie Thomson published the successful culture of human embryonic stem cells, we began our work with stem cells following the ideas by Irv Weissman and colleagues that leukemias are derived from stem cells. Thus the mature, fully differentiated, pigment-producing melanocyte may not be the target for transformation to melanoma. We still have no clear answer to this but in other fields including in brain and breast tumors, it has already been demonstrated that the target for transformation is the lineage-specific stem cell. Melanocyte stem cells had been described in the mouse hair follicle but not in human skin.

Dong Fang therefore started with human embryonic stem cells, figuring that these cells would be toti-potent and could provide us with melanocytes. Culturing human embryonic stem cells took some trial and error but differentiating them into melanocytes was straight forward, surprisingly. We just had to culture the cells with all the right melanocyte growth factors. However, making the cells stop in the differentiation pathway as they become multi-, oligo-, and then uni-potential is very difficult and we are still battling with this. Once we learned to differentiate human embryonic stem cells to melanocytes we collaborated with George (Xiaowei) Xu to isolate stem cells from hair follicles. Indeed we could isolate a cell that is positive for the embryonic stem cell marker Oct 4 and that differentiates into melanocytes, neuronal cells, smooth muscle cells and adipocytes.

The same media conditions that helped us isolate precursor cells from hair follicles were then used to culture cells from melanoma lesions. Quickly we could isolate and culture cells that fulfilled the criteria for tumor stem cells: Self-renewal, differentiation, and high tumorigenicity. Melanoma stem cell populations grow as spheres. However these cultures are not purely stem cells. The true stem cell population represents only approximately 0.1 % of the total. We expect that the percentage of stem cells in tissues is 10- to 100-fold lower. This low percentage has also been found in other tumor systems.

Stem cells are fascinating cells to work with; their plasticity is far greater than we ever anticipated. Can they transdifferentiate into totally new cell types? There was big excitement when Catherine Verfaillie published in 2002 her MAP cells from bone marrow that could differentiate into many cell types. We immediately tried to differentiate bone marrow-derived MAP cells into melanocytes but we failed even after Dong went to her lab for four weeks. The Verfaillie group had difficulties repeating their own work but most recently (Science Feb. 9, p.760, 2007) has become successful again. Thus there is still hope that we can derive melanocytes from bone marrow cells.

Therapy. Our interest in therapy was sparked at an exact date: June 22, 2002, when Mike Stratton's group published on the V600E BRAF mutation in melanoma. The finding that two-thirds of all melanomas have an activating mutation in the BRAF gene electrified the field and changed the landscape of melanoma therapy. Satya and Gary Li had already developed an interest in MAPK signaling. We established collaborations with the Barbara Weber lab to confirm the high prevalence of BRAF mutations in melanoma. Suddenly there was a bone fide target that was 'druggable'. The CRAF inhibitor Sorafenib was serendipitously available, and my colleagues Keith Flaherty and Lynn Schuchter immediately seized the opportunity. After encouraging phase I/II clinical trials, a large Phase III study with 800 patients is currently ongoing using a combination of Sorafenib with chemotherapy. In the meantime several new compounds have been developed that are very specific for mutated BRAF and that show strong inhibitory activity in xenograft models. We have established productive collaborations with several drug companies, all which have provided us their drugs even before they go into clinics. Keiran Smalley is leading this effort and we are even now thinking of screening for our own compounds.

The problem in melanoma is that cells show not only activation of the MAPK pathway but also in others including PI3K/AKT, Stat3, NFκB, Notch, FAK, and likely others. We expect that inhibition of one molecule in one pathway is not sufficient for long-term therapy response. Likely, we need to inhibit two or even more pathways at the same time to inhibit melanoma cells successfully. Finding the

ideal targets (we have so far only mutant BRAF) will be challenging in the next few years, but the field is optimistic that major progress is close in finding the right targets and combinations of drugs that even advanced metastatic melanoma is curable. Can we leave the development of new drugs to companies because they have a long history of producing them or can academia contribute in productive ways? Both settings have specific strengths. Industry has outstanding chemistry and screening experience and infrastructures for medicinal chemistry. Their abilities to push the toxicity and approval requirements is unsurpassed, but industry is weaker than academic institutions in biological models and knowledge of a 'small' cancer like melanoma. Academic investigators are also strong in target identification and validation. An ideal partnership in the development of new drugs would be between industry and academia.

PHILOSOPHICAL VIEWS ON CANCER MODELS

Researchers studying cancer need models because they need to understand the mechanism of physiological and pathological changes in cells. Unfortunately, there is no ideal model and each is the result of compromises. In cancer, the ultimate criteria are whether and how a model contributes to an understanding of the disease in humans and how it helps us in human cancer prevention, detection and treatment. Due to the complexity of cancer and its various forms, cancer researchers have used widely divergent organisms, from yeast to flies, worms, fish, or mammals, as models. The strength and limitations of primitive organisms such as yeast are clear. They divide rapidly, allow easy manipulations and are superb for studying cell growth and death. However, their lack of differentiation limits their ability to mimic disease processes. Thus the value of each model is defined by its limitations.

