International Science Academy

San Francisco 2005

dkfz. Heidelberger Life-Science Lab


POWELL AND MARKET
21

Markus Als — Anna Baum — Tim Böltken — Sibylle von Bomhard
Christoph Fischer — Clarissa Fischer — Elisabeth Fischer
Stefan Gerhardy — Monika Gessat — Alexandra Kahn
Alexander Koch — Annemarie Lüdecke — Philipp Rauch
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The academy participants near the Golden Gate Bridge

Editorial

California Dreaming ...A Dream Come True!

The second group of "San Francisco Academicians" has safely returned home, full of inspiration and excitement – a dream come true, what a trip!

Could we have imagined, when the whole thing started back in 2002, that the project would become so successful. Hard work coupled with perseverance, sharpness, an open mind, lots of faith, and good friendship made this dream come true. Of course, there's nothing wrong with dreams that don't come true, as long as some of them do – we all need dreams, we always do!

I am particularly grateful that Californians are continuing to welcome our German students. As a German-Californian myself, I've always encouraged the two worlds to meet and tried to foster mutual understanding. With the traumatic experience of 9/11 we Americans became concerned with balancing the need for tighter security yet maintaining intercultural exchange on our principles of freedom and tolerance, which did suffer initially, as testified by the decline in foreign students and scholars enrolling at U.S. institutions of higher education – meanwhile this trend has been reversed and we have become reassured that our system has prevailed and proven its merits.

The groundbreaking first academy remains an ongoing success story – all students of the SFA 2003 are making big strides towards their science careers, most having completed their undergraduate studies, and now moving on to graduate school to become physicians, biologists, physicists, mathematicians, and even one lawyer; some have received scholarships and honors and have become members of scientific societies – and one, Samuel Bandara, is now heading back to Stanford for a six-month internship, to the same lab he stayed at during our 2003 Academy. Two short biographies were published[1] resulting from personal encounters with Nobel Prize winners in the summer of 2003 – Sam chatted with Paul Berg in his office down the hall at Stanford Medical School and Michael had lunch at CalTech in Pasadena, with David Baltimore sitting at the same table. Many lab results from the research conducted by our academicians were incorporated in scientific publications. The group still meets regularly and maintains close friendship.

The first project report was a professional production which we all worked very hard for, and are proud to see positively received not only by the local scientific community, but beyond. This has encouraged a continuation of the program and will continue to inspire the next SF Academy in 2007 – to make more dreams come true. This year's project report follows in a good tradition and is a dignified production. Particular thanks to Christoph Fischer, Martin Schorb, and Monika Gessat who arranged this year's academy and accompanied the group to the Bay.

SF Academicians: congratulations to your admirable success, your young professionalism, and unwavering endurance; the positive experiences you've made justify my involvement and pride. Wishing you good luck in the future and endless excitement in discovering the (New) World. May your curiosity be nourished, your efforts and talents perceived and recognized, and you be blessed with lasting friendship.

Yours, Ted

Theodor C. H. Cole, *Consultant to the SFA*

[1] Samuel Bandara (2004) Paul Berg. Biologie in unserer Zeit **34**(2):122
Michael Breckwoldt (2004) David Baltimore. Biologie in unserer Zeit **34**(3):194

Geleitwort

Mit großer Begeisterung berichten die jungen Teilnehmerinnen und Teilnehmer über die Science Academy 2005 in San Francisco, die bereits zum zweiten Mal in der faszinierenden Stadt an der amerikanischen Westküste stattfand. Das *Heidelberger Life-Science Lab* orientiert sich an Superlativen: Die Bay Area hat die höchste Dichte an Bio- und Hightech-Unternehmen weltweit und lockt darüber hinaus mit den herausragenden Universitäten Berkeley und Stanford, in denen exzellente medizinische und naturwissenschaftliche Forschung betrieben wird.

Viel Vorarbeit war für das ehrgeizige Projekt nötig: Die wissenschaftlichen Aufgaben wollten in Theorie und Praxis vorbereitet sein, die Teilnahme an Sprachkursen diente der Verbesserung der Kommunikationsfähigkeit, man bemühte sich um Sponsoren, hatte die Reise zu planen – alle Beteiligten erlebten anstrengende Monate, die eine Menge Eigeninitiative erforderten. Allen, die diese Veranstaltung möglich gemacht haben, gilt unser besonderer Dank.

Die insgesamt 17 Abiturientinnen und Abiturienten, die an der Academy teilnahmen, und ihre drei Betreuer beschreiben nun auf beeindruckende Weise „ihre“ Science Academy 2005 in einer Broschüre. Sie präsentieren sich und ihre wissenschaftlichen Projekte, die sie in den Laboren vor Ort durchführen konnten. Sie stellen die akademischen Lehrer in Berkeley und Stanford vor, die als ihre Gastgeber fungierten und sie in ihre „Labs“ einluden.

Die Wissenschaft lebt vom begeisterungsfähigen Nachwuchs, der schon in jungen Jahren Aufgaben und Verantwortung übernimmt und grundlegende Fragen stellt. Solche besonders motivierten jungen Menschen kommen im *Heidelberger Life-Science Lab* zusammen. Alle, die sich an dieser Initiative und den hoffentlich zahlreichen künftigen Summer Academies beteiligen, sollten auch in Zukunft ihre Neugier und ihren jugendlichen Forschergeist bewahren. Gerne werden wir sie dabei nach Kräften unterstützen.

Prof. Dr. Otmar D. Wiestler
Vorstandsvorsitzender und Wissenschaftlicher Vorstand
der Stiftung Deutsches Krebsforschungszentrum, DKFZ

Translation With great enthusiasm the young participants report on the Science Academy 2005 in San Francisco, which for the second time took place in this fascinating city on the West Coast of the United States. The *Heidelberg Life-Science Lab* is oriented along superlatives: the Bay Area not only features the highest conglomeration of bio- and hightech firms worldwide but is also attractive through its outstanding universities in Berkeley and Stanford, where excellent medical and scientific research is conducted.

Extensive preparation was necessary for this ambitious project: the scientific tasks ahead were to be theoretically and practically well prepared, language training helped to improve communication skills, sponsors were sought, the trip needed to be organized – all participants experienced strenuous months requiring considerable personal initiative. We express our special gratitude to all who made this possible.

In this brochure, the 17 high school graduates participating in the academy along with their mentors impressively present “their” Science Academy 2005. They introduce themselves and their academic professors in Berkeley und Stanford who hosted them in their labs, and describe their scientific projects.

Science is nourished by an enthusiastic young generation, ready for engaging in tasks and assuming responsibility, and asking questions of fundamental importance. Such exceptionally motivated young people meet within the *Heidelberg Life-Science Lab.* All who have become part of this initiative and who intend to participate in the hopefully numerous future summer academies should attempt to retain curiosity and a juvenile explorative spirit in the future. Gladly will we perpetuate in supporting them in their endeavors.

Prof. Dr. Otmar D. Wiestler,
Chairman and Scientific Director of the Management Board
German Cancer Research Center, DKFZ

The Kristin Scott Lab in the summer of 2005

Foreword

It has been a privilege to be a faculty participant in hosting the *Heidelberg Life-Science Lab's* Summer Academy both in 2003 and 2005. I wholeheartedly encourage students and faculty to continue to support this wonderful program.

The students of the *Heidelberg Life-Science Lab* are absolutely the best. I have been extremely impressed with the students who have worked in my lab. They are intelligent, full of curiosity, and very excited about science. They arrive eager to do experiments, and quickly become immersed in the laboratory. It has been a joy to have them here in Berkeley!

My lab has benefited both professionally and personally from the Heidelberg Summer Academy. My graduate students and postdoctoral fellows have had great fun teaching the Heidelberg students how to do experiments and telling them about our research. All of us have enjoyed discussing and comparing science, politics, and life in California and Germany.

The Heidelberg Summer Academy is a unique experience that benefits all of us. It is so important to support the international exchange of students in order to build long-term scientific and social contacts critical for scientific progress. On top of that, the enthusiasm of young scientists is contagious and energizing! I am looking forward to meeting more students in 2007!

Kristin Scott, Ph. D.
Assistant Professor
Department of Molecular Biology
and Helen Wills Neuroscience Institute
University of California, Berkeley

The academy participants Christoph Fischer, Alexander Koch, and Martin Schorb

Preface

Two years after the groundbreaking success of the first International Science Academy to San Francisco 2003, a second group of enthusiastic high school graduates from the *Heidelberg Life-Science Lab* made it to the Bay for a scientific 'dream come true'.

From July 25 to August 19, twenty academy participants had the chance to gather practical experience in doing research internships at the renown University of California at Berkeley and Stanford University, in research laboratories ranking among the leading in their fields – and to experience the academic environment and incredible atmosphere of an American university campus. The four weeks spent in California were both scientifically and personally enriching and though time went flying by way too fast, unforgettable impressions and lasting friendships remain.

The San Francisco Academy cannot be described or understood by only referring to the actual time spent in the labs in the United States. Plenty of tasks had to be accomplished in advance – mainly by the academy participants themselves – to make this dream come true. Often this resulted in a feeling of helplessness in the face of all the problems and obstacles that had to be overcome. Two arduous years were necessary to prepare this academy. After a new group of interested and motivated students had gathered who were willing to engage in the effort of organizing their internships, we needed to find scientists in Berkeley and Stanford who were willing to host us. Especially during this period the planning suffered a lot of setbacks. However, frustration only led to greater determination, knowing that it had successfully been done before – and finally we managed to find labs for each participant. Once the offered lab internships were confirmed, this certainty provided a new wave of motivation to organize the necessary framework that had been left out until this point. Flights and housing had to be booked, insurance contracts signed, and all the other trifles and details which initially one tends not to take as seriously, but turn out to be detrimental in the end in planning such a trip. Of course the experience gathered in planning the academy in 2003 was extremely helpful, but still, every new planning process has its own tricks and obstacles that need to be overcome and new problems (and several previously unsolved ones) pop up in unexpected moments.

The most pertinent of the long list of unsolved problems remained the financing. All International Science Academies are coupled with high costs and San Francisco makes no exception. Fundraising did not prove any bit easier than back in 2003 and the German economy referred us to the hopefully better times yet to come. In the end, the whole project was once again completely financed by its participants and their families.

Looking back now it seems clear that this investment was well worthwhile and every cent (or penny) wisely spent. Once again the International Science Academy San Francisco has turned out to be an enormous success and a great experience for everyone involved. The students were able to choose their internships from a wide range of topics that suited their interests: anywhere from biology (neurobiology, cell and molecular biology, genetics) and medical sciences (pediatrics, human genetics, pharmacology, radiology, and stem cell research) to quantum physics and computing.

On the following pages you will have an opportunity to learn about the fascinating projects and the actual work and achievements of the academy participants as well as their personal impressions. We hope that a spark of enthusiasm jumps over to you and you will be able to feel some of the ardour and excitement of discovery our participants felt during this academy.

Acknowledgements

Our sincere thanks goes to Monika Gessat who has been mentoring this academy for the second time. You have been a great help for this program and we are very sorry you decided to leave the *Life-Science Lab* after this academy. Special thanks also to Theodor C. H. Cole, without your support this publication would not have been possible; your enthusiasm and commitment has been a great motivation for us as much as for all academy participants. Furthermore we would like to thank all the mentors and staff of the *Heidelberg Life-Science Lab*, especially Dr. Thomas Schutz, Dr. Katrin Platzer, Hannah Novatschkova as well as the *Alumni Association of the Heidelberg Life-Science Lab*, especially Dan Choon and Michael Breckwoldt.

Christoph Fischer and Martin Schorb
Student Mentors and Coordinators of the SFA 2005

University of California at Berkeley

The University: The campus of UC Berkeley is ideally located right in the middle of town on a westward slope overlooking San Francisco Bay with beautiful sunsets over the Golden Gate Bridge. The often European-style buildings are widely spaced between lush-green lawns and recreation areas where students often sit and chat under the Californian sun. The tallest building on campus, and a landmark clearly visible from San Francisco, is the Campanile (1914), built after the original in Venice.

Seal of UC Berkeley

The university was founded in 1868, and the present campus inaugurated in 1873, with an enrollment of 191 students. Today, roughly 34,000 students are enrolled in some 40 academic programs, with around 2000 active instructional faculty. At the undergraduate level, there is a highly competitive 25% admissions rate. About 800 doctoral degrees are conferred each year, of which roughly 10% are in biology. The library provides 9 million print volumes, one of the finest research collections in the United States.

The Campanile

Nobel Laureates: Throughout the years 18 Nobel Prizes have been awarded to Berkeley faculty members – presently there are seven active Nobel Laureates among the faculty. Ernest O. Lawrence, who developed the first cyclotron, was awarded the Nobel Prize in 1939 – today his name is commemorated in the Lawrence Berkeley National Laboratory.

Geography and Safety: The particular combination of soft rolling hills with the soft rolling waves of the picturesque San Francisco Bay and its passage to the Pacific, spanned by the architectural masterpiece of the Golden Gate Bridge, marks a breathtaking geographical setting, particularly as viewed from the upper Berkeley campus, and particularly from the Campanile. Some think that the Bay Area is not the most secure place, not only in geographical terms. Prudence and appropriate safety are mandatory in everyday life (as in any big city, after all it's a 3-million metropolis) – but it is also true that the San Andreas fault runs right underneath the Berkeley stadium. Fortunately, there was no damage done to the campus by the big earthquake of 1989, which took down part of the Oakland Bay Bridge. Earthquake drills and emergency training programs are commonplace to California... and an essential part of all lab safety instructions... so, watch yourself, and you'll be alright... you're 'living on the faultline'.

The University Library

Tuition: And now, how 'bout finances? Well, at an average nonresident tuition of about $25,000 per annum, it ranks close to private universities; however, resident tuition (taxpayers) is considerably less, at around $4,000 – and most students in need receive financial aid, on the average covering at least half of their tuition. So, while German universities are still largely non-tuition and tax-financed, American universities are partly so, but the fact that students have to pay their own fare, means that American universities are more customer-oriented and try to offer according services.

Inside the library

Leisure: In order to relax from studying, the Berkeley hills are attractive for hiking and jogging under *non-native* huge eucalyptus and palm trees (beware of the *native* poison oak) – blue jays and squirrels abound, but luckily there are no mountain lions or bears (and very few rattlers, if any!). The Berkeley Botanical Gardens is an oasis of peace and contemplation – and, of course, a nursery for many native Californian and exotic plants. The Bay Area's got everything from music to the arts, how 'bout a symphony concert at Hertz Hall, or a visit to the Museum of Paleontology, Natural History, Anthropology, or Modern Art – or a "Golden Bears" basketball game... or if you just feel like sitting down for a good meal, there are plenty of excellent ethnic restaurants (how 'bout Ethiopian), ...or healthy, organic American food!

Our San Francisco Academy has been very fortunate to be hosted by the friendly and generous Berkeley faculty – it has been an exciting experience for each one of us to meet you and your staff.

http://www.berkeley.edu

Theodor C. H. Cole

Stanford University

In 1876, former California Governor Leland Stanford purchased 650 acres for a country home and began the development of his famous Palo Alto Stock Farm for trotting horses. He later bought adjoining properties to bring his farm to more than 8,000 acres, land that became the Stanford campus.

Hoover Tower

On October 1st, 1891, Stanford University opened its doors after six years of planning and building. Jane and Leland Stanford established the university in memory of their only child, Leland jr., who died of typhoid fever at 15. Within weeks of his 1884 death, the Stanfords determined that, because they no longer could do anything for their own child, they would use their wealth to do something for other people's children. "The children of California shall be our children." Leland Stanford devoted to the university the fortune he had amassed, first by supplying provisions to the mining for California gold and later as one of the "Big Four", whose Central Pacific Railroad laid tracks eastward to meet the Union Pacific and complete the transcontinental railway. They settled on creating a great university, one that, from the outset, was untraditional: co-educational, in a time when most were all-male; non-denominational, when most were associated with a religious organization; avowedly practical, producing "cultured and useful citizens" when most were concerned only with the former. Jane and Leland Stanford determined that the character of the Main Quadrangle and the Palm Drive main entrance would reflect a grand, formal style: California Mission-inspired buildings of local sandstone and red-tiled roofs surround a cloistered quadrangle, the so-called Main Quad, with Memorial Church as its focal point.

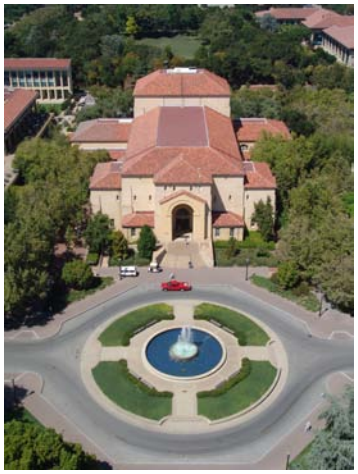
A Teaching and Research University

Seal of Stanford University

Presently, there are approximately 7000 undergraduate and 8000 graduate students inscribed, instructed by about 1700 faculty. With an approximate 7 to 1 student-to-faculty ratio, Stanford emphasizes close interaction with faculty. Of the seven schools at Stanford, three award undergraduate degrees: Humanities and Sciences, Earth Sciences, and Engineering. Working under the supervision of faculty, undergraduates may join laboratory teams on campus, research a topic through Stanford's extensive library or travel to field sites around the world to complete an independent project. Study opportunities are offered in overseas to participate in internships and research projects. Approximately 90 percent of undergraduates receive their degree within five

years. A Stanford student needs a budget of $42,000 a year (tuition $32,000 plus costs for room and board, books etc.). In order to allow admittance to qualified students without regard to their ability to pay, 74 percent of them get financial aid by the university. 94 percent of undergraduates live on campus as do 58 percent of graduate students and 30 percent of faculty members. The housing system for students consists of residentials with traditional dormitories, apartments, suites and residences. Faculty and staff live in owner-occupied housing units or in rental units on the campus.

Graduate students can pursue postbaccalaureate degrees in seven schools: Business, Earth Sciences, Education, Engineering, Humanities and Sciences, Law and Medicine. Exchange programs with the universities at Berkeley and San Francisco complement the courses.



The Quad

Stanford researchers have contributed to many developments over the years, 25 percent of Stanford faculty have been awarded the Nobel Prize since the university's founding. Among the living Nobel laureates are:

- Arthur Kornberg, Nobel Prize in Physiology or Medicine, for the discovery of the mechanisms in the biological synthesis of RNA and DNA
- Paul Berg, Nobel Prize in Chemistry, for his research on recombinant DNA
- three physicists at the Stanford Linear Accelerator Center (SLAC) were honored for their work they conducted at SLAC on elementary particles
- three professors in the Department of Physics
- and eight are faculty in economics or business

The Stanford Medical Center

The Stanford Medical Center includes the Stanford School of Medicine, Stanford Hospital & Clinics and the Lucile Packard Children's Hospital.

The Stanford Medical Center is well known for its breakthrough technologies and treatments, including the first synthesis of biologically active DNA in a test tube, the first construction of a recombinant DNA molecule containing DNA from two different species, discovery of immune response genes and development of the microarray technology that allows researchers to see at once which genes of the thousands present in a cell are switched "on."

We are grateful for this second International Science Academy, made possible by the kind support and welcoming acceptance of our hosting professors and their staff at Stanford. Our young eager students have enjoyed and absorbed every bit of it, and have learned for life!

www.stanford.edu

Monika Gessat

Redwoods at Stanford and the Peninsula

Palo Alto

"The Big Tree" in the logo of Stanford is a familiar sign to every visitor of Stanford University. Palo Alto means "high tree". It was the name given to the place nowadays known as the City of Palo Alto and the campus of Stanford University. In November 1769, Captain Gaspar de Portola's expedition to fortify the port of Monterey for Spain found instead San Francisco Bay. The party camped on the banks of San Francisquito Creek near a giant Coastal Redwood that later travelers came to call El Palo Alto. The twin-trunked giant tree was visible for miles. The little town that started to grow across El Camino Real took the name Palo Alto, and the redwood tree became the symbol of the city and later of Stanford University.

The Redwoods

Among the conifers there is a family of trees known as Taxodiaceae, among them the redwood trees. Redwoods have grown and prospered in many areas of Europe, Asia, and North America since the warm Paleozoic Era over 160 million years ago. Changes in climate have restricted their natural range and eliminated all but three genera of redwoods. There are reasons to believe that they represent the end points of three long lines of separate evolution. Most of the species in those lines are now extinct. The three which remain are the ones known as *Metasequoia*, *Sequoia*, and *Sequoiadendron*. Each of these genera nowadays consists only of one single species: Giant Sequoia *(Sequoiadendron giganteum)*, native to the western slope of the southern Sierra Nevada mountain range in California; Dawn Redwood *(Metasequoia glyptostroboides)*, native to a remote area of central China; and Coastal Redwood *(Sequoia sempervirens)*, native to a narrow belt along the Pacific Coast from southern Oregon to central California, extending not more than 80 km inland.

The Giant Redwood

The entire native range of Giant Redwood is an area only 400 km long by 25 km wide on the western slope of the Sierra Nevada of California. It is only found at elevations between 850 and 2700 m. Its ecology demands moist soil, it gets most of its water from snow that melted and soaked into the ground. Seedlings and young trees up to 400 years old need abundant light. If conditions are right for centuries, a giant grows up. Giant Redwoods are the world's most massive trees. The Giant Redwood reaches its maximum height in about 800 years, and if it lives on to be up to more than 3000 years old the subsequent growth is radial. Though not as tall as a Coast Redwood, Sierra Redwoods have larger trunk diameters, rightly appointed Giant Redwoods. – At Stanford only one young tree has grown. It stands nearby a group of several Coastal Redwood trees (Fig. 1). The tree is easy to discriminate from other redwood species by the scaly, awl-shaped leaves (Fig. 3, *left*).

Fig. 1. The left tree with a round top is a young Giant Redwood, within a group of Costal Redwoods

The Coastal Redwood

Coastal Redwoods (Fig. 2) are the world's tallest known trees (next to certain *Eucalyptus* species in Australia). Average mature trees stand from 60 to 75 m tall, some have been measured at more than 110 m tall. Coastal Redwood needles are stiff, dark green, which stand out from the branches in so-called distichous arrangement (Fig. 3, *right*).

Fig. 2. Coastal Redwood

The Coastal Redwood grows in a small belt along the mountains of the coast line form Oregon to Big Sur. Here, the temperatures are moderate year-round, and heavy winter rains and dense summer fog provide the trees with water they need. Because less rainfall occurs during summer in California, redwoods are dependent upon this fog to get enough moisture to grow and survive. Studies show that fog supplies 13–45% of the total water used annually by redwoods, and that redwood leaves can even absorb some water directly from the fog. In addition, fog blocks the evaporating rays of direct sunlight, reducing the amount of water that redwoods lose by transpiration. When fog comes into contact with redwood trees, it condenses into liquid water and drips off the foliage onto the ground. In this way, redwoods strip water from the fog and drip it onto the ground, where it is used not only by the redwoods but by other plants as well.

Fig. 3. Branchlet of Coastal Redwood with needles in two alternating rows *(left)*; the small scaly leaves of Giant Redwoods, appressed all around the stem *(right)*

In 1850, 2,000,000 acres of redwood forest extended from central coast of California to Oregon. Today, only 80,000 acres remain. Following the discovery of gold in 1848, San Francisco became a key port of entry for miners, and the population multiplied six-fold within four years. The Coastal Redwood trees supplied the building material for the rapidly growing city. Accessible hillsides were stripped off their trees, only some woods in steep terrain were not logged. For instance, Muir Woods in Marin County north of San Francisco were spared because of the steep slopes of Redwood Canyon. To enforce the protection of the trees along Redwood Creek, William Kent and his wife purchased the woods in 1905 and donated the land to the federal government. Kent requested the land not to be named for himself but after conservationist and naturalist John Muir.

