

A Problem of Disordered Development

Neuroblastoma is a notoriously heterogenous disease. A neuroendocrine tumor, it is the most common extra-cranial solid tumor in children. It is known to spontaneously regress and disappear in infants. But it can also metastasize and develop chemoresistance, in which case the options for treatment are limited, toxic, and seldom curative. Carol Thiele, Ph.D., Head of the Cell and Molecular Biology Section in CCR's Pediatric Oncology Branch, has been studying the molecular mechanisms that determine whether neuroblastoma cells proliferate or differentiate since she joined the Pediatric Oncology Branch in 1983. Her insights have led to new therapeutic approaches and the discovery of a novel human gene that is likely to be fundamental both to tumor suppression and to normal development.

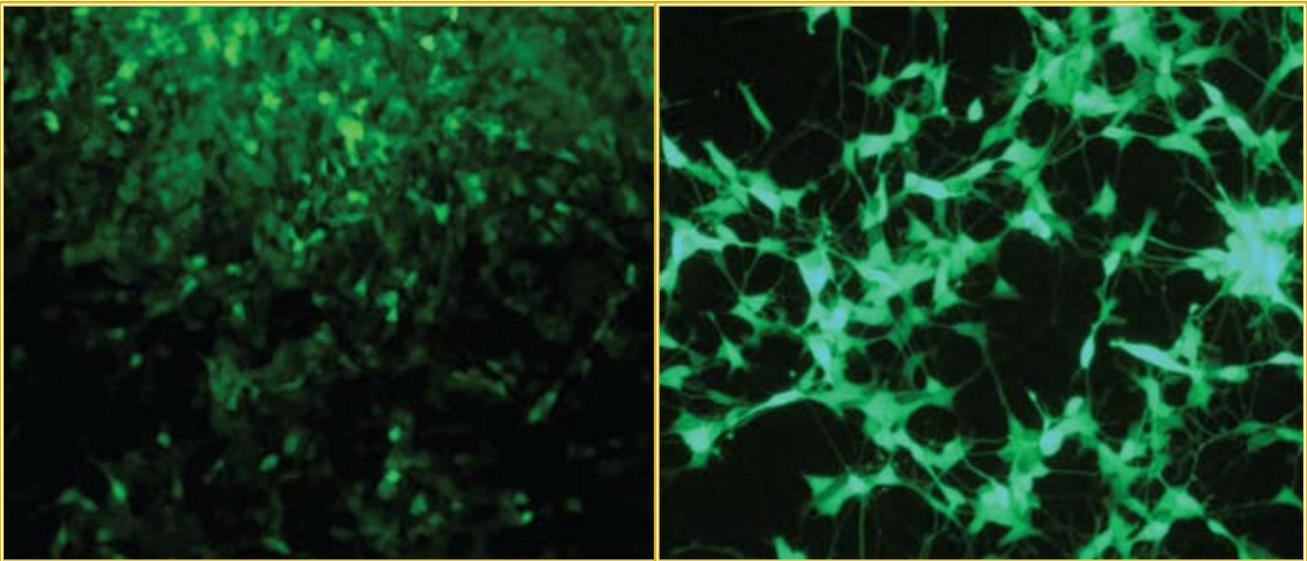
On the bulletin board next to Carol Thiele's desk is a black and white photomicrograph with four panels labeled with the kind of adhesive lettering that was painstakingly applied to such images before the advent of computerized image processing. Each panel shows a collection of neuroblastoma cells that are growing under different conditions. Thiele will eagerly take it down to show visitors the cellular phenomenon that spurred her research career.

Mark Israel, M.D., was Head of the Molecular Genetics Section

of the Pediatric Oncology Branch when Thiele was first working at the NCI as a Damon Runyon Scholar and looking for a full-time position. "Mark presented me with this phenomenon," Carol explained. "If you look at neuroblastoma cells in culture, they are round, undifferentiated, rapidly proliferating cells. But, if you simply add retinoids (a derivative of vitamin A) to the mix, they stop dividing and start to look more neuron-like." Neuroblastoma cells have a number of genetic alterations that render them cancerous. "That you

could impose growth control and induce differentiation with retinoids suggested to me that the compound was acting to bypass or complement the defective mutations." Thiele, a molecular biologist by training, was hooked into delving deeper into this process to understand its mechanisms.

The first use of vitamin A to differentiate cancer cells was pioneered in acute promyelocytic leukemia (APL) by Theodore Breitman, M.D., at the NCI in the 1980s. "They actually put retinoids into a clinical protocol to treat APL," Thiele explained, "but the response



Neuroblastoma cells labeled with green fluorescent protein, before (*left panel*) and after (*right panel*) treatment with retinoic acid. Retinoic acid treatment arrests tumor cell growth and induces differentiation.

wasn't as good as in cell culture." It turns out that only a subset of the APL patients—those with a particular chromosomal translocation—respond to retinoids. Once that was understood, retinoids became frontline therapy for those patients.