Much progress has been made in cancer research when almost seventy years ago, the first permanent cultures of human cancer cells became available. HeLa cells continue to be a staple in many laboratories and someone has calculated that an entire freight train could be filled with HeLa cells that were grown in culture by cancer researchers. Among all human tumors, melanoma cells adapt easiest to a life in culture and several thousand permanent cell lines have been established in melanoma research laboratories over the last three decades. Our laboratory has cultured over 2000 cultures of normal melanocytes, nevus cells, and primary and metastatic melanomas.

Cultured cells from human tumors represent a selection of the entire population of that given tumor. Thus, not all cells from each tumor grow in vitro. Those that do grow undergo phenotypic and genotypic changes in culture due to their genetic instability. Still, even HeLa cells maintain many of the characteristics of the original cervical carcinoma lesion from which they were derived. In the early eighties we compared the chromosomal abnormalities of primary and metastatic melanoma cells when freshly isolated from patients, at passage two in culture, and after several months to years of culture, and found relative stability of all the major chromosomal abnormalities over time in culture, although there were some shifts. Deletion of the p16 gene in over 96% of melanoma cultures is the most prominent culture artifact because the same gene is deleted in few tumors only (but often silenced). Despite some drawbacks, cultured human cells remain a prime model to study signaling pathways and to determine the functional significance of tumor-associated gene expression changes.

Cells under standard culture conditions are driven to proliferate and they continuously migrate while attaching to their self-produced

substrates. Such culture conditions result in gene expression patterns that at least for the normal cells differ from those of cells in the normal organ. The differences are major, and microarray analyses revealed more than 600 genes that were differently expressed when comparing two- versus three-dimensional culture conditions. Normal melanocytes, when incorporated into the skin reconstructs, 'magically' find their right place at the basement membrane that is developing between the 'dermis' and 'epidermis'. Their extension of dendrites and ability to transfer pigment to keratinocytes follow the same patterns as in normal skin. Thus the tissue context is critical for normal cells to function in a proper way. Experimental read-outs from two-dimensionally cultured cells often do not reflect the biology of the same cells in an organ, whereas three-dimensional growth conditions mimic the normal organ conditions much more closely. For our studies in gene functions in melanocytes, we find the three-dimensional growth conditions much more interpretable when compared to conventional cultures. Much of the past work in signaling and pathway crosstalk that has been worked out in conventional cultures may need to be revised because it does not reflect the dynamics in tissues.

Tumors represent in vivo a primitive organ with in- and outgoing vessels, stromal cells and matrix that provide the scaffolding, and inflammatory and immune cells. Thus studying tumor cells solely in monoculture may not reflect their status in vivo. Similarly to normal cells, three-dimensional structures can be established that contain besides the tumor cells fibroblasts, endothelial cells in micro-capillary networks and inflammatory cells. Can such structures replace the study of human lesions? Of course not, but the human lesions can only be maintained in a frozen or fixed state. Such lesions are our reference point to guide us in the selection of genes and pathways, but their static nature has too many limitations. Can three-dimensional in vitro tumor models replace the mouse as the main model for biological studies in human cancer? Not completely. Both the human three-dimensional cancer models and the mouse models for human cancer complement each other. Mouse models are currently the dominant model for all of cancer research. However, all too often investigators can be observed in a 'mouse trap'. They study a pathological condition in a mouse genetic model that does not reflect any pathological phenotype in humans. If interpretations from these models are difficult, then why study them? The human disease should be our guiding principle for investigations in any model.

VISION FOR THE FUTURE

The landscape for cancer research is changing. Much of our work today is technology-driven. Individual labs are hard pressed to cope with the opportunities in new technologies and at the same time to maintain an infrastructure for molecular studies, biochemistry, and biological models, many of which are very time consuming and costly. The pool of funding for single investigator grants (RO1), which has been the main source for generations of researchers, has been shrinking recently despite a surge in new investigators that came into the cancer field starting in the late nineties. It is painful to see during study sections the grants of young investigators fail time after time because they have to compete with the entire pool of established labs. The emphasis on new initiatives such as the Roadmap by the NIH also caught RO1-financed investigators by surprise and they had difficulties adapting to those new opportunities. Large labs with a diverse infrastructure can better deal with the changes in funding opportunities, although the current tightness in funding is also

affecting them. The difficulties in maintaining a balanced budget is disproportionate to opportunities in research, i.e., we cannot afford to follow all promising research leads.

How can we cope with the demands for following new research opportunities under expected tight budgets? Certainly, we can try to open new channels of funding from philanthropy or industry, but opportunities for 'orphan diseases' such as melanoma are limited. The major answer will come from collaborations and interactions between labs within one research field or between multi-disciplinary groups. We have to open our 'niche' to others and then we can expand ourselves. This is easier said than done because there are many real and perceived hurdles for collaborations, including our own hesitation to share results before publication or institutional restrictions on sharing material. NIH- or European Commission-sponsored agreements are increasing, and these cooperative networks and consortia are certainly pointing to the future of academic research. Sharing technologies and resources demands from each of us a new approach as to how we find our new roles and how we can maintain our 'niche'. The rules are not yet established nor will they be for some time in the future because all of us need to learn and adjust.