Literature

Harvey H. Thomas (1978) The Sequoias of Yosemite National Park, Yosemite Association

Monika Gessat

The Hosting Professors

Gregory S. Barsh

Professor
Departments of Genetics and Pediatrics
Stanford University School of Medicine

Color variation is one of the most readily apparent differences among closely related animals, and has been studied extensively as a model for Mendelian genetics over the last 100 years. Our laboratory is interested in the mechanisms that give rise to eye, hair, and skin coloration, both as a tool for studying gene action and interaction, and because many signaling pathways used by the pigmentary system play important roles in human development and disease.

http://barshlab.stanford.edu/

Mina J. Bissell

Distinguished Scientist
Life Sciences Division
Lawrence Berkeley National Laboratory

The laboratory is interested in the role of extracellular matrix (ECM), its receptors and its degrading enzymes as central modulators of tissue-specific gene expression, signal transduction, apoptosis and cancer. We use the mammary gland of mice and humans to study the above processes in breast and breast cancer. Given that all tissues share the same DNA sequences, the question of how tissue specificity is maintained and how it goes awry in cancer is a central problem in biology. Our laboratory has developed "designer microenvironments" in three-dimensional (3D) cultures to study the role of the microenvironment and tissue architecture in normal and malignant cells and how these external factors shape signal transduction in tissues.

http://www.lbl.gov/lifesciences/BissellLab/main.html

Chang-Zheng Chen

Assistant Professor
Department of Microbiology and Immunology
Stanford University School of Medicine

We study the genetic networks controlled by regulatory RNAs, such as microRNAs and small interfering RNAs, and the roles of these RNAs in modulating the development, function and pathogenesis of vertebrate immune systems. Areas of current interests include: miRNAs' roles in the maintenance, self-renewal, and differentiation of hematopoietic stem/progenitor cells; miRNAs as diagnostic and therapeutic targets for leukemias and other blood disorders; miRNA-mediated posttranscriptional gene regulatory networks and their interactions with transcriptional networks.

http://mirna.stanford.edu

Kenneth Downing

Senior Staff Scientist
Life Sciences Division
Lawrence Berkeley National Laboratory

The assembly and disassembly of microtubules at particular times are essential steps in the cell cycle. These processes are closely regulated, and interference with the regulatory mechanisms can lead to cell death. These properties have made tubulin both a fascinating specimen for biophysical studies and a useful target for anti-cancer drugs. It is important to understand how tubulin molecules interact with each other as well as with a large number of other proteins and ligands in these activities in order to have a full understanding of the life of the cell. As a first step in this direction we have determined the structure of tubulin by electron crystallography.

http://www.lbl.gov/lifesciences/labs/downing_lab.html

Rona G. Giffard

Professor
Department of Anesthesia
Stanford University School of Medicine

Brain injury from stroke, head trauma, and chronic neurologic degenerative diseases is a major cause of morbidity and mortality in this country. We are particularly interested in the cellular consequences of brain injury. To study this problem we work with primary cultures of neurons and astrocytes from mice in addition to employing rodent models of stroke. Current work focuses on: the role of astrocytes in brain injury; the interaction of neurons and glia during injury; the role of heat shock response and hsp70 overexpression in neuronal and astrocytic injury and the role of oxidative stress in injury.

http://anesthesia.stanford.edu/giffardlab/

David Goldhaber-Gordon

Assistant Professor
Department of Physics
Stanford University

Our group studies simple, archetypical structures such as a quantum point contact – a narrow constriction between two electron reservoirs, which can be thought of as a short one-dimensional wire. To learn more about how electrons organize themselves in these well-studied (but still poorly-understood) structures, we either develop new measurement techniques or work with new materials in which electrons interact more strongly.

http://www.stanford.edu/group/GGG/index.html

Bruce MacIver

Associate Professor of Neurophysiology
Department of Anesthesia
Stanford University School of Medicine

We investigate the cellular, synaptic and molecular mechanisms of action of central nervous system drugs; especially barbiturates, opiates, anesthetics and other CNS depressants. Electrophysiological recording techniques and pharmacological probes are used to investigate the sites and mechanisms of action for CNS depressants. Most of our studies focus on the CA 1 area in rat hippocampal brain. Neurons in this area are depressed by anesthetics through a combination of pre- and postsynaptic actions on glutamate and GABA mediated neurotransmission.

http://www.stanford.edu/group/maciverlab/

Mark A. Kay

Professor
Departments of Pediatrics and Genetics
Director, Program in Human Gene Therapy
Stanford University School of Medicine

The goals of my laboratory are to establish the scientific principles and develop the technologies needed for achieving persistent and therapeutic levels of gene expression in vivo. While our ultimate aim is to use gene transfer to treat human disease, we plan to address basic biological questions that will be important for rational design of vectors for gene therapy applications. Towards this goal, we are working on developing new non-viral and viral vectors for gene transfer and establishing the cellular and molecular mechanisms involved in gene transduction in animals.

http://www.med.stanford.edu/kaylab/

Kristin Scott

Assistant Professor
Division of Neurobiology
Department of Molecular and Cell Biology
University of California, Berkeley

We study taste perception in the fruit fly, *Drosophila melanogaster*, to examine how sensory information is processed by the brain. We use a combination of molecular, genetic, electrophysiological and behavioral approaches to study taste circuits. Our aims are to understand how different tastes are distinguished by the brain and how taste perception is modified by experience.

http://mcb.berkeley.edu/labs/scott/

Jeremy Thorner

Professor
Department of Molecular and Cell Biology
University of California, Berkeley

Transmembrane and intracellular signal transduction mechanisms are the focus of our group, especially understanding how extracellular stimuli control gene expression, cell growth, cell morphology, and cell division at the biochemical level.

http://mcb.berkeley.edu/labs/thorner/

Gerald Westheimer

Professor
Division of Neurobiology
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The processing of visual information by the human brain is approached by psychophysical experiments, by the study of the primate visual cortex in a collaborative arrangement with the Neurobiology Laboratory at the Rockefeller University, and by models of neural circuits and brain mechanisms. Of particular interest are interactive and plastic changes associated with the response modifications that occur with attention, active perception and learning.

http://mcb.berkeley.edu/faculty/NEU/westheimerg.html

Joseph C. Wu

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My laboratory works on gene transfer and stem cell therapy in the heart. We use various reporter genes and reporter probes in conjunction with novel imaging technologies to study these molecular & cellular events. The ultimate goals are to study cardiac gene expression and stem cell survival noninvasively, repetitively, and quantitatively. My clinical interests are nuclear cardiology, echocardiography, and adult congenital heart disease.

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Salt Perception in *Drosophila melanogaster*

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Introduction

Taste is a very important sensory perception needed by animals to detect nutritious foods and avoid toxic substances. In insects, taste also influences other behaviors like egg laying and mating – female Drosophilas also have taste neurons on their ovipositors, with which they can "taste" whether they are laying their eggs into a nutritious substrate.

Taste is recognized by sensory bristles, chemosensory hairs on the proboscis and legs (even on their wings) that activate proboscis extension (Fig. 1). The bristle consists of four gustatory neurons and one mechanosensory neuron. Gustatory receptor (taste) proteins (GRs) are located within the membrane of the cell. Todate about 70 GR genes have been found in the genome of Drosophila.

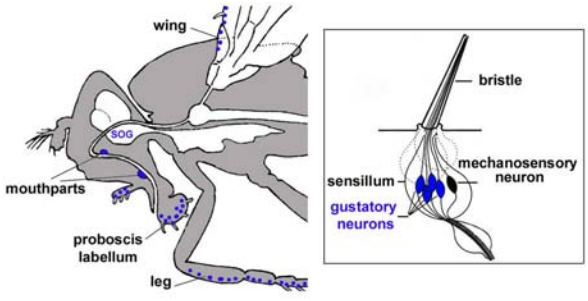

Fig. 1. *Left:* Body of *D. melanogaster* and patches of the chemosensory bristles; *Right:* Structure of a bristle

Every taste quality corresponds to a particular type of taste receptor: a sweet receptor protein recognizes sugar and compounds, a bitter receptor binds to a wide range of plant alkaloids and compounds, salty recognizes sodium and potassium ions and the sour receptor binds to hydronium ions from acids. Mammals also have a receptor for "yummi taste" – "umami" in Japanese – that recognizes the amino acid glutamate, which in nature signals a protein source. A "tastant" getting in contact with a taste neuron will only bind to a sweet or a bitter GR, but never to both.

Kristin Scott's group studies this taste system in *Drosophila melanogaster*. They investigate how taste substances are detected and transduced by the peripheral nervous system and further processed in the brain.

They created two kinds of mutants to characterize taste. In one transgenic line, specific receptor cells are fluorescently labelled by different proteins, for example by a calcium-sensitive green florescent protein (GFP) and so they are able to study alterations in membrane potential anatomically and molecularly. In the other one a diphtheria toxin (dti) is expressed in the specific taste cells. This toxin kills cells, allowing the group to study taste behaviors with flies lacking taste cells.

Drosophila has four main taste modalities: sweet, bitter, salty, and sour. Two taste receptors have already been identified. They mediate sweet (Gr5a) and bitter (Gr66a) (Fig. 2). But which receptors mediate salty and sour perception is still unknown.

Our project involved to attempt to find out the behavioral response to different con-

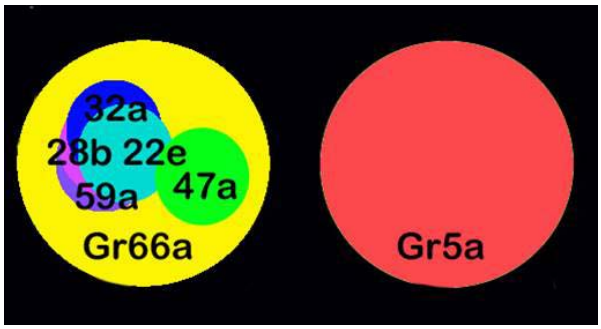

Fig. 2. Composition of Gr66a and Gr5a taste receptors. The GRs "bitter" consist of a wide range of other receptors

centrations of sodium chloride in wild-type flies and which gustatory receptors are responsible for detecting salt.

Method: Box Assay

For our behavioral experiments we used the so-called box assay. This was the most used method in our research about the behavior of fruit flies to different concentrations of salt. This method is normally used for behavioral experiments with salt, sugar, and caffeine as well as for experiments with combinations of these chemicals.

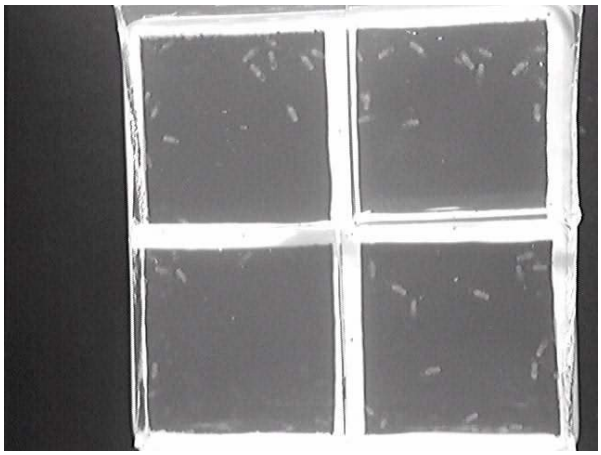

Fig. 3. Box assay

Figure 3 shows a plastic box (about 10 × 10×10 cm) covered by four agarose gel slices. Area 1 and 3 contain a certain concentration of salt, the two others (2 and 4) consist only of water with agarose, used as controls. We starved the flies over night for about 14 to 16 hours, so that they are interested in finding something to eat. The flies got knocked out by CO_2 and were filled into the box. After a ten-minute break, we started to film the flies for 30 minutes. After this we put the flies back into their vials to use them a second time with another concentration of salt after a short starvation time. The movie gets divided into 21 pictures and we were able to analyze the slide show and count the flies in each quarter. The amount of flies on both salt agarose gels slices (n_1) and the amount of flies on the control gel slices (n_2) gives us the preference index: (n_2) subtracted from (n_1) divided by (n_1) plus (n_2).

$$P_i = (n_1 - n_2)/(n_1 + n_2)$$

By using different concentrations we were able to visualize the behavioral response to different concentrations of salt. Mostly we did three trials with each concentration.

First we used wild-type flies without any DNA modifications to find out which concentration shows the most attraction and which one the strongest repulse.

First Results

Figure 4 shows our results of the different concentrations of salt and the behavioral responses of wild-type flies. There seems to be a peak at 25 mM but there is also a large variance. So it is safer to consider the 50 mM as the most attractive concentration. The most repulsive concentration is 1 M. Also, 200 mM does not show any variance, because we only did two trials, with exactly the same result. After using the 50 mM concentration you can say that, the higher the concentration of salt, the more the flies despise it. Our conclusions from this chart have been that the flies cannot detect the lowest concentration of 1 mM. The most attractive mixture seems to be at 50 mM and the most repulsive one is the 1 M concentration. The other concentrations do not show a clear-cut behavioral response.

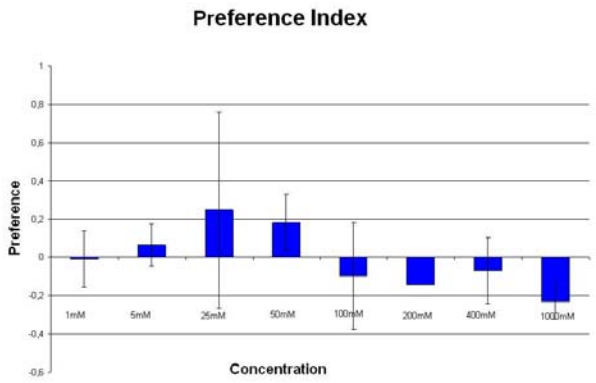

Fig. 4. The graph lists the 8 trials from 5 mM to 1000 mM. The abscissa (x-axis) shows the Preference Index, the ordinate (y-axis) lists the concentration. Negative values illustrate avoiding behavior, positive values show attractive behavior

Method: Mutant Assay

The next step was to use our results for the mutants. We only used the most repulsive and the most attractive concentrations. First we used the Gr5aDTI flies with ablated Gr5a cells. Experiments with proboscis extension showed that these flies cannot taste low concentrations of sugar and salt. We expected a different behavioral response to the 50 mM concentration than the wild-type flies, but the same one at 1 M. Second, we used the Gr66aDTI flies with ablated Gr66a cells. Experiments also proved that these flies cannot taste any bitter nor high concentrations of salt. So we expected the same behavior to 50 mM, but a different response to 1 M salt, than the wild-type flies. If our expectations are true, the Gr5a und Gr66a cells are involved in detecting salt. The Gr5a cells would be responsible for detecting low concentrations and the Gr66a cells would be responsible for detecting high concentrations of salt.

Results

Unfortunately, the mutants showed a completely different behavior than expected. We were not able to lure them onto the slices of agar – they just remained on the bottom of the box, and so we could not film them nor obtain useful results.

Personal Impressions

We were quite disappointed not being able to obtain meaningful results – but looking back at three weeks of hard work we are content with our efforts because we both had the possibility to get involved in interesting fundamental research. We had access to the daily routine of the lab work, learned a lot about our topic and how to set up an experiment on our own. Like the participants from the SFA 2003 had told us, working in the Scott Lab is self-contained. We were taught to get our own ideas and propositions involved into our project.

Even though the work was sometimes a little bit monotonous (repeating the trials several times), it was a lot of fun, because Peter always tried to keep us occupied, Markus flipped some flies from vial to vial or Tim tried to build futile networks from our Apple to the lab Apple. Actually we were very content when we completed our chart with the preference index by the end of the second week.

Of course, there where a few things we were not expecting to find out: most items are used only once and then discarded, Americans (especially Peter) are fascinated about cell phone ring tones (Get low!), and last but not least the rate of consumption of Haribo 'gummi bears' is unbelievable, despite the fact that Martin had warned us early on that our seven packets would probably be gone within a single day. But the variety of the people from all over the world (China, India, Iran, metropolitan Detroit and New York, and of course, from Karlsruhe) created an incredible atmosphere – not only during our splendid lunches or picnics on the Berkeley Green sponsored by the lab. We made a lot of new friends from all over the world and are grateful and proud that Kristin Scott declared that she again would welcome students for the next International Science Academy in San Francisco 2007.

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Psychophysical Retinal and Central Visual Processing

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Introduction

We were hosted in the lab of Prof. Westheimer, one of the most renowned scientists in the field of visual neurobiology. With Prof. Wehrhahn from Tuebingen at leave in Berkeley, and a student from UC Berkeley, Crystal Tan, he gave us a first introduction to the fascinatingly vast field of psychophysics. Psychophysics deals with the interconnections between physical stimuli and their perceptions by human beings. Psychophysics can be related to all different kinds of areas of perception, but in this research it is only related to optical stimuli. All psychophysical set-ups have in common that they try to assign the physical quality of a stimulus to a subjective perception. This takes place during psychological experiments, in which the subjects have to express their perceptions of varying stimuli in numbers. The results of psychophysics are of considerable importance for psychological theories and concepts in perceptional psychology. In addition, they provide key aspects in research and standardization of environmental factors which affect the well being, performance, and security of human beings in their daily life. The most important questions addressed by psychophysics are:

1. How strong does a stimulus need to be in order to evoke a perception?
2. How big does the difference between two stimuli need to be in order to evoke a different perception (just notable difference = JND)?

The main intention of our work was to make a contribution for answering aspects of these questions.

The research of Prof. Westheimer relates to the way in which our brain channels visual images presented to our eyes and how activity in these pathways leads to the way we recognize objects and make decisions based on our perceptions.

Materials and Methods

The series of experiments were performed by using computer programs developed by Prof. Westheimer and Prof. Wehrhahn, on the basis of randomized modules. This enabled the professors themselves to actively participate in the trials as probands. The probands were supposed to decide which one of two lines given on a screen appeared second. This decision was made by clicking on the equivalent mouse button. One test series contained 150 stimuli (one stimulus = one decision). For our trials we used a low-resolution monitor to make the stimuli be more visible. The computer counts the mouse clicks and processes the data. The brightness, color, and distance of the two lines which were presented to us during the trials, as well as the brightness of the monitor were regulated by parameters defined before the onset of the trials. Depending on the kind of trial we were supposed to do, the parameters were modified. Those parameters are expressed in a row of numbers. For example: 45 402 6301 1 is a typical parameter.

- 45 stands for the background brightness.
- 402 means that the two lines are 4 pixels thick and two pixels apart
- 6301 describes the color of the two lines. 63 stands for white and 01 stands for black. So 6301 means that the left line is white and the right one black.
- 1 stands for the scale of the temporal spacing, which indicates three different time separations (see below).

If 1 is used as scale number, it means that the left line will appear 0 ms, 8.3 ms, 16.66 ms, or 25 ms before the right one or vice versa, according to the outcome of the calculations of a randomization module (0 means that both lines appear simultaneously). When 2 is used as a scale number, the time separations are just multiplied with that scale number. Consequently, the time separations with the scale number 2 would be 16.66 ms, 33 ms, and 50 ms. In general we used the scale 1 or 2. At the end of each trial the computer calculates a table of the distribution of the mouse clicks done by the subject and the actual distribution of the 150 stimuli (ideal line). So, the outcome would be perfect if the upper and the lower number would be exactly the same on the three left columns, whereas on the right side the upper row would be zero as the computer counts the left clicks. In the middle column the perfect result would be that the number in the upper row is exactly the half of the number given in the row below.

	-25 ms	-16,66 ms	-8,33 ms	0 ms	8,33 ms	16,66 ms	25 ms	
0508082.f91	11	10	9	7	14	10	8	= Amount of left mouse-clicks
45 402 6301 1	20	15	18	19	26	30	22	= real distribution
		1		2		3		

Fig. 1. The figure shows the outcome of a typical experiment. In total there are 7 different possibilities how the two lines may appear: *1.* Right line before left line; *2.* Both lines at the same time; *3.* Left line before right line

The computer uses statistical calculations for the analysis of the data. They are depicted in 7 different columns: slope, slope error, mean, mean error, the total number of times clicked during one trial, chisq (Chi square), and scale. The slope describes the tangent that is drawn on the learning curve, which is produced by the tests; high numbers stand for a high threshold, low numbers for a low threshold. A low threshold stands for a very steep tangent, which means good learning has been achieved.

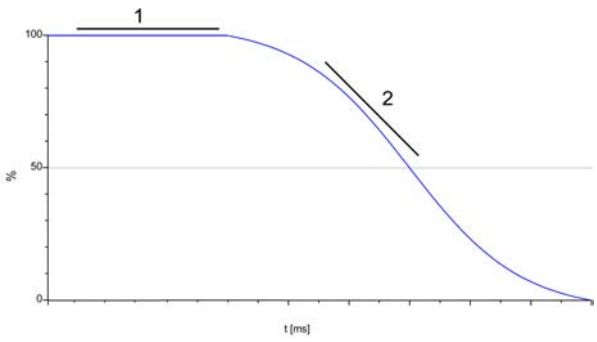

Fig. 2. *1:* very flat; *2:* very steep

The slope error tells how much the learning curve and the tangent, which is the ideal line, are apart from each other. The mean is the location in stimulus space at which the observer's responses are equally distributed between the answers "yes" and "no" to the question "Does the left line appear earlier than the right one?". Because the results are accumulated in batches of 150 responses, this 50% point will be an estimate from the distribution of "yes" and "no" answers. This estimate of the mean has its own distribution, whose error can be calculated. The Chi square is a measure for the quality of the data and the reliability of the fits. The fit measures how good the data fits into a Gauss distribution. The scale shows the temporal spacing (see above).

Results and Discussion

Learning Curves. In our first week in the lab we started with the basic experiment. We had the two lines appearing after each other and had to decide which one came second. For this we used the scales 1 and 2. At the end of the week we wanted to check whether we had learned to recognize the two lines faster and more efficiently and whether we made less mistakes clicking the correct mouse button. To be able to visualize this properly we generated a learning curve with our results. A learning curve is composed of the slope (called 'threshold' in the figure) and the slope error (in the figure: the vertical line through the points). In a perfect case the learning curve would start at a high point (as you never saw that stimulus before and you make mistakes clicking the mouse buttons) and lower down in a rather steep curve, which remains flat afterwards. The two curves we generated with our outcomes are unfortunately not like they are supposed to be.

As you can see in Fig. 2 the curve starts at a very low point and has no resemblance with the above-described perfect learning curve. It seems as if the proband (A. K.) has already had contact with comparable stimuli. This curve could be described as the opposite of a learning curve. We couldn't figure out why this happened or where the subject had seen the stimuli before. The learning curve that can be seen in Fig. 3 (C. F.) starts at a high point as it should, but is too steady to be a real learning curve. In both cases we were not able to tell why the curves did not come out as expected.