Meanwhile, other investigators were testing the effects of retinoids on different cancer cell lines without knowing how they might work. "It was definitely considered something of a touchy-feely area of clinical research," recalled Thiele. Researchers have since demonstrated that retinoids bind to a family of retinoic acid receptors, which are actually nuclear binding proteins that regulate diverse transcriptional programs relating to cell growth and differentiation. It is also now known that retinoic acid (RA) is important for the development of certain neural subtypes. But many questions still remain about how retinoic acid can compensate for the genetic alterations that occur in neuroblastoma cell lines to produce differentiation and how to optimally translate the mechanisms observed in culture into tools for the clinic.

Deconstructing Vitamin A

To follow up on the effect of retinoic acid on differentiation of neuroblastoma cells, Thiele conducted collaborative studies to define the underlying mechanisms. "We were really lucky because one of our collaborators, Pat Reynolds, was able to insert retinoic acid into a neuroblastoma clinical trial that involved high-dose chemotherapy and bone marrow transplantation," said Thiele. The patients were randomized to receive retinoic acid after the intensive treatments and the study showed that the kids who received retinoic acid had a longer event-free survival. As a result, retinoic acid is now part of the standard of care for patients with high-risk neuroblastoma. "It's invigorating and motivating for basic scientists when they can actually see how their research can contribute to the development of a clinical trial."

Continuing with their studies, Thiele and her colleagues found a subtlety in the effects of retinoic acid. They realized that at concentrations normally found in the body (much lower than the pharmacological doses), retinoic acid was turning on another receptor on the

neuroblastoma cell surface—TrkB—which, in normal neurons, is a receptor for brain-derived neurotrophic factor (BDNF). Furthermore, researchers discovered that TrkB and BDNF were expressed in neuroblastoma tumors that had poor prognoses.

BDNF is a survival factor for neurons and when they are physically or chemically disrupted, neurons respond by turning on the TrkB receptor system. "We hypothesized that the expression of TrkB may be how the neuroblastoma fights back against chemotherapy and develops drug resistance," said Thiele.

In 1996, Thiele and her colleagues first showed that BDNF and TrkB could affect the way the cells process cytotoxic drugs—the common chemotherapeutic agents vincristine and vinblastine. They then showed that if a neuroblastoma cell line is incubated with progressively higher levels of a cytotoxic drug, TrkB levels stayed the same but the cellular expression of BDNF increased as the cells became more drug resistant.

The team also took cells that had either low or high expression of TrkB and incubated them with different concentrations of BDNF and then chemotherapy. They found

that high levels of TrkB and low concentrations of BDNF had the same effect as low levels of TrkB and high concentrations of BDNF. In either case, drug resistance increased.

“So we knew that the TrkB receptor was attenuating the effects of chemotherapy in our cell cultures,” said Thiele. “The question was how.”

And the answer was the AKT signaling pathway, a major common denominator for survival factors like BDNF. Thiele and her Research Fellow Zhijie Li, M.D., adopted a strategy to restore chemosensitivity by targeting AKT with drugs. In a recent paper in the *Journal of the National Cancer Institute*, Thiele’s team has shown that neuroblastoma cells are very sensitive to an AKT inhibitor alone. But they now have data demonstrating that the combination of AKT inhibition and a standard chemotherapeutic agent is highly synergistic.

“We’re also excited by a recent phase I clinical trial of the AKT inhibitor perifosine in pediatric cancers,” added Thiele. Among the patients tested, there were four responders, which included two of the three neuroblastoma cases included in the trial. “So our hope is this kind of strategy—high-dose chemotherapy up front integrated with an AKT inhibitor—will get more kids into a complete response. We know that the complete responders have a much lower chance of relapsing over time.”

Thiele notes that they deliberately chose not to work on the translation

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(Photo: R. Baeer)

Zhijie Li, M.D., and Carol Thiele, Ph.D.

of TrkB inhibitors into the clinic. “In studying the biology of neuroblastoma, I realized there were probably several growth factors that could have a similar effect on chemosensitivity. So instead of a therapy that combines multiple specific targets, I felt that the best bet was to go downstream to a common survival signaling node like AKT.” This approach, Thiele hopes, will bypass any relapse due to alterations in a related survival factor.

Hunting for Tumor Suppressors

Alfred Knudson, M.D., Ph.D., is credited with the development of the hypothesis that somatic loss of both alleles of a gene could lead to cancer. The now-famous two-hit hypothesis later merged with the concept of tumor suppressor genes when it became clear that the development of retinoblastoma was associated with mutations in both alleles of the retinoblastoma gene *RB*. “Less well known,” said Thiele, “is that neuroblastoma is the second cancer for which Knudson postulated a tumor suppressor.”