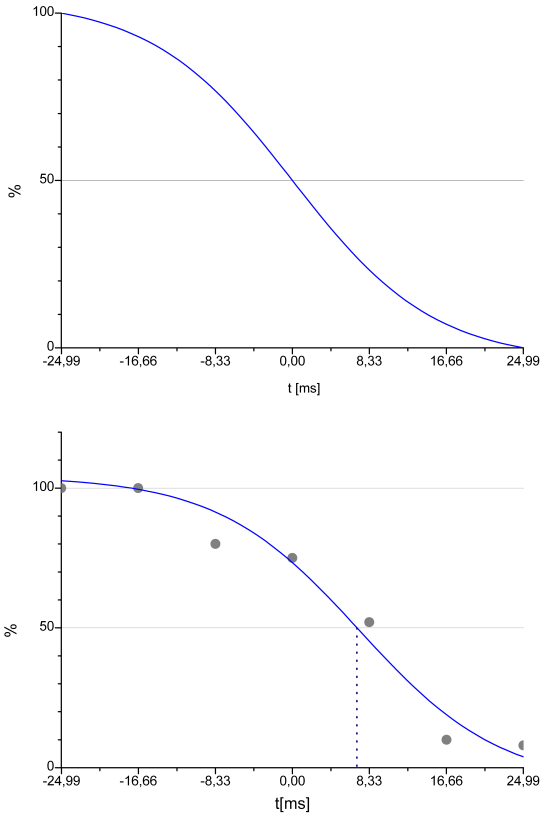

Fig. 3. (A) Learning curve Alexandra Kahn; (B) Learning curve Clarissa Fischer

Adaptation (The "Waterfall-Effect"). The "waterfall-effect" is a well-known phenomenon to scientists in the field of visual neurobiology. If you stare at a waterfall for some time and then move your eyes towards something solid, non-moving, like a rock, the item seems to "flow upwards". It is a visual illusion caused by the monotonous down-stream of the waterfall. In this experiment the question was whether the same thing happens when there is a sideways-directed adaptation of lines (e.g., from the right to the left) between the different stimuli, i.e., a tendency in the opposite direction concerning the decision, which line appears second. In our experiments the two lines appeared inside a circle and between the stimuli there was an adaptation (lines moving constantly from one side to the other). For these experiments we needed two parameters. The first parameter (–0402 63 –140) describes the circle and the adaptation, the second one describes the stimuli as in the other trials. First parameter:

- −0402: adaptation moves from right to left (a change of the mathematical sign changes the direction)

- 63: color of the adaptation lines (white)

- −140: adaptation is shifted 140 pixels from the center to the left circle (positive sign: right circle)

As in the trials before, the proband had to decide which line appeared second by clicking the equivalent mouse button. Finally we screened whether there had been a significant shift to one side or not. Unfortunately our results were not conform with this hypothesis and did not significantly differ from the results of our previous experiments without adaptation. The further research on this issue will show whether there is a "waterfall-effect" to the side and whether this could effect us in our daily lives in any way.

Sweeping Movement. This is the latest project of Professor Westheimer and his lab. The layout of the program is similar to the other experiments, but this time the question is not which line appears second but rather how the proband perceives the appearance of the two lines. In some cases when the two lines appear, it seems as if they were "sweeping" across the monitor and not just appearing one after the other. The question is on what facts and parameters does this perception depend and whether it is possible to make a perceptual distinction between the appearance to the observer of a discrete or jump displacement of a target from one position to a closely neighboring one, and a continuous, sweeping movement between the initial and final positions. We tried to answer this question by changing the parameters and deciding whether there was a sweeping movement of the lines or not. The decision was made again by clicking the mouse buttons. But this time we defined that the left mouse button meant, "sweep", whereas a right click meant "no sweep". The order of the lines was irrelevant. We chose three different distances between the lines: 8, 16, and 30 pixels. The scales for the temporal spacing were 2 or 4 depending on the experiment. In this way we were able to see the influence of both, distance and speed of the lines, on the perception of the proband. In the following graphs one can see that more sweeping movement could be seen with longer time distances (50 or 100 ms) and very short spatial distances. It was quite remarkable that Prof. Westheimer, in performing the same experiment (only one row of the trial was the same), saw less "sweeping" when the lines had longer time distances instead of more. We were not able to determine the reason for this outcome, as we unfortunately were not able to repeat the experiment for time reasons. As our lab time was coming to an end and we were not able to carry out more experiments of this kind with other parameters, we could not find an acceptable answer to the questions we had asked ourselves – but we want to pursue these questions and find an explanation for our results.

Acknowledgements

We want to thank Prof. Gerald Westheimer for generously hosting us in his lab for three weeks and investing a lot of his time in patiently answering our many questions – during and after our stay at UC Berkeley. Special thanks also to Prof. Christian Wehrhahn, who arranged many aspects of our stay in the lab, devoted significant time to introducing us to psychophysics and supervising us in the lab, and Crystal Tan for showing us how to work independently with the programs.

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Molecular Cloning of Putative EGFR Inhibitor Resistance Genes

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Abstract

Cloning of genes is one of the basic principles and techniques in molecular biology. It is used to obtain the gene of interest inside a vector, amplified for further use. Non-small-cell lung cancer (NSCLC) tumors not responding to EGFR tyrosine-kinase inhibitor gefitinib have different gene expression than responders. We cloned three of the genes with significant differences in expression levels (*DUSP3*, *PHLDA2*, and *CORO1C*) into retroviral vectors in order to investigate effects of their expression in T4-2 cells on gefitinib sensitivity. The successful cloning of the *DUSP3* gene containing a flag-tag was verified by sequencing (others are pending).

Introduction

The interactions between cancer cells and their surrounding environment create a context that supports tumor growth and protection from immune defense [1]. As with nonmalignant cells, growth and progression depend to a great extent on the activity of cell membrane receptors that control the signal transduction pathways inside the cell, regulating processes such as cell proliferation or apoptosis [2]. The epidermal growth factor receptor (EGFR), as one of these transmembrane receptors, has been shown to play an important role in tumor growth and survival [3]. It belongs to the family of human epidermal growth factor receptors (HER) and has an intracellular tyrosine-kinase domain that induces cascades of signal transduction. The results of many research groups investigating EGFR as a potential target for anticancer treatment showed the importance of EGFR signaling in development of certain tumors. Gefitinib, an inhibitor of EGFR-tyrosine-kinase, is the first targeted agent to be approved for the treatment of patients with non-small-cell lung cancer (NSCLC) [4]. Although many trials showed encouraging results, the drug – in combination with other treatments – had nearly no effect in treating phase III patients [5]. In comparing gene expression of NSCLC tumors responding to this drug and non-responders Kakiuchi et al. [6] identified 51 genes whose expression significantly differed. Some of the genes with high difference in expression also play an important role in the development of breast cancer. During our internship at the Bissell Lab, we tried to analyze three out of the top ten of these genes: *CORO1C*, *DUSP3*, and *PHLDA2*. The cloning of these genes would initiate a study of the effects of overexpression of these genes in T4-2 breast cancer cells.

Summary of the Investigated Genes as Described on Entrez Gene [7]:

DUSP3 – Dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related). The protein encoded by this gene is a member of the dual-specificity protein phosphatase subfamily. These phosphatases inactivate their target kinases by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues. They negatively regulate members of the mitogen-

Table 1. The primers used for the PCR reactions (concentration: 100 ng/ml)

No.	Oligo name	Sequence ($5' - 3'$)
1	*CORO1C*-R1-Sense	AAAGAATTCATGAGGCGAGTGGTACGACAG
2	*CORO1C-Bam*HI-Antisense	AAAGGATCCTCAGGCTGCTATCTTTGCCATCTG
3	*CORO1C*-Flag*Bam*HI-Antisense	AAAGGATCCTCACCCCTTGTCGTCATCATCC-TTGTAGTCGGCTGCTATCTTTGCCATCTG
4	*DUSP3*-R1-Sense	AAAGAATTCATGTCGGGCTCGTTCGAGCTC
5	*DUSP3-Bam*HI-Antisense	AAAGGATCCTCAGGGTTTCAACTTCCCCTCC
6	*DUSP3*-Flag*Bam*HI-Antisense	AAAGGATCCTCACCCCTTGTCGTCATCATCC-TTGTAGTCGGGTTTCAACTTCCCCTCC
7	*PHLDA2*-R1-Sense	AAAGAATTCATGAAATCCCCCGACGAGGTG
8	*PHLDA2-Bam*HI-Antisense	AAAGGATCCTCATGGCGTGCGGGGTTTGGGCTG
9	*PHLDA2*-Flag*Bam*HI-Antisense	AAAGGATCCTCACCCCTTGTCGTCATCATCC-TTGTAGTCTGGCGTGCGGGGTTTGGGCTG
10	T7 sequencing primer	TAATACGACTGACTATAGGG
11	*Cy3GFP* reverse	TAACGGGAAAAGCATTGAACACC
12	*pEYK*-Insert-5′	TGGAAAGGACCTTACACAGTCC
13	*GAPDH*-Sense	CCCCTGGCCAAGGTCATCCATGAC
14	*GAPDH*-Antisense	CATACCAGGAAATGAGCTTGACAAAG

activated protein (MAP) kinase superfamily (MAPK/ERK, SAPK/JNK, p38), which are associated with cellular proliferation and differentiation. Different members of the family of dual-specificity phosphatases show distinct substrate specificities for various MAP kinases, different tissue distribution and subcellular localization, and different modes of inducibility of their expression by extracellular stimuli. This gene maps in a region that contains the *BRCA1* locus which confers susceptibility to breast and ovarian cancer. Although *DUSP3* is expressed in both breast and ovarian tissues, mutation screening in breast cancer pedigrees and in sporadic tumors was negative, leading to the conclusion that this gene is not *BRCA1*.

PHLDA2 – Pleckstrin homology-like domain, family A, member 2. This gene is one of several genes in the imprinted gene domain of *11p15.5* which is considered to be an important tumor suppressor gene region. Alterations in this region may be associated with the Beckwith-Wiedemann syndrome, Wilms tumor, rhabdomyosarcoma, adrenocortical carcinoma, and lung, ovarian, and breast cancer. Studies of the mouse gene, however, which is also located in an imprinted gene domain, have shown that the product of this gene regulates placental growth.

CORO1C – Coronin, actin-binding protein, 1C. This gene encodes a member of the WD repeat protein family. WD repeats are minimally conserved regions of approximately 40 amino acids typically bracketed by gly-his and trp-asp (GH-WD), which may facilitate formation of heterotrimeric or multiprotein complexes. Members of this family are involved in a variety of cellular processes, including cell cycle progression, signal transduction, apoptosis, and gene regulation.

Materials and Methods

PCR

In order to first amplify our genes from a cDNA template, we performed polymerase chain reaction using specific primers for *DUSP3*, *CORO1C*, and *PHLDA2* with *GAPDH* as a positive control. Each set of primer consists of a sense primer, containing a recognition site for the restriction enzyme *Eco*RI, a non-tagged antisense primer and an epitope-tagged antisense primer, both containing *Bam*HI recognition sites. A flag-tag (sequence: asp-tyr-lys-asp-asp-asp-asp-lys) was used for the purpose of recognizing the resulting proteins on a Western Blot assay. The cDNA sample from HEK-293 (human embryonic kidney) cells turned out to be efficient for *PHLDA2* using Taq DNA polymerase (Sigma), while T4-2 cells were more efficient for *DUSP3* and *CORO1C* using Pfu DNA polymerase (Stratagene).

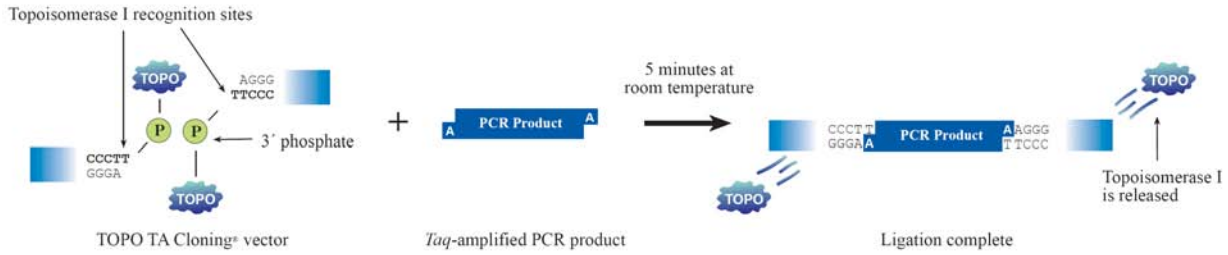

Fig. 1. TOPO TA Cloning® of Taq-amplified DNA

Cloning

We cloned the products of the PCR using Pfu (*DUSP3* and *CORO1C*) into the *pBM-IRES-Puro* vector [8] using *Eco*RI and *Bam*HI restriction enzymes (NEB) and T4 DNA ligase (Roche). The Taq-PCR product *PHLDA2* was cloned into the pcDNA3.1/CT-GFP-TOPO® vector (Invitrogen). This ease-of-use vector has single 3′ tymidine overhangs as well as a topoisomerase I from vaccinia virus covalently bound to it. The 3′ ends of Taq DNA polymerase products have an overhanging adenine residue, fitting to the thymidine overhang of the vector. The covalent bond between the vector and the enzyme (at tyrosyl residue 274) is attacked by the 5′ hydroxyl of the PCR product, ligating the DNA and releasing the topoisomerase.

Fig. 2. Separate colonies on agar plate as tested for cloned genes

Transformation

The resulting ligation products were transformed in chemically competent *E. coli* using 40 s heat shocking at 42°C. Then the bacteria were allowed to recover in LB medium and cultured on agar plates containing ampicillin (100 µg/ml). Single colonies were seperated and checked by a PCR using vector specific primers (see Table 1) to determine if the gene was inserted properly, e.g., in the correct orientation.

Midiprep

From colonies with promising PCR results we extracted the plasmid using Qiagen HiSpeed™ Plasmid Midi Kit. After precipitating the eluted DNA by adding isopropanol as described in the protocol, we centrifuged the sample to get a pellet of the wanted plasmid DNA, washing away unwanted residual salts with 70 % ethanol. We dissolved the plasmid DNA to a final volume of 50 µl. For DNA quantitation we performed UV spectroscopy of a 1/100 dilution using the SpectraMax Plus (Molecular Devices) photometer. Purity was estimated by calculating the $A_{260\text{nm}}/A_{280\text{nm}}$ ratios of our samples.

Results of the experimental steps were electrophoresed on 1.5% agarose gel (Invitrogen) and visualized on UV transilluminators (Alpha Innotech). Plasmid and digested DNA was purified out of gels or reaction digests using Qiagen purification kits. Sequencing of the obtained DNA was performed by SeqWright, Houston, TX with primer 12 (see Table 1).

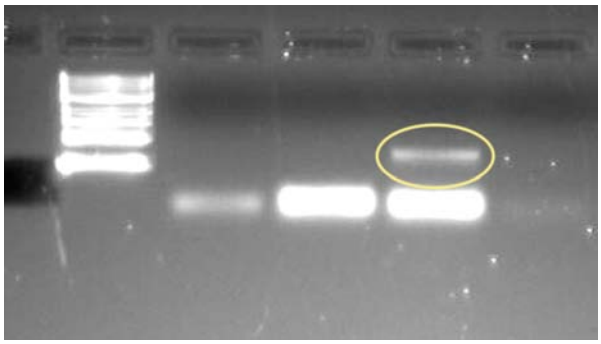

Fig. 3. Agarose gel electrophoresis image of a PCR testing the success of *DUSP3* cloning. Cell culture 3 shows the significant bar. For reference, a 1 kb DNA ladder was used in lane 1

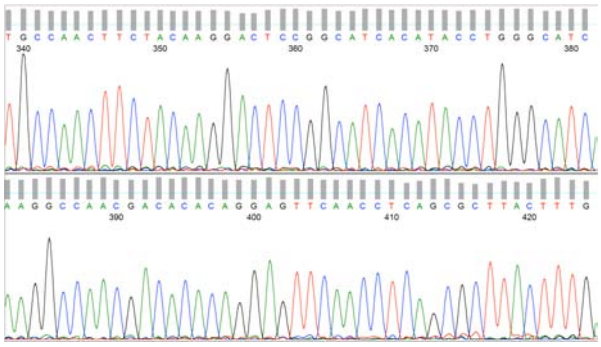

Fig. 4. Excerpt of the sequencing result of the selected colony, validating the successful cloning of DUSP3. Chromatograph visualized using FinchTV v.1.3.1 by Geospiza, Inc.

Results and Discussion

The purified plasmids containing *DUSP3* and *PHLDA2* had a concentration of 2.175 and 3.45 µg/µl, respectively. The purity of the *DUSP3* sample was 1.422 that of the *PHLDA2* was 1.462. The total yield of DNA turned out to be 108.75 µg (*DUSP3*) and 172.5 µg (*PHLDA2*).

Sequencing of the gained and purified plasmid containing *DUSP3* showed, that the cloned gene definitely is *DUSP3*. In this short time of a three week internship we managed to successfully clone one of 'our' genes for the first time into human breast cancer cells.

The next step would be to overexpress those genes in T4-2 cells and measure sensitivity to EGFR inhibitors. A further aim would be to investigate the system of interactions between the genes involved in the sensitivity to gefitinib and develop a general describing plan leading to predict effects of expression changes on the T4-2 cells.

Acknowledgements

We want to thank Mina Bissell for giving us the opportunity to visit this great lab and get an insight in this fascinating field of science. Very special thanks to Paraic Kenny who was a great supervisor during these three weeks and gave us a lot of really helpful hints and advices. It was an unforgettable experience and we had an absolutely great time.

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Influence of MAP2c on Microtubule-Polymerization by Electron Microscopy

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Abstract

Two microtubule binding sites have previously been identified for the microtubule-associated protein MAP2c, one on the exterior and one on the interior of the microtubule "tube". However, it has not yet been determined which of these sites, if any, is biologically relevant. To further study that question we added MAP2c to microtubules at different times during polymerization. During that work we recognized that the two different ends of microtubules behaved quite differently in the presence of MAP2c as shown in some pictures taken with an electron microscope. Until now there has been no easy way of distinguishing between the plus and minus end of microtubules. But then our observations showed that the adding of MAP2c during polymerization might actually be that 'easy way'.

Introduction

Microtubules assembled from α, β-tubulin heterodimers are labile polymers unless stabilized by other molecules. In living cells there is a balance between polymerization and depolymerization called dynamic instability [1]. The dynamic instability accords for several of the vital functions microtubules have in cells, such as the formation and degradation of the spindle-apparatus, both of them of vital importance for the process of mitosis. As cancer cells all have the one property in common that they divide uncontrolled, an efficient way of combating cancer is interfering with the process of mitosis. Most of the commonly used anti-cancer drugs disturb either the assembly of microtubules (such as epothilone [2]) or their degradation (such as taxol). Therefore a lot of the scientific work done in the area of microtubules is related to the detection of new anti-cancer agents. Another function of microtubules is the transport within a cell, where motor proteins such as kinesin wander along the protofilaments of microtubules and thereby transport molecules attached to them and also different kinds of cells are propelled by cilia or flagella containing microtubules. For various reasons the detection of binding sites of anti-cancer agents as well as of transport molecules is important. First the occupation of different binding sites would probably allow the prediction that the binding of one material would not affect the binding of another material. Also, the exact knowledge of the binding site can lead to predictions of new agents that would occupy the same binding site.

Our work included testing whether kinesin and MAP2c occupy the same binding site and therefore whether the binding of MAP2c would affect the binding of kinesin. Other parts of our work involved assaying of the stability of MAP2c-stabilized microtubules under various conditions [3]. Another interesting aspect of MAP2c binding to microtubules is that it is not known whether it binds on the outside or the in-

side of microtubules. Two groups working on that question came to different conclusions (see [4] and [5]). Using different methods, the one group detected MAP2c on the outside of microtubules adding the protein after polymerization with taxol, while the other group added MAP2c before polymerization. As these differences might accord for the variant results, we tried to find differences in the appearance of microtubules when MAP2c was added at variable times.

Methods

Microtubule Polymerization

Microtubules were assembled using 25 µl of "tubulin minus glycerol" purified from bovine brain and provided by cytoskeleton. Then, 1 mM of GTP was added to the tubulin and both were spun at 100 K, 4°C for 10 min. After the spin, the supernatant was incubated at 37°C for 20 min. Stabilizing proteins were either added before, during, or after the incubation which is synonymous to polymerization. When taxol was used as a stabilization agent [6], the microtubules were spun through a cushion of glycerol to further purify them. Therefore, a mixture of 0.4 ml "glycerol and PEM buffer" and 20 µl taxol were used as a cushion which was carefully placed on top of the incubated tubulin and then spun at 100 K, 37°C for 10 min. Afterwards the cushion was washed with PEM buffer and then removed. The remaining pellet was washed with a mixture of 200 µl PEM buffer and 20 mM taxol two to three times and afterwards diluted in 15 µl of the PEM/taxol mixture.

EM-Grid Preparation

Carbon-coated grids were placed under UV light for at least 10 min. A mixture of 0.4 µl of microtubules and 3 µl of PEM buffer was added to the carbon side of the grid which was then flipped over and the microtubules droplet was mixed with a water droplet, 20 µl. The procedure was repeated. The surplus of liquid was removed by touching a piece of filter paper at an angle of approximately 45°. After that, a 10 µl droplet of uranium acetate was added to the carbon side of the grid which was removed after approximately 15 seconds by pressing the grids downside down against a piece of filter paper. Electron microscopy of the resulting preparation yielded Fig. 1.

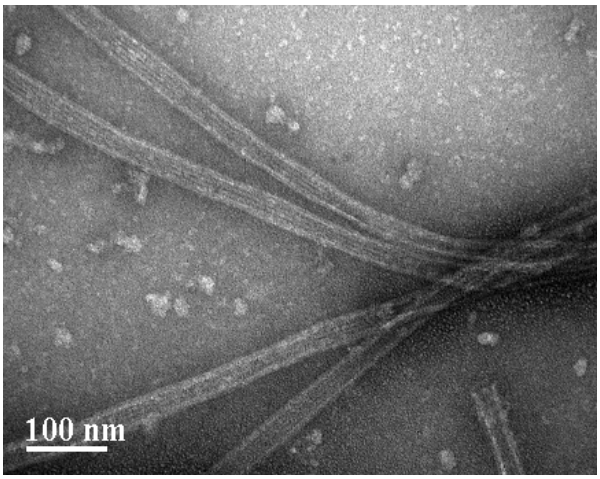

Fig. 1. Polymerized microtubule

Binding Kinesin to Microtubules

Kinesin concentration was estimated using "advanced protein assay 5x" (cytoskeleton) and compared to the equally tested concentration of microtubules. The molar ratio used was approximately 1 mol kinesin to 1 mol tubulin. Kinesin, microtubules, and a small amount of apyrase, which is used to maintain an ATP- and ADP-free environment, was mixed at room temperature and rested for 5 min.

Binding MAP2c to Microtubules

The concentration of MAP2c was tested the same way we tested kinesin but only a very small amount of MAP2c was added to the highly concentrated microtubules. The MAP2c was added at different points of time. It was either added before polymerization, after 10 min of polymerization, or after the process of polymerization.

Results and Discussion

To find differences in the binding of MAP2c to microtubules, MAP2c was added at different points of time, and we tested the stability under cold stress: no differences were to be found. While taxol stabilizes microtubules when they are put on ice, the stabilizing properties of MAP2c are not as pronounced: all tested MAP2c-stabilized microtubules depolymerized.

Further experiments showed slight differences between microtubules polymerized with or without MAP2c. However, the differences decreased when the microtubules were given some time before examining them under the electron microscope with negative stain. We observed that microtubules polymerized with MAP2c, appeared to be relatively similar to those polymerized with taxol: they were straight and had mainly normal ends, both of them looking quite alike.

On the other hand, when MAP2c was added after the polymerization had begun, the two ends of individual microtubules looked very different from each other. One end came apart gradually, with individual filaments coming apart (i.e., it appeared "crumbly") while the other end terminated abruptly and was not fibrous (Figs. 2–4).

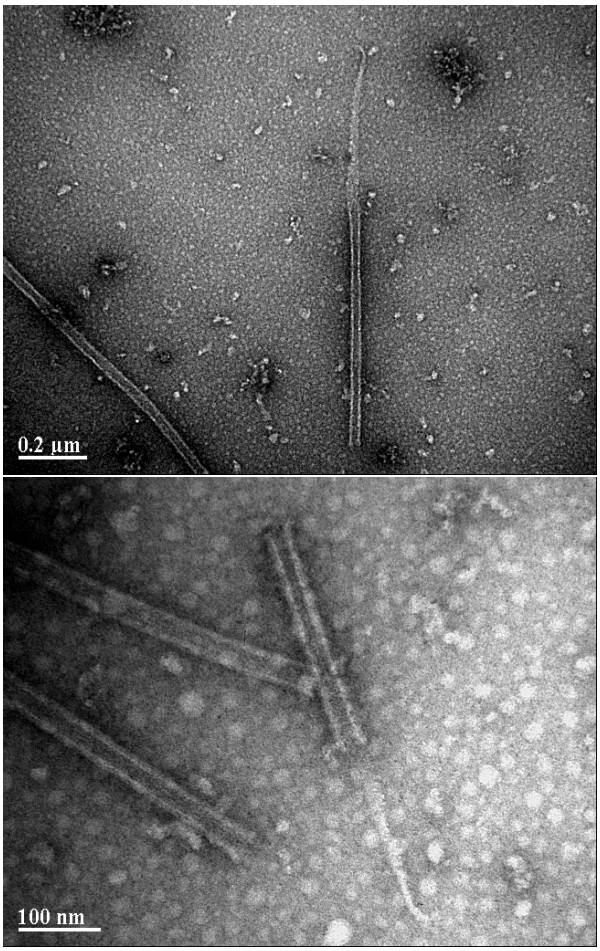

Fig. 3. Adding of MAP2c to microtubules during polymerization (two frames; different magnifications)

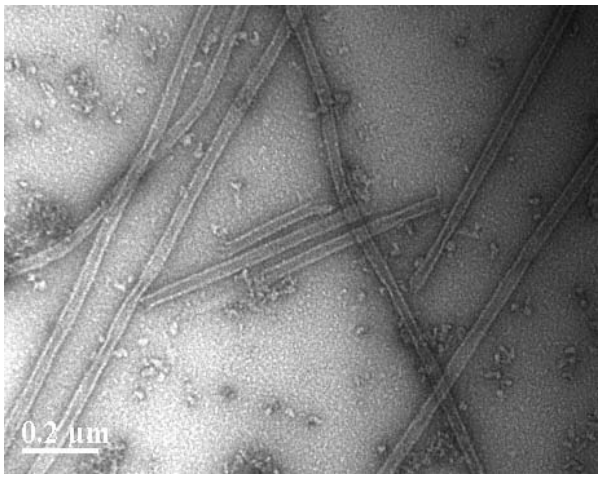

Fig. 2. Adding of MAP2c to microtubules before polymerization

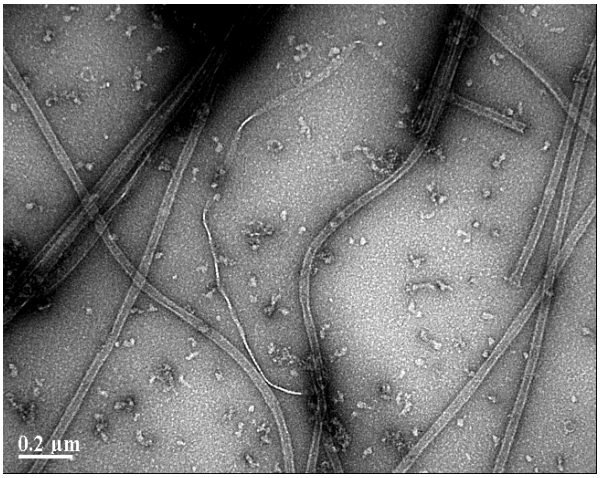

Fig. 4. Adding of MAP2c to microtubules after polymerization

We suppose that the microtubules polymerize in a more stable way in the presence of MAP2c and that this difference is shown in the crumbly end. Our assumption is, as microtubules have a plus and a minus end, that MAP2c stabilizes one end more than the other end. We cannot tell which one is which by this experiment alone, but to detect it will be a future goal.

Another phenomenon we realized was that the difference between the two ends shrank when the sample had been abandoned for a while. As early as after 30 min most of the differences were gone. Our hypothesis is that MAP2c does not stabilize the microtubules so much that their polymerization and depolymerization is inhibited. So the balance they are normally in is maintained. Another possible suggestion is that the crumbly end was unlike the rest of the microtubules not stabilized and therefore depolymerized while the rest stayed intact. But again one would need to do further experiments to answer that question.

Acknowledgements

We would like to thank Ken Downing for giving us the opportunity to be a part of his great lab, even if only for a short while. We also really enjoyed the annual summer lab party to which Ken and his wife Linda invited us and which again prooved to us that the atmosphere at his lab is always friendly and welcoming. Another person in the Downing Lab we would very much like to thank is Chuck Sindelar who gave us a lot of support and who was never impatient and always encouraging. We also very much appreciate the effort of our tutors Martin Schorb, Christoph Fischer, and Monika Gessat to arrange this year's academy, it was a great experience for all of us. Special thanks to Christoph and Martin who supervised the Berkeley group and were never short on jokes or pieces of advice. Last but not least we wish to thank our parents who have always endorsed us and who covered all our costs, making the trip possible after all.

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Localization of the Transcription Factor SMP1 in *S. cerevisiae* is Dependent on YPK1 and Extracellular Osmotic Conditions

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The following report is a description of my research carried out in the lab of Jeremy Thorner under the supervision of Françoise Roelants.

The first part of the project comprises the cloning of the *SMP1-GFP CEN* plasmid, producing several single and double deletions, as well as corresponding microscopic observations, all part of Dr. Roelants' ongoing research.

The second part consisting of osmotic shock assays with the *ypk1Δ* mutant is based upon the first, but arose from my own initiative and ideas.

Introduction

Cell wall integrity signaling in *Saccharomyces cerevisiae* has more and more moved into the center of research interest during the last years. Early views of the yeast cell wall as a static structure have given way to a new recognition of its highly dynamic nature (Levin 2005). One of the signaling pathways that has been identified as being implicated in cell wall integrity comprises the Pkh1/2 (orthologs of mammalian protein kinase, PDK1) and Ypk1/2 protein kinase pairs. Within this pathway, Pkh1 preferentially targets Ypk1, while Pkh2 appears to phosphorylate primarily Ypk2 (Roelants et al. 2002; Casamayor et al. 1999). It was also found that Ypk1 has a more prominent role in mediating the shared essential function of Ypk1 and Ypk2. A *ypk1Δ* mutant grows slowly at 30°C and displays an actin depolarization effect (Chen et al. 1993), immediately noticeable by an abnormal cell shape. Roelants et al. (2002) could also show that Ypk1 is exclusively localized to the cytosol, and clearly excluded from the nucleus and the vacuole. One of the dosage suppressors of the temperature-sensitive lethality of *ypk1-1^{ts} ypk2Δ* cells carries the SMP1 gene, an Mcm1, Agamous, Deficiens, Serum Response Factor-box transcription factor related to Rlm1 with which Smp1 shares its DNA binding specificity. As a result, it was suggested that the Pkh1/2 and Ypk1/2 protein kinase pairs form a signaling cascade mediating its effect through Smp1, working as a parallel pathway to the MAP kinase cascade mediating its function through Rlm1. Moreover, it was observed that Smp1 stimulates expression of osmoresponsive genes like *STL1* in a manner dependent of the Hog1 MAP kinase cascade, explaining the increased sensitivity to osmotic shock displayed by the *smp1Δ* mutant. It could also be shown that Smp1 accumulates in the nucleus in stationary phase, which was again shown to depend on *Hog1* expression (De Nadal et al. 2003).

This report explains an approach at finding cellular reasons for the genetically established connection between Ypk1 and Smp1 and at combining these observations with the influence of the Hog1 MAP kinase. Moreover, I will describe an osmotic shock

assay directed at elucidating the dependence of Smp1 localization on extracellular osmolarity in different strains.

Methods

Strains and Media

The *S. cerevisiae* strains used in this study are listed below. Cultures were taken from frozen glycerol stocks at –80°C, grown overnight on YPD plates at 30°C, resuspended in the medium and incubated on a shaker.

Strain	Genotype	Source
BY 4741 (parent)	*MATα his3Δ leu2Δ met15Δ ura3Δ*	Commercial
ypk1Δ	Isogenic to BY4741, except *ypk1::KANMX4*	Commercial
ypk1Δ	Isogenic to BY4741, except *ypk1::HIS3*	The present study
hog1Δ	Isogenic to BY4741, except *log1::LEU2*	The present study
ypk1Δ hog1Δ	Isogenic to BY4741, except *ypk1::KANMX4* and *hog1::LEU2*	The present study
ypk1Δ hog1Δ	Isogenic to BY4741, except *ypk1::HIS3* and *hog1::LEU2*	The present study

Deletion of the *HOG1* Gene

Gene disruption was carried out by homologous recombination, replacing the *HOG1* gene in the genome with a cassette carrying an insertion of *LEU2* into *HOG1*.

The *LEU2* gene (on a plasmid) was amplified by PCR using *HOG1*-primers at both the 5′ and 3′ end, and checked on an agarose gel, yielding a band approximately 3.5 kb in size. This is consistent with the length of *LEU2* and the chosen primers together. The PCR product was then gel-purified using a commercial kit.

Yeast strains BY4741 and *ypk1Δ (ypk1::KANMX4)* were grown to mid-logarithmic phase and transformed with the purified PCR product, using the high-efficiency lithium acetate method: After washing the cells once with water and once with 0.1 M lithium acetate, 240 µl of a 50% solution of polyethylene glycol, 36 µl of 1 M lithium acetate, 25 µl of salmon sperm DNA and 25 µl of the purified PCR product were added to the pelleted cells in layers. After incubating at 30°C for 30 min, the now competent cells were heat-shocked for 24 min at 42°C. Following the heat shock, the cells were resuspended in water, streaked out on SC-Leu selection plates and incubated overnight at 30°C. Colonies were checked by colony PCR. Electrophoresis on agarose gel yielded a single band about 475 bp in size, the expected length being 469 bp, thereby verifying the deletion.

Deletion of the *YPK1* gene

Gene disruption was carried out by homologous recombination, replacing the *YPK1* gene in the genome with a cassette carrying an insertion of *HIS3* into *YPK1*. The deletion cassette (as described by Schieders 1999) was cut out of a plasmid and gel-purified from the vector using a commercial kit.

Yeast strains BY4741 and *hog1Δ (hog1::LEU2)* were then grown to mid-logarithmic phase and transformed with the purified cassette, using the high-efficiency lithium acetate method: After washing the cells once with water and once with 0.1 M lithium acetate, 240 µl of a 50% solution of polyethylene glycol, 36 µl of 1 M lithium acetate, 25 µl of salmon sperm DNA and 25 µl of the purified PCR product were added to the pelleted cells in layers. After incubating at 30°C for 30 min, the now competent cells were heat-shocked for 24 min at 42°C. Following the heat shock, the cells were resuspended in water, streaked out on SC-His selection plates and incubated overnight at 30°C. Colony PCR was run, using one *HIS3*-primer and one *YPK1*-primer, and afterwards checked on a gel. A very distinct band about 965 bp in size was detected for several colonies. This matched the predictions assuming that the deletion cassette was integrated into the genome, thereby replacing the original *YPK1* gene.

A CEN Plasmid Expressing SMP1 with GFP at the C-terminus Under Its Own Promotor (pPR1)

Both the plasmids *YCplac33* (yeast marker: *URA3*, *E. coli* marker: Ampicillin resistance gene) and *pMB1*, containing *SMP1* with *GFP* at its C-terminus (Roelants, unpublished data), were double-digested with *Eco*RI and *Hin*dIII (restriction sites in the multiple cloning site of *YCplac33*, in *pMB1* flanking the *SMP1-GFP* fragment.) The cut vector (*YCplac33*) and the *SMP1-GFP* fragment were gel-purified with a kit and ligated using T4 ligase (subcloning). Afterwards, competent *E. coli* cells (DH5alpha) were transformed with the ligation product employing a heat-shock method (45 s at 42°C.) After transformation, the bacteria were streaked out on LB plates containing Ampicillin and incubated overnight at 37°C. Several positive colonies were detected, two of which were resuspended in LB Amp and incubated overnight again. Plasmid DNA was then purified using a commercial Miniprep kit. Finally, a control digest (*Eco*RI and *Hin*dIII) of the Miniprep product from eight different colonies was carried out and checked on an agarose gel. The correct plasmid was found in three of the colonies, confirmed by two bands on the gel, the longer with the length of the empty vector, the shorter with the length of the *SMP1-GFP*-fragment.

GFP Fluorescence Microscopy

Localization of GFP-tagged *Smp1p* within the cell was determined by fluorescence microscopy: Nuclear DNA was stained by adding Hoechst dye directly in the medium at a final concentration of 1 µg/ml for the last 30 min of growth. Yeast cultures at different phases of growth were concentrated by a short spin-down at 1500 g. Samples of each culture were then viewed directly with a TE300 fluorescence microscope (Nikon) equipped with a 100×/1.4 Plan-Apo objective and a 1.4 numerical aperture condenser.

Cell Extract Preparation and Western Blot Analysis

Preparation of cell extracts was carried out as described by Roelants et al. (2002). Cell cultures at different ODs were harvested by brief centrifugation, washed twice by resuspension, and resedimentation in ice-cold lysis buffer (50 mM Tris-HCl *p*H 7.5, 5 mM EDTA, 3 mM DTT) and resuspended in 200 µl of the same buffer. Pre-chilled glass beads (0.45–0.6 mm in diameter) were added to the meniscus of the cell suspension, and lysis was achieved by vigorous vortex mixing for six 1-min intervals, with intermittent cooling on ice. To remove the glass beads, the bottom of the Eppendorf tube was punctured with a syringe needle (< 0.5 mm in diameter) and inserted into another tube; the lysate was collected into the fresh tube by brief centrifugation. The crude extract was then subjected to centrifugation at 16,000 g for 15 min to remove unbroken cells and large debris. The protein concentration was measured using the Bradford method. For immunoblot analysis, samples (50 µg of total protein) were diluted into SDS-PAGE sample buffer (Laemmli 1970), subjected to electrophoresis in an 8–12% gel, and then transferred to nitrocellulose. Analysis was carried out using the Odyssey® Infrared Imaging System, following the accordant commercial Western Blot Analysis protocol. Blots were probed with a mouse anti-GFP primary antibody and afterwards with a fluorescently (IR)-labeled goat anti-mouse secondary antibody.

Osmotic Shock

Wild-type and *ypk1Δ* yeast cells, all carrying the *SMP1-GFP CEN* plasmid *pPR1* described above, were exposed to an osmotic shock by incubating them in 1 M NaCl. This was achieved by adding one volume unit of SC-Ura 5 M NaCl to four volume units of the yeast cells growing in SC-Ura. After another hour of growth, the cells were examined by fluorescence microscopy.

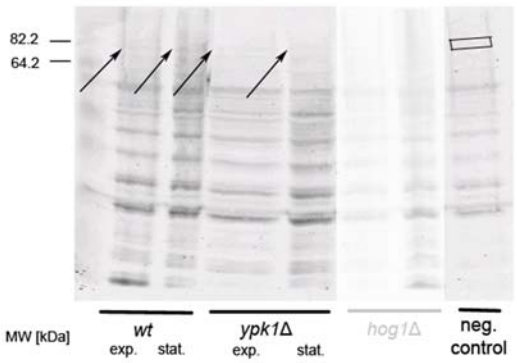

Fig. 1. Western Blot of extracts from *wt* and *ypk1Δ* cells transformed with the plasmid *pPR1* at different stages of growth reveals normal-level expression of the SMP1-GFP hybrid protein (under its own promotor)

Results

Nuclear Localization of SMP1 is Dependant on YPK1

Wild-type cells as well as *ypk1Δ* mutants were transformed with the CEN plasmid *pPR1* carrying *SMP1* with a GFP-tag at its C-terminus. Equal expression levels of the tagged protein could be verified by western blot analysis of extracts of the screened cells, using a mouse anti-GFP primary antibody (Fig. 1, cf. Methods). Although the blot yielded a lot of background, only one band was present in all cell extracts, but not in the negative control. This band run at a length of approximately 75 kDa, almost perfectly matching the expected size of the Smp1-GFP hybrid protein (molecular mass of Smp1 49 kDa, GFP 27 kDa). Moreover, none of the positive bands exceeded size and intensity of the others in an ostentatious way. Fluorescent microscopy at different stages of growth revealed that SMP1 accumulates in the nucleus of *ypk1Δ* cells already in exponential phase, whereas in wild-type cells, accumulation can only be observed in stationary phase. This conclusion is based on the combination of two independent experiments, each including counting of 75 cells: The percentage of cells showing nuclear localization of SMP1 in stationary phase is persistent, accounting for 79% in the case of the *ypk1Δ* mutant and for 80% in wild-type cells. In exponential phase, *ypk1Δ* cells still show a percentage of 72% nuclear localization, while the number drops to 21% in wild-type cells.

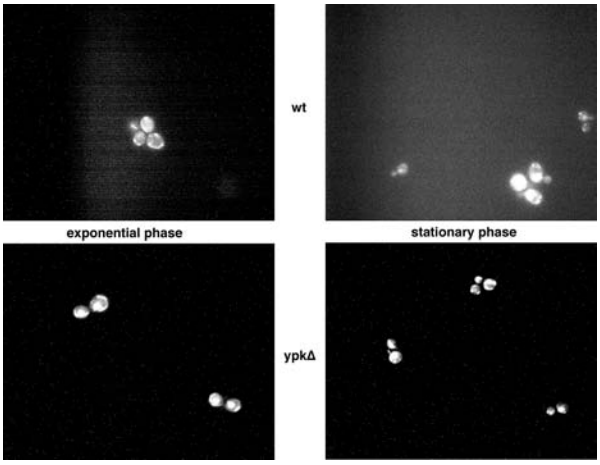

Fig. 2. *wt* and *ypk1Δ* cells at exponential and stationary phase, exposition time 20 s for both. Further description to be found in the Results section

Nuclear Localization of SMP1 is Fostered by Osmotic Shock in ypk1 Deletants

After one hour of osmotic shock, i.e., cultivation in SC-Ura 1 M NaCl, exponentially growing wild-type and *ypk1Δ* cells carrying the CEN plasmid *pPR1* were looked at under the fluorescent microscope. The wild-type cells showed no difference between 1 M NaCl and normal osmotic conditions, meaning nuclear localization figures around 20% for both (data not shown). In contrast to that, the nuclear localization percentage of exponentially growing *ypk1Δ* cells increased from 72% in SC-Ura (see above) to 92% in 1 M NaCl. Furthermore, the striking intensity of the fluorescence signal after the osmotic shock points at a possible overexpression of Smp1 under the described conditions.

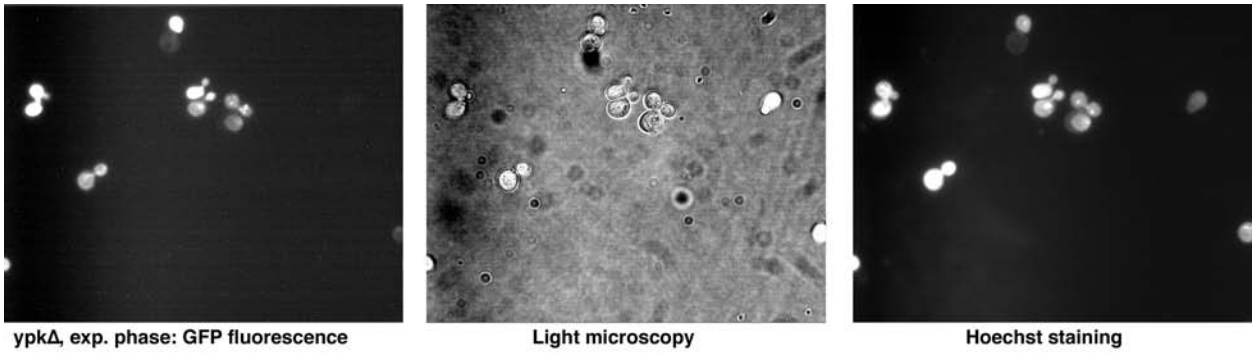

Fig. 3. DNA-specific Hoechst staining proves accumulation of GFP in the nucleus (example, not shown for the other fluorescence microscopy pictures in this report)

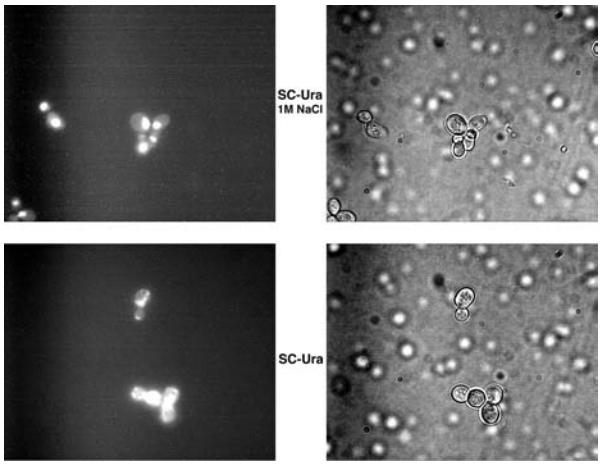

Fig. 4. *ypk1Δ* yeast cells with the plasmid pPR1 after 1 h osmotic shock in 1 M NaCl show almost complete nuclear localization at a high fluorescent intensity. Control cells from the same strain grown in SC-Ura for the same span of time show less distinct localization at a reduced intensity (exposition time 20 s both). For interpretation see Discussion

Discussion

The observations with the different *SMP1-GFP CEN* plasmid transformants confirm, in combination with the results of the Western blotting, that deletion of *YPK1* induces nuclear localization of *SMP1* as early as in exponential phase of growth, as was observed for cells carrying a 2μ plasmid also containing the GFP-tagged *SMP1* gene (Roelants, unpublished observations). While SMP1 accumulates in the nucleus of all strains only in stationary phase (except for *hog1Δ*), deletion of *YPK1* seems to go along with the transcription factor SMP1 moving to its place of effect, i.e., the nucleus, during earlier phases of growth. To fortify this point, further studies with *ypk1Δ smp1Δ* double mutants are necessary to determine whether SMP1 is essential for growth of cells lacking functional Ypk1. In order to find out whether the mechanism leading to SMP1 relocation and activation in the context of *ypk1Δ* strains is dependent on Hog1-catalyzed phosphorylation, further assays on the *hog1Δ ypk1Δ* double deletant generated in my project are underway.

The observations in my experiment related to *ypk1Δ* cells exposed to an osmotic shock verify the *ypk1Δ* phenotype of my transgenic strain, since the osmotic support was able to partly rescue the slow growth phenotype of the cells, which has been described before (Roelants et al. 2002). This was immediately visible by their standard cell shape. Furthermore, it at first seems logical that nuclear accumulation of SMP1 (which is known to induce expression of osmoresponsive genes) increases under conditions of osmotic stress.

But why does the percentage rise in the *ypk1Δ* mutant whose phenotype is actually complemented by high extracellular osmolarity? My results imply that exactly this effect of rescue might be mediated through Smp1. The high levels of Smp1 expression

that I was able to observe in 1 M NaCl could mean that the destabilizing effect on the membrane produced by the osmotic shock is added to the effect of the *ypk1Δ* mutation, thereby being sufficient to trigger a signaling cascade leading to Smp1 overexpression and nuclear localization.

Further experimentation is required to elucidate whether it is Ypk1 itself that prevents Smp1 from nuclear localization under physiological conditions during the phase of exponential growth. This would imply its direct involvement in the mechanisms triggered by high-osmolarity I could observe. Another possibility is that both proteins work in independent pathways both supporting cell wall integrity. Especially exposing a *ypk1Δ smp1Δ* double mutant to an osmotic shock and a following visual phenotype screen could supply further evidence here.

Acknowledgements

Warmest thanks go to Jeremy Thorner and Françoise Roelants for providing me with this wonderful research opportunity.

I would also like to thank Christian Ungermann, Biochemistry Center, University of Heidelberg, for the comprehensive preparation in his laboratory. Further thanks go to Patrick Westfall for primers, as well as to all present members of the Thorner Laboratory for their advice and warm welcome.

Last but not least, I would like to thank the entire group of ISA San Francisco 2005 for this incredible month we spent together in the United States.