“One of the genetic alterations in neuroblastoma cells is the loss of

chromosomal region 1p, and the kids with that signature have a very poor prognosis,” explained Thiele. Garrett Brodeur, M.D., postulated that this loss of 1p could be associated with the loss of a tumor suppressor gene. “So people have been looking for *the* neuroblastoma tumor suppressor gene for 25 years.”

Two years ago, Brodeur published the identification of a candidate neuroblastoma tumor suppressor gene, *CHD5*, in the right region of chromosome 1. And while that may have seemed the long-awaited end of the search, Thiele and her colleagues have also identified an entirely different tumor suppressor candidate in that same vicinity.

“It was the kind of discovery that arises out of knowing people around the NIH campus who are doing interesting things,” said Thiele. Her colleague, Beverly Mock, Ph.D., Deputy Chief of CCR’s Laboratory of Cancer Biology and Genetics, was sequencing genes on chromosome 4 of the mouse in a region that is syntenic (corresponds in location) with human 1p when she came across a neural gene. “Bev asked if

I would be interested in studying it,” said Thiele. “Meanwhile, Ward Odenwald, Ph.D., at NINDS had studied the same gene in *Drosophila* and thought it was important from a neurodevelopmental perspective.”

“What we knew from the fly,” explained Odenwald, “was that the gene *Castor* is expressed during nervous system lineage development

it into neuroblastoma cell lines to test its tumor suppressor activity. Liu persevered and eventually succeeded in cloning *CASZ1* and making stable neuroblastoma clones in which *CASZ1* gene expression could be induced.

“Now we think we have developed a very nice story,” said Thiele. Her team has found that *CASZ1* is deleted in 98 percent of neuroblastoma tumor

Nobody has really studied this gene in the mammalian system.

(Photo: M. Li)



Zhihui Liu, Ph.D.

but only in a very narrow time window within the neural precursor cell.” *Castor* is part of a dynamic temporally regulated network of genes that are markers for particular developmental programs.

“We all got together and talked about cloning the human homolog of *Castor*,” said Thiele. “I had a research fellow coming to the laboratory and I thought this would be a really easy project for him to get started on.” But it took the work of two talented fellows, Xuezhong Yang, Ph.D., and Zhihui Liu, Ph.D., to actually perform the critical experiments to characterize *CASZ1*.

“At the time,” remembered Liu, who had just arrived from China in 2004 to work in the Thiele laboratory, “cloning the gene was really hard.” The Human Genome Project was in progress, but that particular region was not well annotated. Furthermore, it is a large gene and even once it was cloned, it was difficult to transiently transfect

cells that have the 1p deletion. Their studies also show that transfecting *CASZ1* back into neuroblastoma cells reimposes growth control and induces differentiation of these cells. More recently, they have shown that the remaining copy of *CASZ1* in neuroblastoma cells undergoes epigenetic remodeling to suppress its expression.

Working with neuroblastoma tumor samples from the Children’s Oncology Group, they have also seen a correlation between the levels of *CASZ1* expression and disease prognosis. High *CASZ1* expression is associated with a very good prognosis and with differentiated cells when examined histologically, consistent with its putative role as a tumor suppressor.

Liu is currently writing up several papers for publication describing what they have learned so far about *CASZ1* and their plans to continue working on this gene.

They are in the process of generating genetically engineered mice with *CASZ1* deletions. “There are so many things to do,” he explained. “Nobody has really studied this gene in the mammalian system.”

“*CASZ1* must be playing some basic fundamental role in vertebrate nervous system development,” added Odenwald. “In all the vertebrates we have examined, there are remarkable pockets of sequence conservation—both in the DNA binding domain and outside the open reading frames.”