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The Role of MicroRNAs in T-Cell Development

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Introduction

The first glimpse on some unknown, very small RNA molecules displaying significant impact on gene regulation was revealed more than ten years ago. In a seminal study conducted by Victor Ambros and colleagues, a gene known as *lin-4* was discovered to play a critical role in controlling the timing of worm development of *C. elegans* [1–2]. Interestingly, *lin-4* does not code for a protein but rather transcribes a pair of small, non-coding RNAs (ncRNAs). This study subsequently sparked a new concept of genetic circuitry by which gene expression is modulated at the post-transcriptional level. It is now believed that several hundreds of these small RNAs are present in the mammalian genome, most of which are proposed to play important functional roles in animal and plant development [3].

The current paradigm suggests that these microRNAs control gene expression at the post-transcriptional level through imperfect base pairing to the complementary region of the untranslated target mRNA [4].

Although the mechanisms by which small RNAs exert their effects are still undefined, this new perspective in cell biology certainly leads us to re-evaluate the longstanding dogma in biology: DNA makes RNA makes protein.

MicroRNAs (miRNAs) are small, about 20–25 nucleotides (nt) in length, single-stranded RNA molecules encoded in the genome of plants and animals. The generation of the mature functionally active miRNA involves a multi-step process. By binding to the messengerRNA (mRNA) via imperfect complementary base pairing, miRNA intervene directly in mRNA stability and translation. Due to such promiscuous binding, each miRNA have the potential to bind many targets, which can be located within a single gene or across multiple genes. As such, miRNAs are able to exert global control of gene expression. The potential impact of miRNA in the gene regulation of higher eukaryotes has been compared to that of transcription factors. Currently, it is estimated that approximately 200 to 500 miRNAs are present in the mammalian genome, regulating the expression of at least one third of all genes.

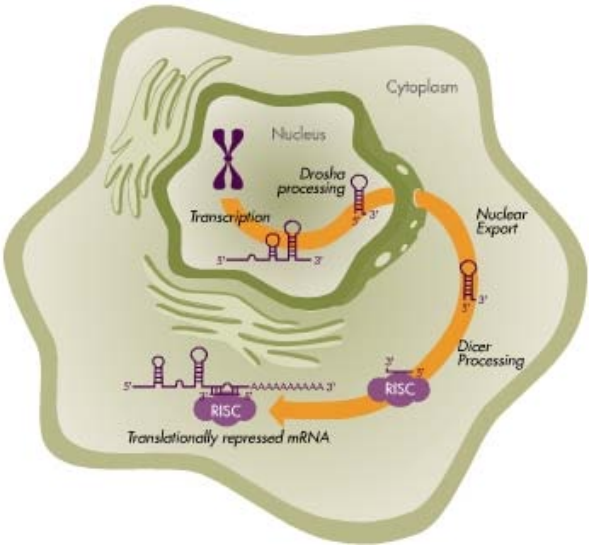

Fig. 1. The biogenesis of mature microRNA, including transcription, Drosha processing, nuclear export by Exportin, Dicer processing, and interaction with mRNA

The biogenesis of mature microRNA is a multi-step process (Fig. 1). The information encoded in the DNA of the organism is transcribed by polymerase II into the primary miRNA. Approximately more than 60% of miRNAs are expressed independently, 15% are located in clusters and 25% in introns [5]. The primary miRNA transcript forms a hairpin or a loop, including areas of recognition of specific proteins. The endonuclease Drosha processes the primary miRNA and releases the 70–100 nt long precursor, hairpin miRNA (pre-miRNA), which is directly exported from the nucleus to the cytoplasm by Exportin [6]. Further processing by Dicer cleaves the pre-miRNA into a double-stranded duplex and one of the strands will be loaded into the RNA-Included Silencing Complex (RISC), while the other strand is degraded by endonucleases in the cytoplasm. It results into a single-stranded mature miRNA, which has a length of about 20–25 nt.

The RISC-complex is able to interact with expressed mRNAs, which are also located in the cytoplasm. The miRNA will guide the RISC to complementary sites on the mRNA targets and result in the destabilization of the target mRNAs or inhibit translation initiation (Fig. 2). In plants the RISC-complex, including the miRNA, seems to act predominantly to initiate the degradation of mRNA [7]. Both mechanisms lead at least to a decrease of the protein level in the cell, which lead to large-scale changes, e.g., in cell development. Thus, cells are able to control the expression of protein by an additional system of regulation, after the transcription into mRNA. The regulation of proteins with miRNA is an elementary mechanism in the cell, especially at the beginning of development and in stem cells.

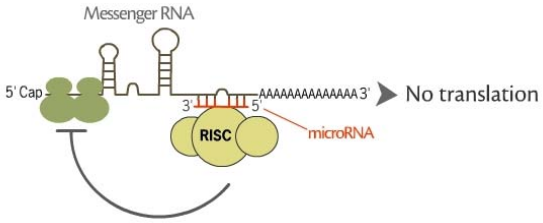

Fig. 2. Interaction between miRNA and mRNA inhibits the translation of mRNA into protein by polymerase III *(green)*

All cells have the same genetic information, only the different expression of proteins is responsible for the over 220 different types of cells and tissues. Moreover, miRNAs can regulate large numbers of target genes, which means, that they play diverse functional roles by regulating a broad spectrum of protein-coding genes. Nevertheless, the biological function of relevant target genes of the majority of discovered miRNAs remain elusive.

The laboratory of Professor Chen is especially interested in the role of miRNA in mammalian hematopoiesis. They focus on the role of miRNA in the post-transcriptional gene regulation circuitry, with the objective of understanding the modes of miRNA-mediated gene regulation during the generation, maintenance, and differentiation of hematopoietic stem and progenitor cells. MicroRNAs are abundantly present in various immune system cell populations, and are believed to significantly influence the development of immune cells, as well as modulating the differentiation of hematopoietic stem/progenitor cells. Moreover, several lines of evidence link miRNA to leukemia and other cancers [8].

In summary, understanding the normal biological function of miRNAs in hematopoiesis will shed light on the roles of miRNA in hematological disorders and may lead to the development of novel therapeutic strategies to correct hematological disorders, such as leukemia.

Material and Methods

The specific aim of our project was to setup a functional assay for examining the role of miRNA in the T-cell development. Here we describe an in vitro culture system which

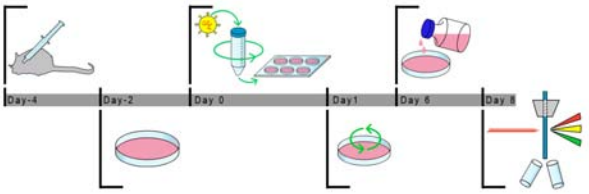

Fig. 3. Schematic diagram of the T-cell assay in time flow. For further information about the single steps see the detailed description below

allows us to define the effects of particular miRNAs of interest in the development of thymocytes (immature T-cells). Thus, we are able to examine the role of miRNA in hematopoietic lineage differentiation in an isolated system. To stably express miRNA in hematopoietic cells, we use a retroviral vector that included a murine stem cell retroviral backbone and a polymerase III expression cassette, containing the human H1 promoter to express the miRNA efficiently in the thymocytes. The miRNA insert must include special flanking sequences to form the hairpin precursor transcript, which can be processed into mature miRNA. Additionally, to trace cells expressing our miRNA of interest, we incorporated a green fluorescent protein (GFP) marker, which is expressed along with the miRNA.

The whole experiment spans over 12 days, beginning with the treatment of mice and ending with the analysis of the cells by FACS staining.

T-Cell Development

Immature T-cells are formed in the bone marrow and home in on the thymus, where they are called thymocytes. T-cell development involves a progressive maturation of immature thymocytes into mature T-cells. Each step is characterized by a specific subpopulation of thymocytes that can be monitered based on the expression of several surface markers as well as their location in the thymic microarchitecture. A typical thymic lobule consists of an outer cortical region and an inner medullary region. The earliest thymic progenitors populate the thymus in the cortical region. At this stage, these immature T-cells are considered non-functional as they lack the expression of a T-cell receptor (TCR) complex that includes coreceptors known as CD4 and CD8. For this reason they are called double-negative (DN; $CD4^{-}CD8^{-}$) thymocytes. As they mature in the cortical region, they differentiate into double-positive (DP; $CD4^{+}CD8^{+}$) thymocytes. Only a limited amount of DP thymocytes (5%) are then selected and allowed to mature into single-positive ($CD4^{+}$ or $CD8^{+}$) T-cells within the medullary region. The DP cells that are fortunate enough to survive such stringent selection processes will eventually represent the T-cell repertoire that can not only respond to foreign pathogens in the periphery, but at the same time, prevented from responding to self [9].

Prearranging the T-Cell Assay

Transfection

An important step in the T-cell assay is the infection of the thymocytes with the miRNA. However, prior to infection of thymocytes, we must produce viruses. To produce a high titer of retroviruses, we transfect BOSC 23 cells with plasmid DNA containing our miRNA construct. In order to obtain a high level of transfection, and thus a high titer of virus, it is pertinent to prevent BOSC cells from clumping, as well as ensuring that 50–80% of the cells are confluent prior to starting the transfection. The transfection process itself is aided by Roche FUGene 6 reagent. In addition, a high titer of retroviruses can be reached with the pCLeco helper construct. After 48 h of incubation (37°C, 5% CO_2) we collect the supernatant, including the virus particles, and stored them at −80°C until infection of thymocytes.

Day −4: 5-Fluorouracil Treatment

The assay starts with the injection of 5-fluorouracil (5-FU) into a mouse via the retro-orbital vein. We treat 6-week-old male C57BL/6 mice (Jackson Laboratories)

with 5-FU at a dosage of 15 mg/kg body weight. 5-FU is a chemotherapeutic drug which induces apoptosis in murine thymocytes. Apoptotic cell death peaked in thymocytes at 18 h after administration of 5-FU. The weight of the organ and the total number of cells in the thymus were reduced by approximately 40%. The number of apoptotic cells correlated with the decrease of weight and number of cells of the organ. The specific aim of this step was to induce apoptosis in the cycling hematopoietic and stimulate the quiescent stem cells into cell cycle. The treatment causes a decrease of double-positive cells ($CD4^+CD8^+$) from over 80% to approximately 10%. Treatment with 5-FU essentially enriches for double negative thymocytes and allows us to clearly define the effects of miRNAs on the development T-cells. After the treatment we get mostly double-negative cells, which are immature T-cells that can differentiate into $CD4^+/CD8^+$ double-positive and subsequently into single-positive cells ($CD4^+$ or $CD8^+$). The total number of intact thymocytes should be around $5–8\times10^6$ cells per mouse after 4 days of 5-FU treatment [10].

Day −2: OP9-DL1 Cell Culture

The development of the T-cells requires a specialized microenvironment, which is naturally provided by the thymic microarchitecture. To mimic the support provided by the thymus, we use an in vitro co-culture system where we seed thymocytes infected with our miRNA constructs on a layer of OP9 stromal cells expressing the Delta-like 1 (DL1) Notch ligand. To achieve optimal conditions for T-cell development, we supplement the culture media with Il-7 and Flt3/Flk2 ligand [11]. This system allows in vitro experiments that were prohibitorily difficult before. The OP9-DL1 cells do not promote completely normal T-cell development, but they enable terminal differentiation of T-cells into $CD4^+/CD8^+$ double-positive and single-positive T-cells. Two days before the initiation of the thymocytes you need to split the stromal cells. The OP9-DL1 cells can be detached from the petri dish with trypsin, a protease that prevents the cells from adhering to the plate. For cultivating T-cells, OP9-DL1 were resuspended in a culture medium with 20% FCS, and 20,000 cells were seeded into each well of a 24-well plate. It is important that the cells are confluent by the time of culture initiation. If you split the OP9-DL1 cells in shorter time before the initiation, the number of seeded cells must be increased.

Day 0: Initiation of the Culture

Preparation of Thymocyte Suspension. The thymus must be extracted from the 5-FU-treated mice after 4 days. The small thymus is located under the sternum, next to the heart. The thymus is then transferred to a petri dish containing cold PBS (phosphate-buffered saline). PBS is a non-toxic and isotonic buffer solution, which is used as a washing solution or to disrupt clumped cells. To disrupt the thymus into single-cell suspension, we gently press the thymus between the frosted ends of two sterile glass slides. Afterwards, glass slides were washed with cold PBS and the solution was filtered through a 70 μm nylon mesh cell strainer. Next, we assess the total number of resuspended thymocytes by staining with Turk's solution and spin the thymocytes to obtain a cell pellet (1700 rpm/7 min).

Spin Infection. Resuspend the thymocytes in OP9-DL1 medium to a cell density of 1.2×10^6 cells/2 ml solution. The resuspended cells will be infected with the virus, containing the miRNA construct. Add 2 ml of thymocyte suspension and 2 ml of virus in a 5 ml FACS tube and 4 μl of polybrene for a final concentration of 4 μg/ml polybrene. The use of the polybrene reagent is necessary to increase the number of infected thymocytes. It can significantly improve the efficiency of successful retroviral vector-mediated gene transfer into primary cells in general. Finally, spin the tube in the

centrifuge at 2000 rpm for 2 hours at room temperature. After a few days, one can check the percentage of infected cells, indicated by the GFP expression. Typically more than 50% of the thymocytes were infected with the miRNA vector.

Culture Set-up. After the spin infection, pour out the supernatant and resuspend the cells in the T-cell culture medium and mix well. For 12 replicates, resuspend cells in 6 ml of medium in a 15-ml Falcon tube. Remove the old medium from the OP9-DL1 culture by gentle aspiration and carefully seed 0.5 ml of the infected thymocytes onto the OP9-DL1 stromal layer. Mix the cells from time to time to prevent settlement of the cells to the bottom. Incubate the plates at 37°C, 5% CO_2 human incubator.

Day 1: Changing the Medium
After 24 hours, the medium of the OP9-DL1 culture should be refreshed to guarantee optimal conditions for T-cell development. Remove culture medium by gentle aspiration without touching the bottom of the plate and add 0.5 ml of fresh, prewarmed T-cell medium.

Day 6: Adding Medium
After 6 days, add 0.5 ml fresh T-cell medium to the culture.

Day 8: Terminating the Culture and Determining Cell Development via FACS Staining
Finally, we analyze the T-cell assay by Fluorescent Activated Cell Sorting (FACS). FACS is extensively used in immunology to assess various subpopulations of cells based on expression of surface markers. This technology is based upon specific light scattering and fluorescent characteristics of modified cells and measures multiple parameters of cells or particles as they pass through a light source in a fluid stream [13]. The flow cytometer is used to monitor the expression of $CD4^+$ and $CD8^+$ T-lymphocytes using monoclonal antibodies. For the T-cell assay, special antibodies, PE-conjugated anti-CD4 antibody and PE-Cy5-conjugated anti-CD8a antibody, have been designed to mark the T-cell development. We collected the non-adherent cells and transferred them into FACS tubes. To harvest the remaining adherent cells, we added PBS containing EDTA to each well. We concentrated all collected cells by spinning them down and resuspending them in cold FACS buffer. For the calibration you need three control tubes from each sample: one unstained (GFP^+), one with PE (GFP^+; PE^+), and one with PE-Cy5 (GFP^+; PE-$Cy5^+$). Separately, mix 0.5 µl of anti-CD4-PE and 0.5 µl of anti-CD8a-PE-Cy5 in 25 µl PBS and add 10 µl of the diluted antibodies into the correct control tube.

For the analysis of the cells, prepare the antibody master mix, including 0.2 µl anti-CD4-PE antibody and 0.2 µl of anti-CD8a-PE-Cy5 per tube. While preparing cells for the FACS staining, keep cells on ice. After the preparation, incubate at 4–8°C for 15 min and vortex. To finish the preparation, add FACS buffer and spin the tubes again. Aspirate the FACS buffer, resuspend the cells in 50–100 µl FACS buffer, and analyze samples with the FACalibur.

Stefan's Results

In this project, Kosal, an undergraduate student from Stanford, and I worked to detect whether special miRNAs have any effects on T-cell differentiation. We tested several miRNAs, including miRNA-321, miRNA-130b, miRNA-155, miRNA-185, miRNA148b, miRNA-33, miRNA-196-2, miRNA-30b, miRNA-30d, miRNA-346, miRNA-124a-1, miRNA 17, miRNA 208, miRNA 9-2, and miRNA 7-1, with the methods described in the T-cell assay.

Figure 4 shows the FACS output. The first picture in the upper left corner shows the separation of the thymocytes from other cells, impurities, and debris or dead cells.

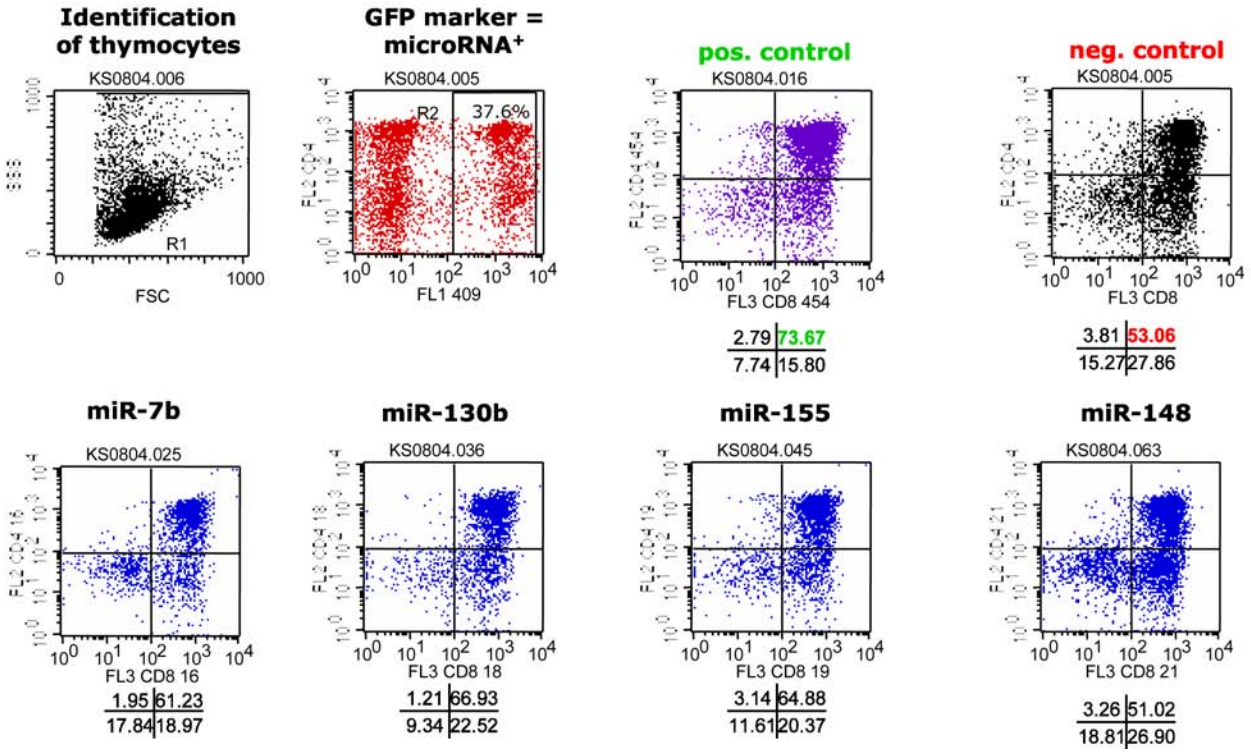

Fig. 4. Examples of the FACS output. Tested samples of miR-7b, miR-130b, miR-155, miR-148, compared to a positive control (miR-181) and a negative control (empty vector)

The Forward Scatter (FSc = abscissa) and the Side Scatter (SSS = ordinate) are used for the preliminary identification of the target cells. It is based on the approximate cell size and the complexity or granularity of the cell. Both values can be used to separate the thymocytes with a gate. Only the cells inside the gate (R1) are tested in the next step. The second graph differs between infected thymocytes and non-infected cells. The cells including the vector express the GFP marker and can be detected. Only the cells on the right side (R2) carry the GFP marker and consequently the miRNA. After these two steps, we know which cells are the thymocytes and carrying the vector. Thus we can be sure that these cells express the inserted miRNA.

The last step, the fluorescent labeling, allows drawing conclusions to the development of the T-cells. Immunofluorescence involves staining of cells with antibodies conjugated to fluorescent dyes. We use the anti-CD4 and anti-CD8 antibodies for the FACS analysis. The four charts at the bottom show examples from the FACS analysis in reference to the development of the thymocytes including miRNA-7b, miRNA-130b, miRNA-155, and miRNA-148b. The charts are divided into four quadrants and display the interaction with the CD4/CD8 antibodies. The first quadrant *(upper right)* illustrates the percentage of double positive cells, binding to both antibodies. A high number of double positive cells are a sign for a positive effect of the miRNA on the development of the T-cells. Only these cells develop both CD4 and CD8 and can mature to an intact T-cell. The second quadrant *(upper left)* presents the CD4 single-positive cells, binding only to the anti-CD4 antibody. The fourth quadrant shows also single-positive cells, regarding the interaction with the anti-CD8 antibody. The third quadrant *(lower left)* displays the double-negative cells that develop neither CD4 nor CD8 and do not interact consequently with any antibody.

Figure 5 shows the average of the double-positive cells, visualized as a box plot. By

these means one can compare the amount of double-positive cells to other constructs. As a negative control, we used an empty vector which does not express any miRNA. Consequently, this construct has no effect on T-cell development and the number of double-positive cells remains on a low level. The average of double-positive cells, treated with the empty vector, is about 55–60%.

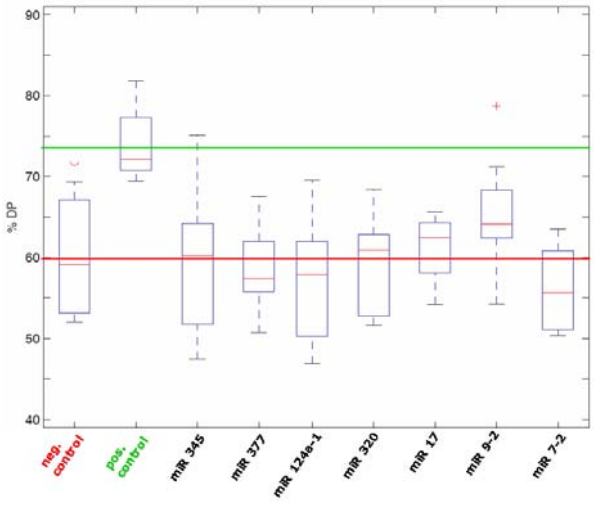

Fig. 5. All data form the FACS analysis, compared to the positive control and a negative control. The red line marks the percentage of double positive cells of the negative control, the green line for the average of DP cells of the positive control

To identify samples with a positive effect on the T-cell development, we compared the results to the miRNA-181 construct. It has been proven that miR-181 has a positive effect on T-cell development [12]. The number of double-positive cells ranges between 75 and 80%. Compared to the negative and the positive marker we determine the number of double-positive (DP) cells of our samples. All tested samples only have a very low average number of DP cells, which shows that none of the cloned miRNAs have any effect on T-cell development. In conclusion, it seems certain that miR-345, miR-377, miR-124a1, miR-320, miR-17, miR-9-2, and miR-7-2, have no effect on T-cell development. The percentage of DP cells is in some samples even below the negative marker, which does not express any miRNA.

Anna's Results

My research was assisted by Tin, a postdoc, who has been working in the Chen lab since July of this year. Our studies were based mainly on the T-cell assay and on the most important part of this experiment, FACS analyses. Tin's field of research is on the different miRNAs, their origin and classification, and their levels of expression. Figure 6 shows a set of data that we obtained from one T-cell assay. Obtaining usable results can be quite cumbersome, due to experimental errors or inaccurate data which require respawning of the assay.

A, B, and C represent the isoforms of the same miRNA families. Figure 6 describes our obtained data on the different activity of the A, B, and C isoforms. The activity of A1 is lower than that of A1+ B1 together. It is quite intriguing that there is such a big difference between the two miRNA constructs. How A and B isoforms can work together to promote T-cell development is one of the questions that members of the Chen Lab are working on.

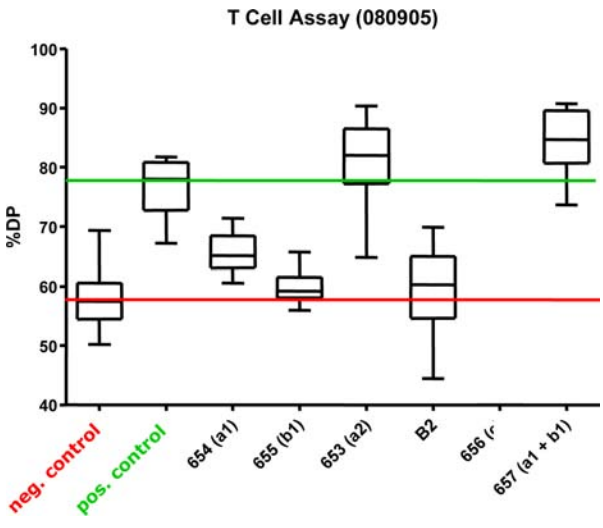

Fig. 6. Box plot: the ordinate shows the percentage of the grade expression, the x-axis shows the different family members of the miRNA

Acknowledgements

Our sincere appreciation is expressed to Professor Chang-Zheng Chen, who made this extraordinary experience possible. Moreover, we like to thank all members of the Chen Lab for their support and their acceptance. Within the short four weeks, we learned many different research methods and got well integrated into the group. Special thanks to Rosalyn, who helped us to prepare the visit in Stanford as well as to Tin, Hyeyoung, and Kosal for their assistance. We are sure that this gain of experience will influence our careers in a most decisive way.

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Fig. 1 & Fig. 2: Ambion Inc. "Introduction to microRNA" (Flash). www.ambion.com/main/explorations/mirna.html

Mouse Embryonic Stem Cells (mESCs) for Investigations in Cardiovascular Disease

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Abstract

Joseph C. Wu and the members of his lab work on gene transfer and stem cell therapy in the heart. They use various reporter genes and reporter probes in conjunction with novel imaging technologies to study these molecular und cellular events. The ultimate goal is to be able to study cardiac gene expression and stem cell survival noninvasively, repetitively, and quantitatively. Clinical interests are in the field of nuclear cardiology, echocardiography and adult congenital heart disease [1].

On the one hand, it was unfortunately not possible to have an own research project during my internship because cell culturing requires a lot of time and, especially, stem cells demand years of operating experience. But on the other hand, this made it possible for me to gain insight into every part of the techniques and research in the laboratory. I learned many aspects of molecular and cellular biology. These include cultivation of mouse embryonic stem cells, transduction of stem cells with lentiviral vectors, and isolation of differentiated cardiomyocytes. Furthermore, I followed small animal cardiac surgery involving stem cell transplantation and heart transplantation and learned about the survival and proliferation of stem cells in living animals by using various novel technologies, e.g., the techniques of molecular imaging.

Introduction

It is not only the dream of researchers and medics, but also millions of people, to find means of repairing specific tissues or even for growing entire organs! The hopes of regenerative medicine is to be able to treat various human diseases by stem cell therapy.

The human body is composed of 200 trillion single cells which belong to 200 unique cell types. All these cells are derived from an estimated 20–30 stem cells (Fig. 1). Mouse embryonic stem cells (mESC) are derived from the inner cell mass of the blastocyst from 3-day-old mouse embryos. They are undifferentiated pluripotent cells, which means they are able to grow into any cell type in the body. Later they become a specialized cell type under appropriate conditions and produce an entire organism in vivo. ESCs can differentiate into ectoderm (skin, neurons, eyes, ears), endoderm (pancreas, liver, thyroid, lung, bladder), and mesoderm (bone marrow, muscles, myocardium, vessels). ESCs are capable of self-renewal – which is the reason for their potential in research.

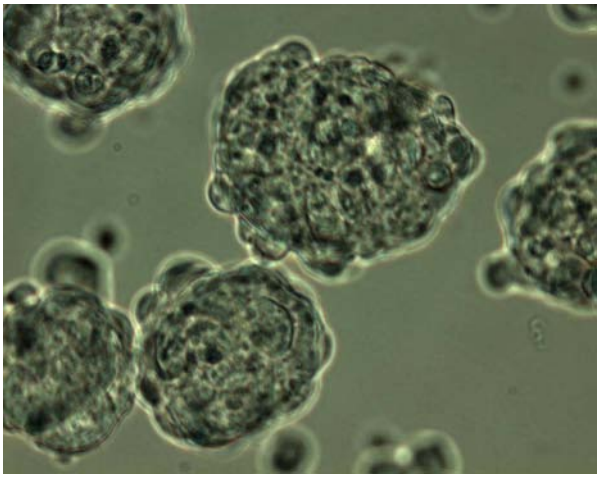

Fig. 1. Mouse embryonic stem cells

Embryonic bodies (EBs) are derived from ESCs. These differentiated cardiac cells appear as "beating clusters" in cell culture and have similar metabolic and electrophysiological properties as adult muscle cells. EBs are used for specific differentiation into different cell types, including cardiomyocytes. Mouse embryonic stem cells (mESCs) are only able to survive for about 25 days after differentiation. The reason is the absence of the necessary in vivo microenvironment. Because of the laborious process involved with in vitro differentiation of ESCs into beating cardiomyocytes, the current goals of ESC research are to figure out what happens to these cells when they are injected into the heart of living animals.

Do the ESCs survive or die? Do they differentiate into cardiac cells? Do they stay where they were injected or can they migrate throughout the body? Does a host immune response reject these cells?

For all these questions, it is necessary to know how to culture and differentiate ESCs first. This is the precondition to get an adequate amount of cells which is readily available for in vivo experiments. Afterwards the survival and proliferation of stem cells in living animals is observed by using molecular imaging techniques. It includes small animal positron emission tomography imaging using the Concorde MicroPET, bioluminescence imaging using the Xenogen IVIS system, fluorescence imaging using the Maestro camera, and nanotechnology using quantum dot imaging.

Methods

Culturing of mESCs

To be able to work with cells especially with embryonic stem cells, it is necessary to culture them outside the organism which is cumbersome because of sensitivity and fragility to environmental effects. In embryonic stem cell culturing a major concern is uncontrolled differentiation. Avoiding this requires high skill and sterile working. The recovery of the state of pluripotency requires constant monitoring of the cultures. Once the cells have started differentiating it is impossible to stop the process and above all, impossible to reverse it. Unwanted and uncontrollable differentiation begins when the cells are plated too sparsely ($< 0.5 \times 10^5$ cells/cm^2), too confluent ($> 4 \times 10^5$ cells/cm^2), or if the pH decreases too much. To maintain the pluripotent state of the mESCs they should have a density of 1.5×10^5 to 4×10^5 cells/cm^2 and they have to exist as a single-cell suspension. The state of pluripotency can be maintained by culturing either on mouse embryonic fibroblasts (MEFs) or using leukemia inhibitory factor (LIF) [2].

Culturing mESCs means to feed them, take care of their condition and confluency, split them and freeze them when there are more than needed. A proper culturing of the cells is the basis for a successful and specific differentiation.

The following describes the different techniques of culturing and guiding the differentiation of mouse embryonic stem cells as carried out in the laboratories of J. C. Wu and which I were able to witness and learn about during the internship.

Thawing mESCs

"Freeze slowly – thaw quickly": first, the tube with the frozen cells is taken out of the liquid nitrogen and immediately placed into the 37°C water bath. The cells must remain there for ∼3 min until they have melted. Then the cells are pipetted with 5 mL mESC medium into a 15 mL tube. Afterwards, 1 mL of these are transferred into the tube in which the cells were originally frozen. Pipetting needs to be done carefully up and down to ensure that all cells have become resuspended. This suspension is transferred back into the 15 mL tube. The cells must be pelleted by centrifugation (800 rpm, 3 min, 4°C) and unhinged of the frozen media. During this time, 6 mL of the mESCs medium is pipetted to a gelatine-coated 100-mm dish. It is important that the gelatine

has been removed by aspiration. The supernatant is then carefully sucked off. Now, the pellet is resuspended in 4 mL of the mESC medium, pipetted into the prepared dish, and swayed gently. At last, the cells are placed back into the incubator [2].

Changing the Medium

The medium must be changed daily. The color of the mESC medium is an indicator for the *p*H value: fresh mESC medium with an optimal *p*H is red. During growth the cells consume nitrogen and *p*H decreases. To change the medium the old medium is first suctioned off. If many dead cells or sediments are present, it needs to be washed with 10 mL PBS and again aspirated. Then 10 mL of fresh mESC medium is added and the cells are returned to the incubator.

Splitting mESCs

mESCs must be split when reaching ∼80 % confluency, generally every second day. First the medium must be sucked off. Then 10 mL of PBS are added to wash the cells; the dish is gently swayed and the PBS removed. After this, 3 mL 0.25 % trypsin are added with EDTA to detach the cells from the gelatine bottom and obtain a single-cell suspension. The dish is then placed in the incubator for 3 to 5 min until all cells are released. Then, 5 mL mESC medium are added to stop the trypsinization. The suspension is pipetted into a 15-mL tube. Centrifuging pellets the cells and unhinges the trypsin. Then, 9 mL of the mESCs medium are pipetted to a gelatine-coated 100-mm dish. Again the supernatant is aspirated. To split the cells with a rate of 1:4, 4 mL mESC medium is added to the tube and the cells gently resuspended by pipetting up and down. Thereafter, 1 mL of the suspension are taken and added to the dish with gently swaying and the dish is then returned to the incubator [2].

Freezing mESCs

"Freeze slowly – thaw quickly": mESCs can be frozen at any time, but is generally done when they reach ∼80 % confluency. At this state cells on a 100-mm dish can be frozen down into 1 mL and split up into 2 to 4 100-mm dishes by rethawing. Deal with The mESCs are handled the same way as described above ('Splitting mESCs' – trypsinizing and pelleting by centrifugation). After this, the supernatant is sucked off. Then 0.9 mL serum and 0.1 mL DMSO are added to resuspend the cells. Now, they are placed into the frozen vial. The tube is stored for 30 – 60 min at −4°C, then 2 h (no more) at −20°C, and thereafter overnight at −80°C in the freezer. If the cells are needed within the next 6 – 8 weeks they can remain there for that time period at −80°C. Stored in liquid nitrogen (−140°C) they keep for several years [2].

Culturing mESCs is not the actual intention. It is the necessary base of operation for a controlled cell differentiation. In order to obtain as good as possible results, i.e., a low as possible percentage of uncontrolled differentiated cells, the cell culturing must be performed very accurately and under perfectly sterile conditions. Now, the cells can be affected (with special additives to the medium) to start controlled differentiation into cardiomyocytes.

The ultimate goal would be to grow cardiomyocytes out of embryonic stem cells to inject them into the body. There they are intended to repair damaged heart tissue all by themselves and animate original cells to do the same. The hope is to prevent surgery and have a natural remedy for tissue damages strokes. In order to reach this distant goal more basic research is needed.

Cardiomyocytes differentiated out of mESCs are observed with the technique of molecular imaging to search out how the cells act inside the body:

Differentiation into Cardiomyocytes

The mESCs are handled the same way as described above under 'Splitting mESCs' – trypsinizing and pelleting by centrifugation. Now the cells are resuspended in complete medium and 30 µL of this suspension

is used. This is mixed with 30 µm Tranpan blue and placed in the cell-counter and adjusted to 1×10^6 cells/mL. Then 54 µL of the cell supension are added to 2 mL mESC differentiation medium. Afterwards 15 mL PBS are added to a 150-mm plate. A multipipette is used to place single drops (18 µL per drop = 370–400 cells per drop) on the top cover of the dish. The top cover is carefully turned over and placed into the lower part. Two days later, the lid is turned over. There must be an embryonic body (EB) in every drop. Now transfer the EBs to a 96-well plate, 1 EB in each well with 100 µL differentiated medium. Three days after the last step, transfer the EBs to a 48 gelatine-coated well plate. The medium must be changed every other day.

Nine days after the differentiation, the beating areas will be visible. Up to day 25 after the differentiation the beating rhythmic will increase from 60 beats per minute to 200–300 beats per minute – the same frequency as the heartbeat of a mouse! Then it will disappear because an intact real natural microenvironment is absent.

Molecular Imaging in Living Subjects

The following describes how the survival and proliferation of stem cells in living animals can be followed by using molecular imaging techniques. An example of the options available using the new technique is shown in Fig. 2.

Molecular imaging is a multidisciplinary field that requires close collaboration among basic cell and molecular biologist, clinician-scientist, chemist, pharmacologist, medical physicist, and biomathematician. It can be defined as "non-invasive, repetitive, and quantitative imaging of cellular and molecular events in living subjects" [4]. It can also help to define the in vivo mechanisms of diseases in the physiologically authentic environment and to provide the potential of understanding evaluation of treatment [3, 4].

Important goals of the biomedical research in molecular imaging involve the development of a non-invasive in vivo imaging method, the reflection of specific cellular and molecular processes (e.g., gene expression, drug effects, or complex molecular interactions). Furthermore, it is a means for monitoring molecular events near-simultaneously, to observe trafficking and targeting of cells, and a method to help to optimize drug and gene therapy [3, 4].

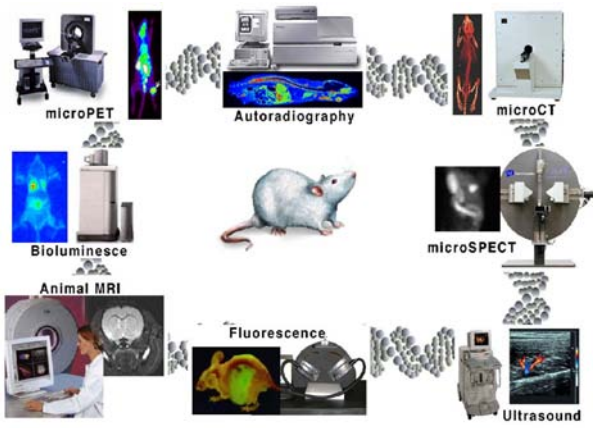

Fig. 2. Various techniques of molecular imaging

Molecular imaging is rapid, reproducible, and quantitative. It has become possible to arrange time-depended experiments and follow them over a longer period of time in the same animal. This new source of imaging techniques is useful for phenotype screening of transgenic and gene-targeted animal models. In-vivo molecular imaging in the same animal at different time points and the use of diverse assays reveals the possibility to observe progressive changes and temporal development under real physiological conditions and without the exigency of its death [3, 4].

Maestro for Quantum Dot Imaging

Maestro [5] is a fluorescent-based in-vivo imaging system. It can be used for multispectral acquisition and analysis. Its advantages are its high sensitivity and solution, multicolor flexibility in the excitation wavelengths, quantitative accuracy in the visible and near-infrared, labels which can be detected in the same sample, and the multispectral unmixing of the autofluorescence.

Furthermore, spectrally overlapping labels can be eliminated and unmixed signals can show and quantify individual signal activity. Labels with emissions from 420 nm to 720 nm and 500 nm to 950 nm can be used (e.g., eGFP, dsRed, and quantum dots) [6, 7]. Figure 3 shows a series of measurements made by Maestro.

Quantum dots are a new class of in-vivo biomolecular and cellalur labels using fluorescence. They are tiny light-emitting semiconductor crystals on the nanometer scale (2–8 nm) and have lots of unique optical and electronic properties: the light emittion depends on their size, they improve the brightness of the signal, are nearly resistant against photo-bleaching, several thousand times more stable than organic dyes, and are able to simultaneously elicite multiple fluorescence colors. So it becomes possible to image and track multiple molecular targets simultaneously, each labelled with a different color. Labelled structures and molecules can be directly observed. Quantum dots are very stable under complex in-vivo conditions and are nontoxic to cells and organisms, but long-term studies of in-vivo toxicity and degradation are necessary. In addition to their ability to absorb a wide spectral range, they have a high level of brightness (10 to 20 times brighter than organic dyes) and photostability. The longer excited lifetimes of quantum dots make it possible to separate the fluorescence of the quantum dots from background fluorescence. Another important appropriation is the binding specificity or the targeting abilities of the quantum dots. A combination of therapeutics and quantum dots can lead to a simultaneous treating and monitoring of diseased tissue [6, 7].

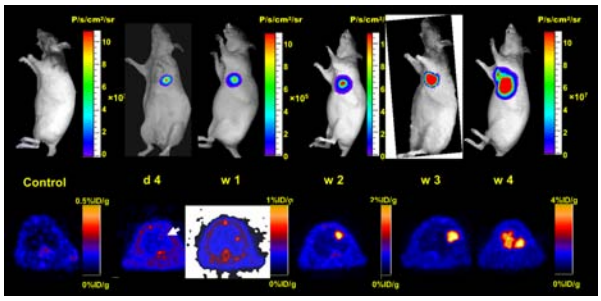

Fig. 3. (A) Molecular imaging of cardiac cell transplantation into rat. The cardiomyoblasts expressing Fluc reporter gene emits cardiac bioluminescence at day 4, week 1, 2, 3, 4 imaged with IVIS system. Control rat has only a background signal. (B) MicroPET imaging of cardiomyoblasts expressing HSV1-sr39tk reporter gene into the same rat by quantifying the [^{1}8F]-FHBG reporter activity

Labeling mESCs with Quantum Dots
In order to label cells they should have a density of $2{\times}10^4$ cells/well (in an 8-well Lab-Tek chambered coverglass system) and be incubated at 37°C under 5 % CO_2 overnight. Now, 1 µL of each Qtracker Reagent A and B is premixed in a 1.5 mL microcentrifuge tube and incubated for 5 minutes at room temperature to prepare a 10 nM labelling solution. Then 0.2 mL of fresh full growth medium are added to the tube and vortexed for 30 seconds, followed by addition of 0.2 mL of labelling solution to the well with the cells (for cells in suspension, $1{\times}10^6$ cells can be added to this labelling solution). Afterwards it is incubated at 37°C for 60 minutes and stirred every 5 min. Then the cells are washed twice with full-growth medium. Now the labeled cells can be observed under confocal microscopy [8].

Discussion

In further experiments, J. C. Wu and his collaborators will attempt to further understand the behavior, proliferation, and influence of stem cells within the body using reporter gene imaging techniques. Also, research efforts are concentrated on the molecular and cellular mechanisms of ESC differentiation in cardiac cells. Answers to these questions may eventually help clinical investigators repair damaged heart tissue and thus may help to save millions of lives.

Stem cell research has provided a great promise for medical and clinical treatment in the 21st century. However, dreams of

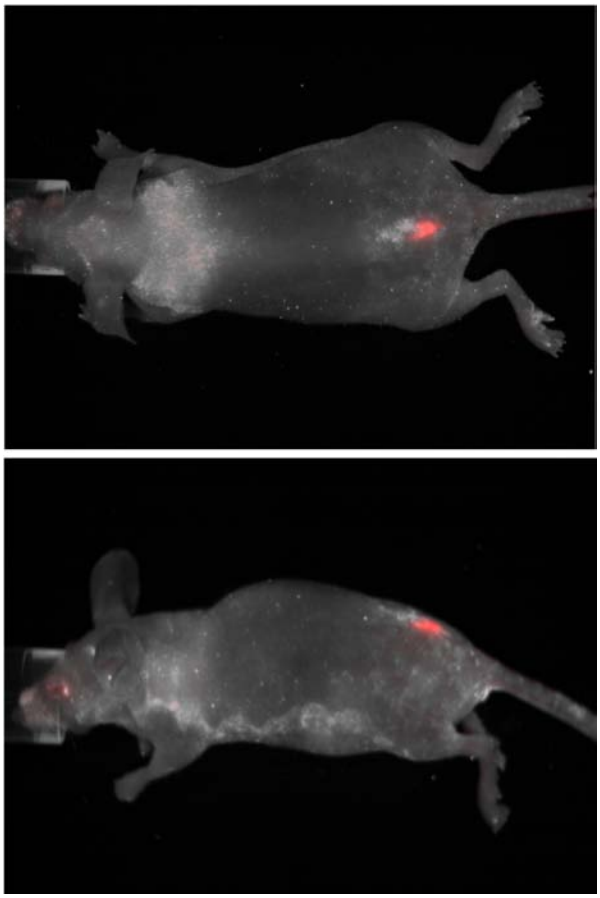

Fig. 4. mESCs labels with quantum dots and injected in the back of a nude mice. Photos taken with Maestro

growing entire organs and tissues, and healing of diseases and regulating dysfunctions will require intense efforts now and in the future.

Acknowledgements

Many thanks to Joseph C. Wu for inviting me to his lab and for his kind support. In addition to learning about the culturing and differentiation of mESCs, I learned much about the various molecular imaging techniques. I am indebted to all members of the Wu Lab, especially to Feng Cao, Shu An Lin, and Yaohung Yang for accepting and integrating me, taking time to supervising me, making me fell welcome, and patiently answering all my questions. During the four weeks I was able to gain insight into state-of-the-art methods and scientific concepts, I learned a lot – my internship at Stanford University opened new horizons and will remain an unforgettable experience.

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Figures 1–4 are kindly provided by members of the Wu Lab

Generating an AAV5 Vector for the Treatment of Hemophilia B

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Abstract

Hemophilia B is a rare but severe inherited disease. It has an incidence of about 1:30,000 live male births and 400 babies that suffer from this disease are born in the USA every year (Miao et al. 2000).

Hemophilia B is a bleeding disorder which is caused by a defect in the *Xq27.1* gene located on the X chromosome. This gene usually codes for a so-called clotting factor, a protein which is involved in the blood clotting process. Current gene therapy research is aimed at developing different gene therapy methods for the treatment of hemophilia, for example, by replacing the defective gene by a factor IX coding gene sequence that is transfected into the liver by a vector and expresses the clotting factor in vivo (Fig. 1).

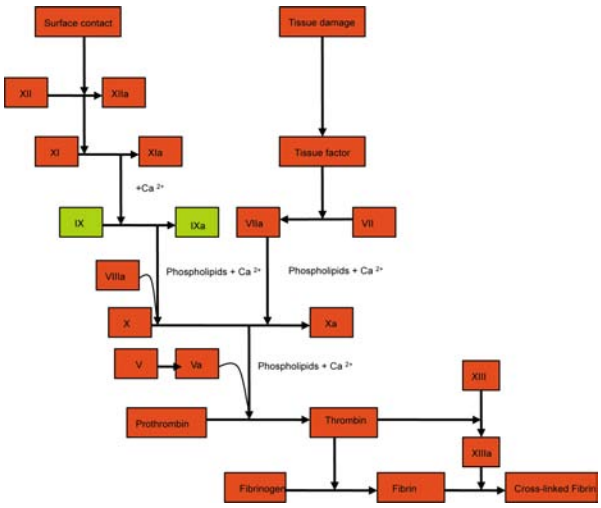

Fig. 1. The blood clotting process

Introduction

To transfect liver cells researchers make use of the viral ability to penetrate cells and integrate their genome into the DNA of the host cell. Therefore the gene of interest (coding for factor IX) is cloned into viral plasmids and transfected into tissue culture cells (packaging cell line) where the DNA replicates and is packed into viral capsid envelopes forming the final viral vectors. This process requires replication genes, the packaging information, and a complex system of promoters and enhancers are necessary.

For gene therapeutic approaches to treat liver diseases adenoviruses were most commonly used. The wild type of adenoviruses causes respiratory diseases and appears rather frequently in the human organism. The viral vector is derived from the infectious genes but there always exists the risk of recombination between the vector and the wild type virus which could lead to an excess of viruses in the human body and finally causes multiorgan failure. Many people have antibodies against several adeno serotypes due to their wide dispersal among human beings. This reduces the safety and efficiency of adenovirus vectors and is one reason why research nowadays concentrates rather on a different family of viral vectors: the adeno-associated viruses (AAV) which belong to the parvoviridae family and have been used successfully to provide long-term expression of the genetic target sequence protein (Grimm et al. 2003).