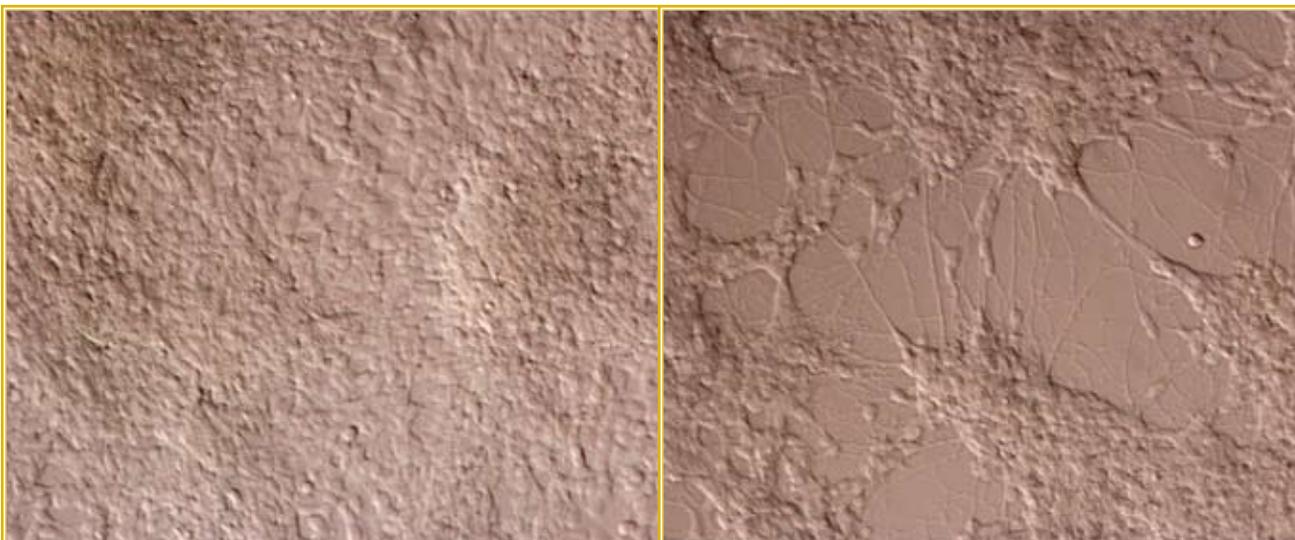
“It is a fun project because we can bring a lot of NCI resources to bear on studying this completely novel human gene,” said Thiele. “We have worked with the CCR’s Office of Science and Technology Partnerships to develop antibodies against *CASZ1* and with the NCI Core Facilities in Frederick to identify *CASZ1* interacting proteins.” It also turns out that there are *CASZ1* mutations in the germline, so Thiele is working with her colleague in the Pediatric Oncology Branch, Javed Khan, M.D., Head of the Oncogenomics Section, and Stephen J. Chanock, M.D., Chief of the Laboratory of Translational Genomics in NCI’s Division of Cancer Epidemiology and Genetics, to determine the frequency of the polymorphisms and functionally analyze them.

Putting It All Together

Intriguingly, retinoic acid turns on *CASZ1* expression in neuroblastoma cells. But researchers are still a long way from understanding how retinoic acid differentiates these cells.

“Our biggest limitation,” noted Thiele “is not having access to good

(Image: C. Thiele)



Induction of *CASZ1* gene expression in neuroblastoma cell lines (*right panel*) arrests tumor cell growth and induces differentiation.

There is an interaction with development and neuroblastoma that we don't quite understand.

primary tumor tissue because of the rarity of the disease. I value *in vitro* models, but we need to develop more physiologically relevant *in vitro* models. That would also help with drug discovery." She also noted that although there is one transgenic mouse model of neuroblastoma, it is specific to one particular subtype of the disease.

In collaboration with her colleague Chand Khanna, Ph.D., Thiele and a Clinical Fellow, Amy McKee, M.D., were able to develop an orthotopic model of the disease by placing neuroblastoma cells under the adrenal fat pad in mice. "Amy was able to put as few as five cells into a fat pad and recapitulate the tumor. So these cells are highly tumorigenic."

On the other hand, there is intriguing epidemiological data to suggest that many potential neuroblastomas resolve themselves during the course of development. When doctors have autopsied

the adrenal glands of children for other reasons, they find wreaths of neuroblasts that look for all intents and purposes like neuroblastoma. The incidence is about one in 500 children. But the incidence of neuroblastoma itself is only one in 500,000, suggesting that the body is mostly capable of regulating developing cells that go awry. Furthermore, in children under one year of age, very dramatic cases of neuroblastoma appear and then spontaneously regress. As long as there are no bone metastases, it seems that the skin lesions are able to resolve on their own.

"There is an interaction with development and neuroblastoma that we don't quite understand," said Thiele. She cited additional evidence from a screening procedure instituted in Japan. "Neuroblastoma was one of the first tumors to be screened," Thiele explained. It was discovered that in children with neuroblastoma, catecholamines

were secreted in the urine. As a result, the Japanese were screening every infant for the disease. "The idea was to catch the disease as early as possible," said Thiele. However, these very early cases would simply go away and there was no decrease in the incidence of late-stage disease, suggesting that they were seeing two distinct diseases over time.

"For me it is really fun to see how many people have gotten into this field," said Thiele. "When I first entered the field, the neuroblastoma scientific meetings included maybe 30 people. Now, at this year's meeting on Advances in Neuroblastoma Research in Stockholm, there were over 500 people." Among the attendees was Nobel Laureate Elizabeth Blackburn, Ph.D. "She really learned about neuroblastoma. I welcome great minds getting really interested in unraveling the enigmatic biology of neuroblastoma. I know this will be key for us to develop more effective and less toxic therapies."

To learn more about Dr. Thiele's research, please visit her CCR Web site at <http://ccr.cancer.gov/staff/staff.asp?Name=thiele>.