They appear to provide high target specificity and no toxicity but cannot replicate on their own once they penetrated the host cell. AAV lack several early genes which are involved in the replication and packaging process which makes them dependent on a helper virus. This virus provides the proliferation genes (the rep gene for replication, the cap gene for capsids that are needed for packaging). This helper virus is an adenovirus (→ adeno-associated virus). Without the helper viruses AAVs integrate into chromosome 19. As soon as the helper appears, AAVs begin to replicate. Because AAV are not associated with human disease, they are rather useful for human gene therapy. Furthermore, they are very stable when they are transfected into non-dividing tissues (Thomas et al. 2003).

One of the disadvantages of AAV is that they have a rather small genome which is flanked by two ITRs (inverted terminal repeats). In between the ITRs there is just space for cloning in a 4.7 kb genome.

Material and Methods

Packaging Cell Line

We used 293-T human embryonic kidney cells whose genome had been transformed with parts of the Adenovirus 5 genome so that they are permissive for the growth of Adenovirus 5 and similar serotypes. The cells were ordered at the American Tissue Culture Center (ATCC) and stored in stocks at 37°C. We grew them in tissue culture flasks in a medium that principally consists of DMEM (Dulbecco's modified Eagle medium), FBS (fetal bovine serum), L-glutamine, and antibiotic-antimycotic (penicillin-streptomycin).

In order to provide a sufficient amount of cells for the transfection with an ideal confluence of about 7×10^4 cells per flask, an immediate cell split (passage I) and a non-immediate cell split (passage II) were necessary.

The cell splits were carried out according to a tissue culture standard protocol during which the cells are loosened with trypsin, washed off with phosphate buffered saline, and counted with a hemocytometer (Fig. 2).

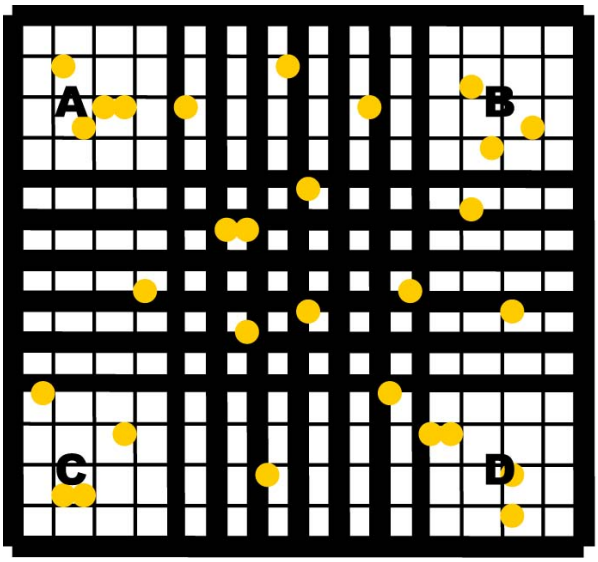

Fig. 2. Counting cells (yellow spots) with a hemocytometer

Preparation of the DNA Plasmids

We used two different types of DNA plasmids for our transfection. The CM1 plasmid, containing the *Xq27.1* gene flanked by the AAV inverted terminal repeats and the *pdF5* plasmid containing the adenoviral helper genes as well as the genes for replication and encapsulation (Rep and Cap). In addition, both plasmids contained an RFP gene (Red Fluorescent Protein).

In order to prove that the DNA plasmids used for the transfection were still in a proper condition after the period of storage, we carried out a restriction enzyme digest. After taking the OD values of all samples with a spectrophotometer (Beckman DU 530 Life-Science UV/Vis) the plasmids (CM1 and two different types of pDF 5 plasmids) were digested with a combination of the restriction enzymes *Eco*RI and *Hin*dIII. Then, the samples and a standard marker were loaded on a 1% agarose gel with ethidium bromide and run for about 30 min at 10 Volt (cf. Results).

Transfection
We performed a double transfection with 25 µg of CM1 and 50 µg of pDF5. Therefore, a transfection solution with 250 µl CM1 and 667 µl of pDF5 (volumes determined with OD values), 80 ml of a 0.3 M calcium chloride solution and 80 ml of 2x HBS was set up and distributed to the tissue culture flasks. After an incubation period of about five hours, the 40 ml of media in each flask were changed to FBS-free DMEM medium to stop the cell growth. One day after the transfection we observed the cells with an electronic fluorescent microscope (cf. Results).

Harvest
Three days after the transfection we were able to harvest the virus vectors. Then 0.5 ml EDTA (pH 8.0) were added to each flask. After an incubation time of about 5 – 10 min, cells were collected and spinned at 3000 rpm for about 15 min. The remaining cell pellet was resuspended in Benzonase buffer.

Purification
Three freeze-thaw cycles were performed with the sample, switching between 10 min in a 37°C water bath and 10 min in a dry ice/ethanol bath.

Benzonase endonuclease was added to the sample and incubated at 37°C for one hour. Afterwards the solution was spinned three times at low temperatures to stop the activity of the endonuclease. After each spin the supernatant was transferred and mixed with a calcium chloride solution (first spin) or a 40% PEG8000/2.5 M NaCl solution (second spin). In the end, the pellet was resuspended in Na-HEPES/EDTA suspension buffer overnight.

On the following day, a cesium chloride precipitation of the viral vector was carried out. After addition of cesium chloride, the refraction index (RI) of the suspension was adjusted to 1.3710 with Na-HEPES/EDTA suspension buffer and a refractometer, transferred to an Optiseal tube and topped off with CsCl solution (RI= 1.3710). To separate the virus fraction from defect particles the solution was spinned at 45 K for 23 hours in a high-speed 70 Ti rotor.

When the first cesium chloride gradient was completed, the fractions were collected through a needle in a hole at the bottom of the centrifuging tube. The fractions with an RI between 1.3711 and 1.3766 were pooled because the viral vector fraction is located there due to its density and a second precipitation was carried out to achieve a further purification. The fractions were collected according to the following standard table:

Table 1. Standard table for cesium chloride precipitation (Nakai et al., 1998) with RI average values compared to the actually measured RIs. The maximum amount of pure viral vectors is located at a RI of 1.3710. Therefore, fraction #14 is collected and used for the following steps

9	1 ml		
10	1 ml	RI Average	Actual RI Values
11	0.5 ml	1.3781	1.3770
12	0.5 ml	1.3760	1.3750
13	0.5 ml	1.3755	1.374
14	0.5 ml	1.3737	1.371
15	0.5 ml	1.3720	1.369
16	0.5 ml	1.3710	1.367
17	0.5 ml	1.3700	
18	0.5 ml	1.3698	

Ultrafiltration
This step was necessary to get rid of the CsCl and further purify with the help of a filtration cartridge (100,000 Dalton MW). The virus complex is very large and passes through (∼1,000,000), CsCl and proteins smaller than 100,000 are removed through the permeate line. A circuit for UF/DF was conducted and the virus was purified under sterile conditions. The circuit was primed with 100 ml sterile water without back pressure (closed permeate line, but open efferent line). The pump drive was adjusted to a speed of 385 rpm, measured with a

tachometer. Then the circuit was primed for a second time with 50 ml sterile water and a back pressure of 13.5–13.9 psi. During a third prime with 100 ml 5% sorbitol/PBS the back pressure was adjusted to 13.5–13.9 psi. This back pressure was kept constant during the actual ultrafiltration/diafiltration circuit. Just before all the vector solution was taken up, another 12 ml of 5% sorbitol/PBS were added into a 15-ml tube and the cycle was repeated. Just before the vector solution was completely taken up, the pump was stopped and operated in the backward mode to collect the concentrated vector solution into a 15-ml tube. The vector solution was filter-sterilized through a 0.22-µm filter and we received 2–2.5 ml of the final product.

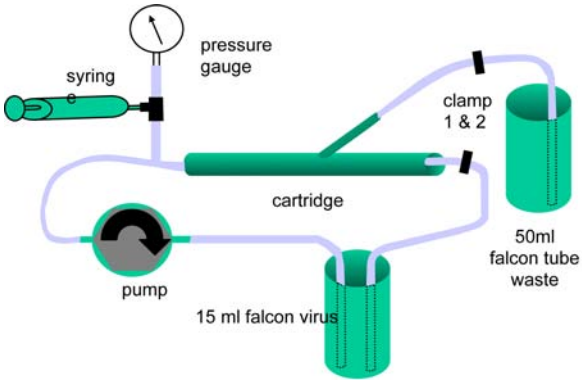

Fig. 3. Ultrafiltration cycle with pump, cartridge, and pressure gauge

Dot Blot

To determine the titer of our viral vector particles, four different dilutions of our final sample were set up in duplicate (1:10, 1:20, 1:40, 1:80) and compared to samples of the original pAAV DNA (standard curve) in a dot blot analysis.

These samples were blotted by using the Bio-Rad dot blot apparatus. The dot blot was processed like a Southern blot with 32p-labeled DNA probe, overnight hybridization, washed and exposed to a film to check the quality of the spots. Finally, the blot was quantified by a phosphoimager.

Results and Discussion

The DNA bands in the agarose gel that we ran before the transfection (to test if our plasmids were in a proper condition) (Fig. 4) were unequivocally distinguishable. This showed that the genome was still intact. If it had been degraded, small DNA particles of various sizes would have produced an indistinguishable smear across the lanes. The comparison with the standard marker and the OD values of the samples provided clear evidence that our DNA sample solutions had a proper concentration of DNA plasmids.

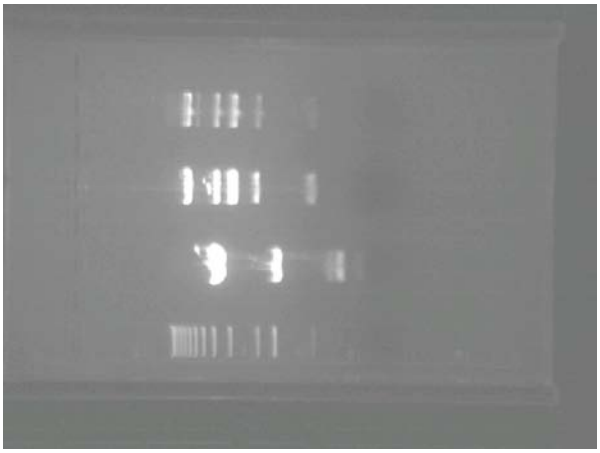

Fig. 4. Agarose gel after restriction enzyme digest. Lanes (from *top* to *bottom*): standard marker, *Cm1* plasmid, *pDF5* plasmid I, *pDF5* plasmid II. Compared to the standard marker, the plasmid lanes show wide, bright but clearly distinguishable bands. This implies that the samples contained great amounts of intact plasmid DNA

Under the electron microscope we detected a large amount of red fluorescent substance which could be easily identified as the glow of the Red Fluorescent Proteins (RFP). For the fluorescent proteins were expressed in the host cells we knew that the transfection had worked and the genes were expressed in vitro. Due to the amount of fluorescent proteins which was rather high we could see that in a lot of cells the genome had been replicated and expressed (Fig. 5).

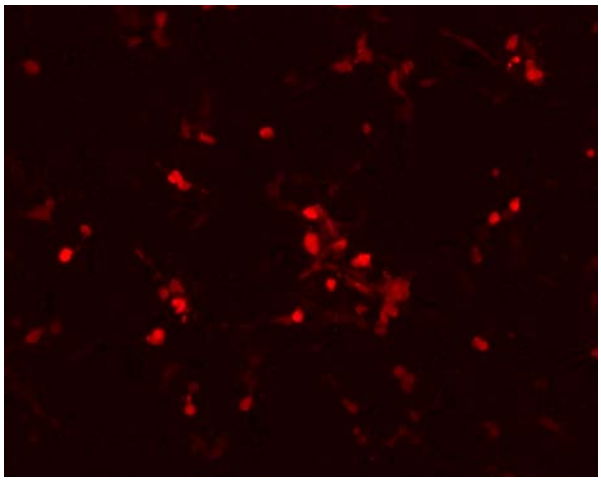

Fig. 5. One day after the transfection: the cells glow red under the fluorescent electron microscope

When we finished our internship, the result of the dot blot was not available yet, because the radioactive hybridization was still in progress. But our lab informed us that the dot blot did not provide clear results. Although it showed a differentiated standard curve, the wells of the samples were rather hard to recognize.

Since the first two control steps (restriction enzyme digest and RFP expression) proved that the transfection process had been carried out successfully up to this point we have to consider which of the following steps finally led to the unexpected low virus titer.

Regarding the packaging cell line we could neither find the reason for the low titer before nor after the transfection. The cell line grew vigorous under proper conditions and was in a good confluence until we cracked the cells up to get out the virus capsids. Although the genes were replicated and expressed, a mistake might have occurred during the packaging process, so that mostly defective vectors were produced.

Another possibility might be that the purification was not carried out properly. Since the measured RI values differ from the ideal average RIs it might be possible that the fractions were shifted slightly so that we could only collect a small part of the actual virus fraction.

Furthermore, we could have made a mistake during the ultrafiltration process. It is rather difficult to catch the exact moment when to finally close the cycle with the clamp. If it is done too early, the concentration of the vector solution is still too low. If it is closed too late, a part of the vectors is washed off into the trash tube together with the rest of the cells and the cesium chloride.

One final possibility would be that we got a rather high virus titer but did something wrong during the dot blot so that just the result of the dot blot was defective.

Acknowledgements

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Molecular Identification of the Tortoiseshell Gene in the Syrian Hamster

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Subcloning and RT-PCR Amplyfying of Mitochondrial Hsp75 into a Retroviral Backbone and Expression Studies in C6 Cells

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Introduction

Anti-apoptotic Effects of Hsp70

The 70 kDa heat shock protein (Hsp70) is induced in response to a variety of insults in the central nervous system (CNS), including ischemia. It is thought to act as a molecular chaperone to prevent protein denaturation and facilitate correct protein folding [1]. Overexpression of inducible cytosolic Hsp70 has been shown to protect from cerebral ischemia in animal models of stroke as well as in cell culture models [2]. Hsp70 has been shown to protect from both necrotic and apoptotic cell death [3, 4, 5] in both global and focal ischemia [2, 6].

Role of Mitochondria in Cell Death

Mitochondria are important regulators of cell death by means of energy production, cellular calcium homeostasis, production of reactive oxygen species (ROS), and the capacity to release apoptotic proteins such as cytochrome-c and apoptosis inducing factor into the cytosol [7]. The loss of glucose and O_2 in ischemia leads to dysfunctions in the respiratory chain [8, 9], especially in Complex IV (cytochrome oxidase) where electrons are transferred to O_2 as the final electron acceptor. This results in alternation of mitochondrial membrane potential (MMP), inversion of the F_1F_0 ATPase, now consuming ATP to pump H^+ into the intermembrane space, and release of cytochrome-c into the cytosol. Hence mitochondria are an important regulator of cell death in ischemia. The effects of Bcl-2 family proteins on mitochondria in ischemia have widely been studied in the past [9, 10] as well as the neuroprotective effects of cytosolic Hsp70 in ischemia [3, 11, 12]. However, the role of mitochondrial Hsp75 (mtHsp75) in ischemia has not yet been studied. By subcloning the gene for human mthsp75 into the retroviral backbone *pBABEpuro* and transfecting it into primary cultures of astrocytes and neurons we now want to study potentially neuroprotective effects of this protein in animal and tissue culture models of stroke and ischemia.

Material and Methods

Purification of Plasmid-DNA

Plasmids-DNA was purified from 2 – 5 ml bacterial cultures using the Plasmid Mini Kit by *QIAGEN* (Catalog No. 12125). Plasmids of 200 ml bacterial cultures were purified using the HiSpeed™ Plasmid Maxi Kit by *QIAGEN* (Catalog No. 12663).

Cultivation and Transformation

For bacterial growth *E. coli* from the *OneShot® MAX Efficiency® DH5α™-T1®* kit by *Invitrogen* (Catalog No. 12297-016) were used. LB-agar plates were prepared using *LB-agar Medium* capsules from *Q · BIOgene* and adding ampicillin to a final concentration of 50 µg/ml. Liquid LB/Amp medium was prepared using *LB-broth, Miller* powder from *Becton Dickinson* and adding ampicillin to a final conentration of 50 µg/ml.

For transformation 1 – 5 µl of plasmid DNA were added to 25 µl of *DH5α™-T1®* *E. coli*, incubated on ice for 30 min, and heat shocked for 30 sec at 42°C. 250 µl of the SOC-medium (included in the *Invitrogen*-kit) were added, incubated at 37°C for 1 h, then plated out on LB-Agar/Amp plates, and incubated at 37°C overnight.

Clones were then transferred from the plates into starter cultures of 4–5 ml LB/Amp medium and incubated for 8 h at 37°C. For Mini-Prep 3 ml of the starter culture were centrifuged at 6000 rpm for 15 min at room temperature, and the resulting pellet was purified using the kit mentioned above. For Maxi-Prep 300 µl of the starter culture were transferred to a 200 ml LB/Amp-culture, incubated at 37°C overnight, centrifuged at 6000 rpm for 15 min at 4°C in a Sorvall SLA-3000 rotor, and the resulting pellet was purified using the kit mentioned above.

Isolation of RNA from Animal Tissues

RNA was isolated from fresh rat brain or from brain samples frozen at −80°C in RNAlater Stabilisation Buffer by *QIAGEN*. Tissue was disrupted and homogenized by processing it 20 – 30 times through a 23G needle. Lysate was then centrifuged for 10 min at full speed (13,000 rpm) in a Heraeus Biofuge pico. Supernatant was used for RNA isolation with the RNeasy® Protect Mini Kit from *QIAGEN* (Catalog No. 74124).

Reverse Transcription of RNA and PCR Amplification of cDNA

Reverse transcription was performed using the *SuperScript III Cells Direct cDNA Synthesis System* by *Invitrogen* (Catalog No. 18080-200). For DNase I digestion 20 µl of RNA were mixed with 5 µl DNase I and 3.2 µl 10 DNase I Buffer, incubated for 5 min at room temperature, 2 µl of 25 mM EDTA were added, and then incubated at 70°C for 5 min. Subsequent first strand cDNA Synthesis was performed according to the protocol provided with the kit.

Rat brain cDNA was PCR-amplified using two different pairs of primers (mitoHSP70-F 5′ ATCGGATCCGTTTGTTCTTGCCCTCGTA 3′ and mtHSP70-R 5′ ATCGTCGACCTACTGCTTCTCTTCCTTT 3′; MtHSP70-F 5′ ATTGGATCCTCTGTGGGGTGTTTGTTCTT 3′ and MtHSP70-R 5′ GTAGTCGACCTACTGCTTCTCTTCCTTTTGATC 3′) both intoducing a *Bam*HI site (G/GATCC) at the 5′ end and a *Sal*I site (G/TCGAC) at the 3′ end. PCR was performed with *Platinum® Taq DNA Polymerase High Fidelity* by *Invitrogen*(Catalog No. 11304-011). Additonally, different concentrations (0, 0.5, 1, 2, 3, 4) of *PCR$_X$ Enhancer Solution* by *Invitrogen* (Catalog No. 11495-017) were tested. For a positive control of cDNA, primers for *GAPDH* were used.

Gel Purification of DNA

DNA fragments from restriction digests designated for ligation were separated by gel electrophoresis (1% agarose gel, 1X TAE buffer) and then isolated from the gel using the MinElute™ Gel Extraction Kit by *QIAGEN* (Catalog No. 28604).

Subcloning of Human *mtHSP75* into Retroviral Vector *pBABEpuro*

A clone of human *mtHsp75* cDNA in *pBluescriptSK* (a kind gift of Dr. Richard I. Morimoto) was digested with *Bam*HI, *Sal*I and *Pvu*I. *pBABEpuro* was digested with *Bam*HI and *Sal*I and dephosphorylated using CIP alkaline phosphatase by *New England Biolabs*. The 2.8 kb *Bam*HI - *Sal*I *mtHsp75* cDNA fragment resulting from the digestion of *pBluescriptSK-clone 13 (mthsp75 cDNA)* was then ligated with the *Bam*HI - *Sal*I fragment of *pBABEpuro*, using T4 DNA ligase by *New England Biolabs*, thus creating the new vector *pBABEpuro-humtHSP75*. The construct has been verified by sequencing.

Cultivation of C6 Cells

C6 cells for transfection were cultivated in *Minimum Essential Medium* (*Invitrogen*, Catalog No. 11575-032) supplemented with 23 mM $NaHCO_3$, 18 mM D-glucose, 2 mM L-glutamine, and 10% equine serum (Hyclone, Logan, UT, USA) and incubated at 37°C, 5% CO_2.

Transfection and Immunostaining of C6 Cells

C6 cells were transfected with Effectene Transfection Reagent from *QIAGEN* (Catalog No. 301425) according to the protocol in the accompanying handbook.

For immunostaining cells grown in 24-well plate were washed 2 times with ice-cold PBS, fixed on ice with acetone/methanol (1:1 mixture) and washed with PBS: then 0.1% Triton X-100 was added and incubated for 15 min and the cells were incubated with blocking buffer (PBS supplemented with 0.1% Triton X-100 and 3% bovine serum albumine) for 1 h at room temperature. Cells were incubated with a 1:50 dilution of primary antibody (Anti-Grp75 Mouse Monoclonal, Stressgen, Catalog No. SPA-825) in blocking buffer, washed 3 times for 10 min with blocking buffer, incubated with a 1:50 dilution of GFP-tagged secondary antibody (Donkey Anti-Mouse IgG, Jackson ImmunoResearch Laboratories, Catalog No. 715-025-150) in blocking buffer, washed for 10 min with blocking buffer and 2 times with PBS. Cells were then observed with a fluorescence microscope.

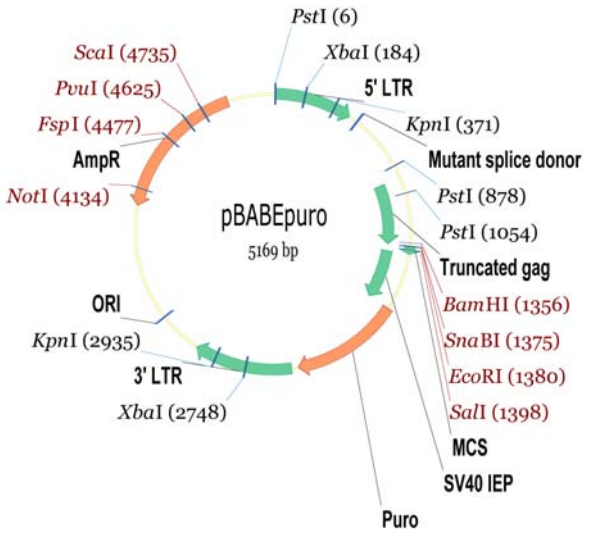

Fig. 1. Plasmid map of *pBABEpuro*

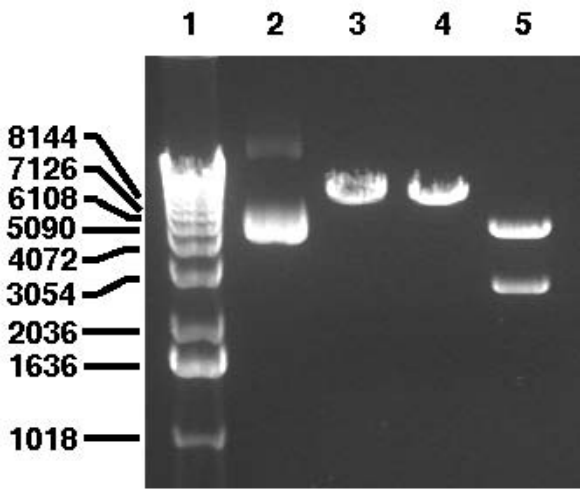

Fig. 2. Control digestion of *pBABEpuro-humtHsp75* (1% agarose, 1X TAE-buffer) (1) 1 kb DNA Ladder (*Invitrogen*); (2) *pBABEpuro-mtHsp75* undigested; (3) digested with *Bam*HI; (4) digested with *Sal*I; (5) digested with *Bam*HI and *Sal*I

Results

Human *mtHsp75* has been successfully subcloned from *pBluescriptSK-clone 13* (*mthsp75*) into the multiple cloning site of *pBABEpuro* (Fig. 1), which was verified by restriction digest and gel electrophoresis (Fig. 2). Unfortunately RT-PCR amplification of rat *mtHsp75* from rat brain RNA did not lead to any results. However, control PCR with *GAPDH*-primers indicated that cDNA was at least present in the sample. Further adjustments to PCR conditions and testing out of different primers will be necessary to solve this problem.

C6 cells have been transfected with *pBABEpuro-humtHsp75* to verify expression of *mtHsp75* and to confirm its localization in mitochondria (Fig. 3).

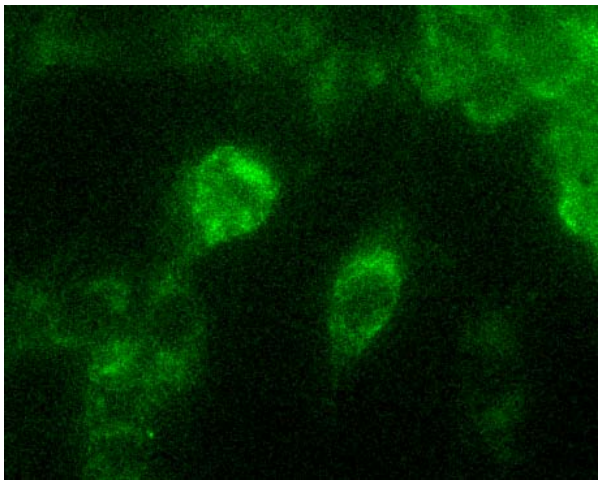

Fig. 3. Immunostaining of C6 cells transfected with *pBABEpuro-humtHsp75*

Discussion

Since expression of human *mtHsp75* in *pBABEpuro-humtHsp75* transfected cells and its localization in mitochondria could be verified (Fig. 3) further experiments can now be performed to study the effects of *mtHsp75* in animal and tissue culture models of global and focal ischemia. It is hypothesized that overexpression of *mtHsp75* will provide protection from ischemia. This hypothesis can now be tested. It will also be worth looking at the effect of *mtHsp75* overexpression on changes in ATP with ischemia.

Acknowledgements

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Tunneling Spectroscopy of Two-Dimensional Electron Gases

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Introduction

The Scale of Mesoscopic Research

In between the macroscopic scale of our daily experience and the microscopic world of single atoms, there is the regime of mesoscopics. Mesoscopics research is carried out at a scale in which thousands of electrons are involved, but in which the rules of quantum physics still apply. The usual scale of mesoscopic devices is in the range of ten nanometers to ten micrometers at low temperatures.

By placing a Si-doped AlGaAs layer onto a GaAs layer it is possible to create a surface between these layers on which electrons can move freely, yet not being able to escape the flat, two-dimensional area. This is what we call a two-dimensional electron gas (2DEG). By using very clean materials, the electrons can be considered as if they were moving through empty space. This enables us to study the intrinsic properties of electrons at certain potentials. Figure 1 shows a cutaway view and a conductance band diagram of a common GaAs/AlGaAs 2DEG heterostructure.

Different kinds of potentials can be created on a 2DEG by planting gate-eletrodes on top of a 2DEG chip. We use electron-beam lithography to create gate structures at the scale of 10 nm to 10 µm on the surface of the AlGaAs/GaAs chips. By applying negative voltages to those gate electrodes one can create depletion-regions in the 2DEG, which backscatter electrons. This enables us to create different structures like 2D quantum wells, 1D quantum wires, or 0D quantum dots whose dimensions can be changed *in situ* just by modifying the voltage applied to the gate electrodes. One device that is studied by the Goldhaber-Gordon group is the so-called "Quantum Point Contact" (QPC). A QPC consists of two gates depleting the 2DEG, creating a very narrow channel, which can be considered as a very short, 1D wire. An interesting property of the QPC is, that conductivity vs. width (i.e., gate voltage) is quantized into discrete steps, which shows us that we already have left the macroscopic regime. Figure 2 shows a 2DEG chip.

0D quantum dots are often called "artificial atoms" (see [1] and [2] for further information). What they have in common with real atoms is that the characteristic properties of those "atoms" are quantized. But in contrast to a hydrogen atom, for example, whose energy-levels, potential function, etc. are fixed by nature, we can engineer our quantum dots in ways so that, for example, a slight variation of charge can create major changes in quantities like conductivity, which then can easily be measured. It is also possible to build classical interference devices such as double slits.

In order to obtain spatial information about the electron-density within certain regions of a 2DEG, we use an atomic force microscope (AFM) which scans the surface of the 2DEG with a negative voltage applied to the microscope tip. This creates a moving, circular depletion region which backscatters electrons. If we now measure the conductivity in respect to the tip's position, we are

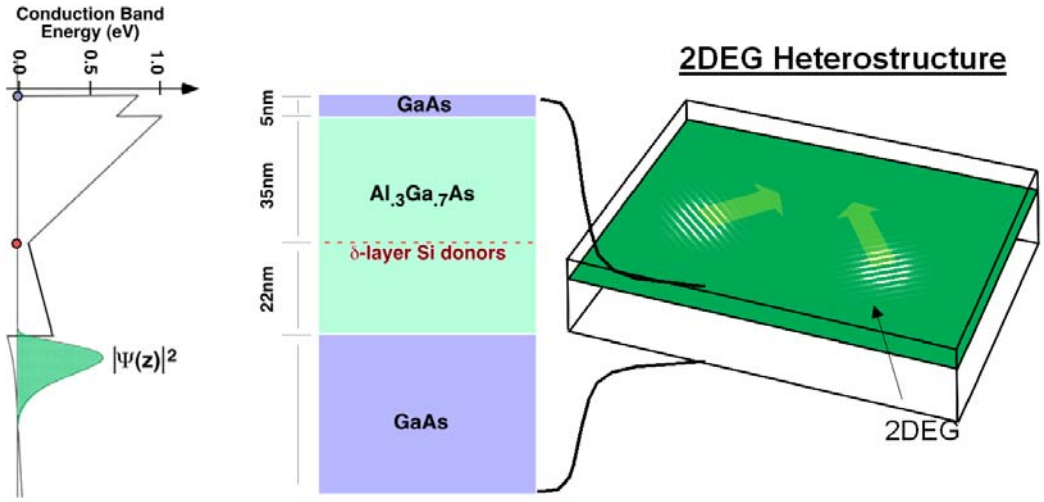

Fig. 1. Cutaway view and conductance band energy diagram of a 2DEG heterostructure

able to see it decrease tremendously when we are over an area with high electron density. This means that we are backscattering a lot of the conductance electrons. If we are over a region with a very low electron density, the decrease in conductivity will be considerably smaller (see [3] and [4] for further information). Figure 3 illustrates the imaging process.

Scanning probe microscopy (SPM) is an excellent technique for imaging surface structures with resolutions down to the size of single atoms. However, until now there are only few methods which allow one to get a glimpse beyond the surface. By using the technique described above, it is possible to obtain information about the electron density of a 2DEG, but still, there are few feasible techniques to investigate the energy levels of the electrons confined in a 2DEG. In this paper we present a theoretical investigation of a possible technique for spectroscopy of 2DEGs.

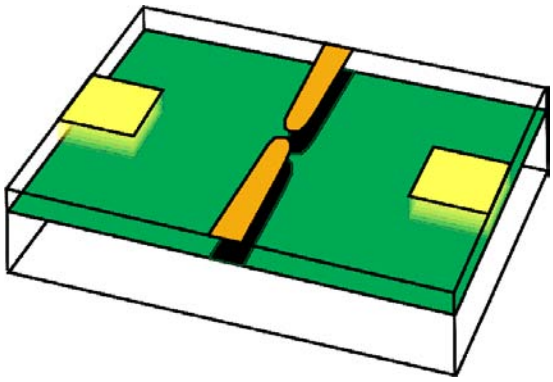

Fig. 2. Schematic view of a 2DEG chip. There are two omacs directly connected to the 2DEG. Those are used to apply a source-drain voltage and to measure the conductivity of the two dimensional electron gas. On top of the chip, seperated from the 2DEG by a semiconductor layer, there are two gate electrodes, which create depletion regions beneath them

Methods

There are already several techniques available that allow researchers to have a look at structures well beneath the surface of a sample. One of them, called ballistic electron emission microscopy (BEEM), applies a large voltage between a sharp metal tip and a thin metal sheet on the sample surface. This fires electrons like ballistic bullets deep into the substrate (see [5] for further information on BEEM). Unfortunately it cannot be used for spectroscopy of delicate electronic states, which would require lower tip-substrate bias voltages. Another method in this field involves the use of single-electron transmitters on an AFM tip capacitively coupled to the 2DEG beneath

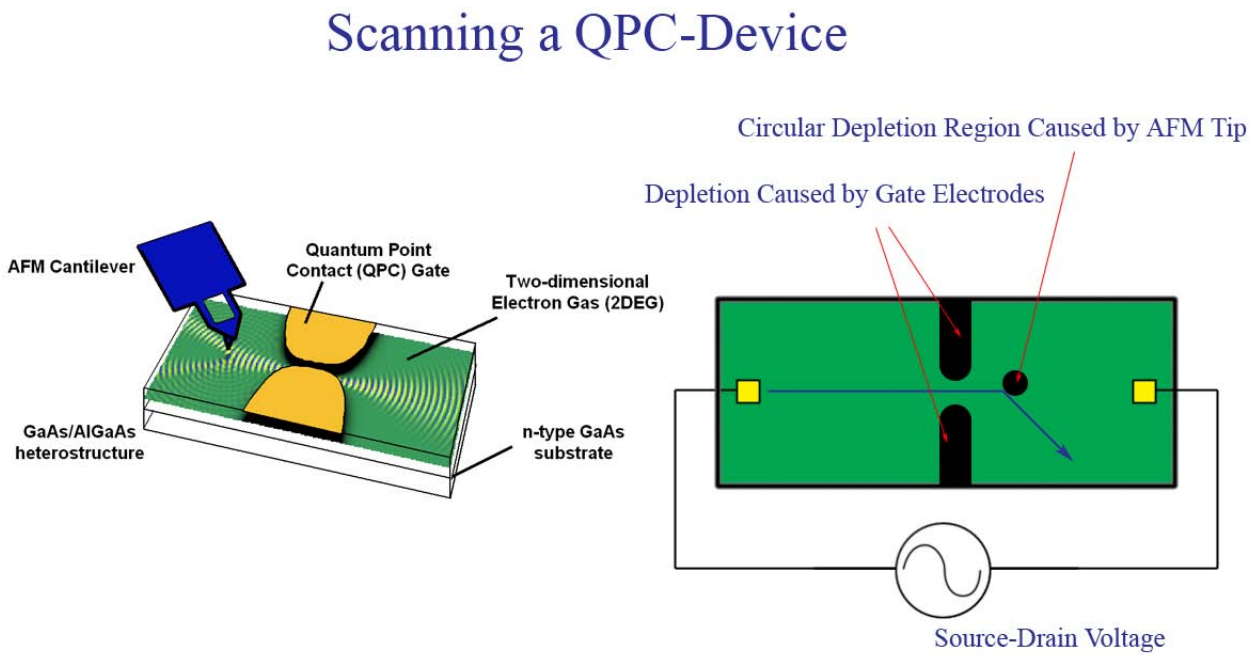

Fig. 3. Schematic of the AFM imaging technique. The green region is the 2DEG. The two big depletion regions are created by gate electrodes on the surface, to form a narrow quantum point contact between the regions on the left and the right. The conductivity is measured through the omacs which are directly attached to the 2DEG. The black circle indicates a depletion region created by a negatively charged tip above the surface of the heterostructure. The blue arrow indicates the path of an electron which is backscattered by the depletion region of the AFM tip, therefor decreasing the conductivity of the 2DEG

the surface (see [6] and [7] for further information). This method allows measuring the charge and therefore the electron density of the 2DEG, yet it cannot directly probe the energy levels of the electrons on the 2DEG.

Our idea outlines a semiconductor – namely a GaAs/AlGaAs heterostructure – which confines two two-dimensional electron gases, seperated by a graded tunneling barrier. The idea is to apply a bias voltage between the two 2DEG layers while moving a tip charged by a negative voltage over the surface of the heterostructure. This pushes electrons from the upper 2DEG through the tunneling barrier into the lower 2DEG. By controlling the energy and momentum of electrons tunneling between the two 2DEGS and measuring the resulting tunneling conductivity we should be able to acquire information about the semiconductor heterostructure and the two 2DEGS. For example, we will be able to examine the different energy levels of quantum dot structures on the lower 2DEG.

In order to be able to apply this kind of electron microscopy the barrier separating the 2DEGs should have certain characteristics concerning the relation between bias voltage and tunneling rate: First, a rise in tunneling rate should occur at voltages that are small enough not to disturb electron spectroscopy and this rise should yet be sufficient to get a fair amount of electrons through the barrier. At last it should be robust in a way that small changes or noise in the bias voltage should not affect the tunneling rate. One type of barrier-structure that incorporates all these features is a

triangular-shaped barrier, which keeps the tunneling rate constant for a broad range of voltages, until a certain tunneling voltage is reached, where the tunneling rate rises very rapidly. To keep the tunneling rate constant beyond this tunneling voltage of the triangle barrier, there is a short, high, square-shaped barrier connected to it.

In reality, these graded barriers are created by slowly varying the percentage of Al in a thick AlGaAs layer (see Fig. 4). But

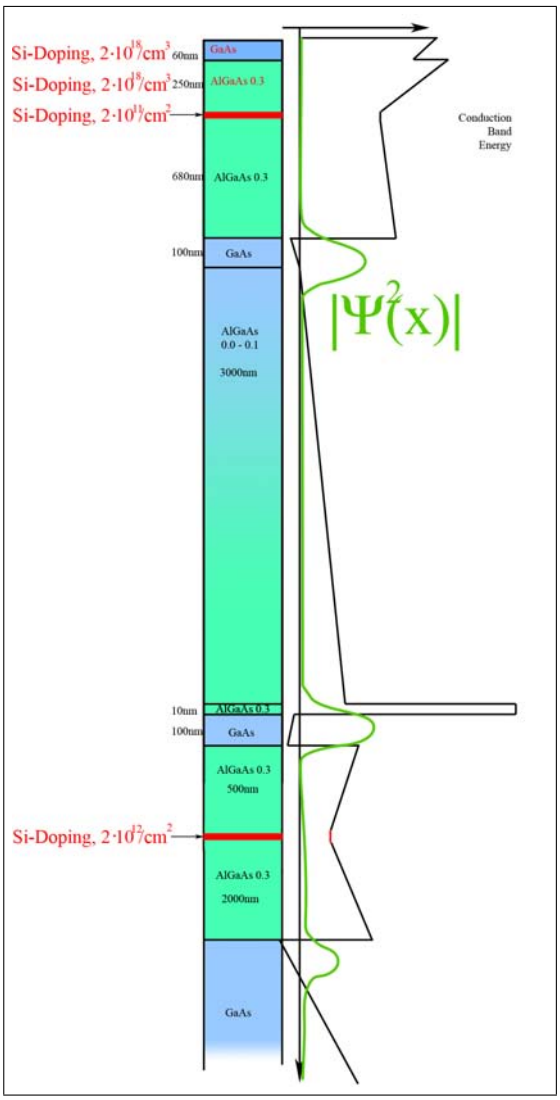

Fig. 4. Conductance band energy and $|\Psi^2(x)|$ of a graded-barrier, two 2DEG heterostructure. Using this design we create a third 2DEG below the other two. There may be further refinements which allow using the same kind of barrier between the two 2DEGs yet not creating an undesired third one

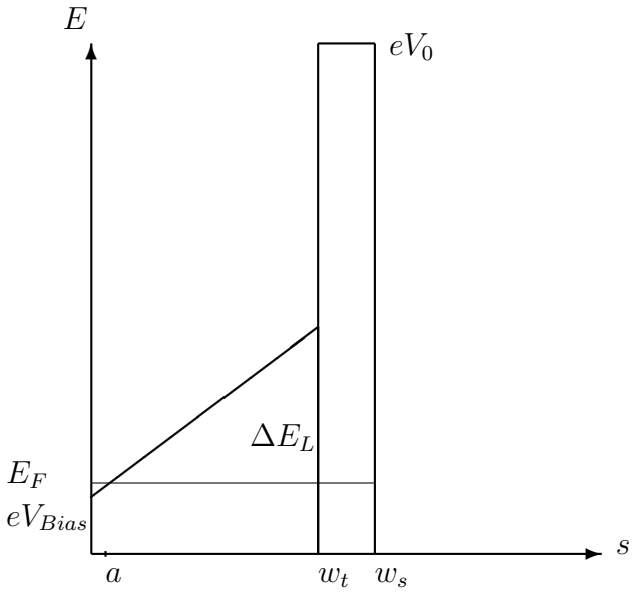

Fig. 5. A possible wave barrier to seperate the two 2DEGS

what exactly would tunneling through this kind of barrier look like? Hereby, I deliver an attempt to creating a $T(V_{Bias})$ function of a triangular-shaped barrier connected to a high, square-shaped barrier (as shown in Fig. 5).

One can divide the problem into two domains: one deals with the triangular-shaped and the other with the square-shaped barrier. The geometry of the triangular barrier, concerning the energies and the bias voltage, appears as shown in Fig. 6.

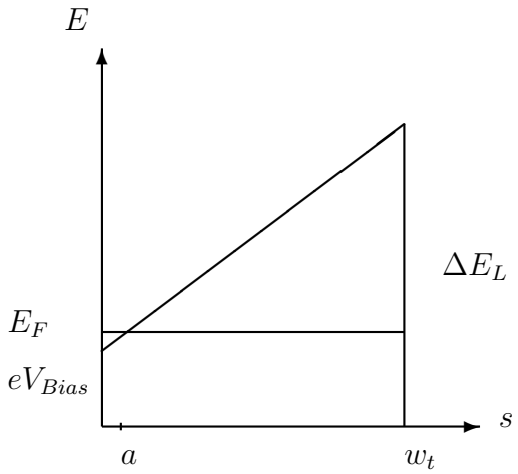

Fig. 6. A triangle shaped wave barrier

For modelling the tunneling barriers I have employed the following WKB-

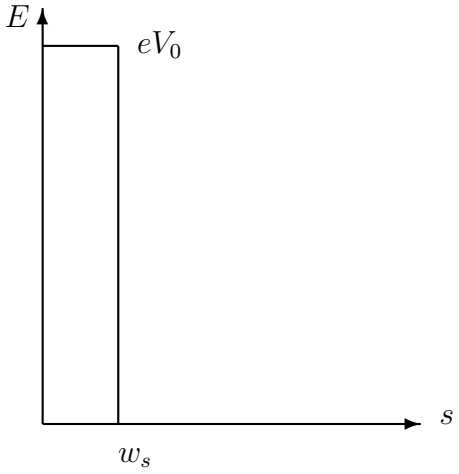

Fig. 7. A square shaped wave barrier

approximation (see Chapter 8 of [8] for further information):

$$T \approx e^{-2\gamma}, \gamma = \frac{1}{\hbar}\int_a^{w_t} |p(x)|\mathrm{d}x$$

The absolute of the momentum p is

$$|p(x)| = \sqrt{2m(V_t(x) - E)}$$

, with

$$V_t(x) = eV_{Bias} + \frac{\Delta E_L - eV_{Bias}}{w_t}x$$

and

$$E = E_F + eV_{Bias}$$

Additionaly, the geometry shown in Fig. 6 gives:

$$a = w_t \frac{E_F}{\Delta E_L - eV_{Bias}}$$

Solving this integral gives the following solution:

$$T \approx e^{-\frac{2w_t}{\hbar}\sqrt{\frac{8}{9}m(\Delta E_L - eV_{Bias})}\left(1-\frac{E_F}{\Delta E_L - eV_{Bias}}\right)^{\frac{3}{2}}}$$

Similarly, I use the WKB approximation to solve the tunneling problem through a high, square shaped barrier (see Fig. 7).

Here it is:

$$T \approx e^{-2\gamma}, \gamma = \frac{1}{\hbar}\int_0^{w_s} |p(x)|\mathrm{d}x$$

The absolute value of the momentum p in this case is:

$$|p(x)| = \sqrt{2m(V_0 - eV_{Bias} - E_F)}$$

We get:

$$T \approx e^{-\frac{2w_s}{\hbar}\sqrt{2m(V_0 - eV_{Bias} - E_F)}}$$

Multiplying the two functions gives the following $T(V_{Bias})$ function for the intervall $I[0; \frac{\Delta E_L}{e}[$

$$\begin{aligned} T &\approx e^{\alpha} \cdot e^{\beta} \\ &= e^{-\frac{2}{\hbar}(\gamma+\delta)} \end{aligned}$$

with

$$\begin{aligned} \alpha &= -\frac{2w_t}{\hbar}\sqrt{\frac{8}{9}m(\Delta E_L - eV_{Bias})} \cdot \\ &\quad \left(1 - \frac{E_F}{\Delta E_L - eV_{Bias}}\right)^{\frac{3}{2}} \\ \beta &= -\frac{2w_s}{\hbar}\sqrt{2m(V_0 - eV_{Bias} - E_F)} \\ \gamma &= w_t\sqrt{\frac{8}{9}m(\Delta E_L - eV_{Bias})} \cdot \\ &\quad \left(1 - \frac{E_F}{\Delta E_L - eV_{Bias}}\right)^{\frac{3}{2}} \\ \delta &= w_s\sqrt{2m(V_0 - eV_{Bias} - E_F)} \end{aligned}$$

For $V_{Bias} \geq \frac{\Delta E_L}{e}$ we get the usual, exponential increase in tunneling probability:

$$T \approx e^{-\frac{2w_s}{\hbar}\sqrt{2m(V_0 - eV_{Bias} - E_F)}}$$

Results

To get a $T(V_{Bias})$ function one simply plugs in some real values, as for example:

$$m = \frac{500\,\mathrm{keV}}{c^2},\ w_t = 2 \cdot 10^{-8}\,\mathrm{m},\ \Delta E_L =$$

$0.15\,\mathrm{eV}$ (30 % AlGaAs), $E_F = 0.02\,\mathrm{eV}$

A sample graph of tunneling rate vs. voltage is shown in Fig. 8.

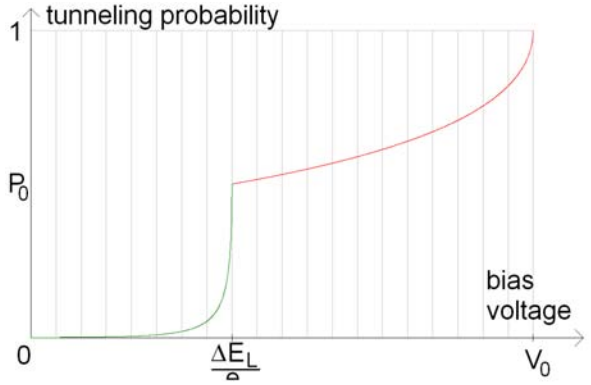

Fig. 8. Tunneling rate T vs. bias voltage V_{Bias}: This is the product of the tunneling-rate vs. voltage functions of the triangle and the square barrier. As voltage reaches $\frac{\Delta E_L}{e}$, $T_{triangle}$ becomes one, and we get the well known graph of a high, square shaped barrier *(red)*. While $T_{triangle} \neq 1$, due to the geometry of the wave barrier, we have a very flat graph that rises very steeply when voltage comes close to $\frac{\Delta E_L}{e}$ *(green)*

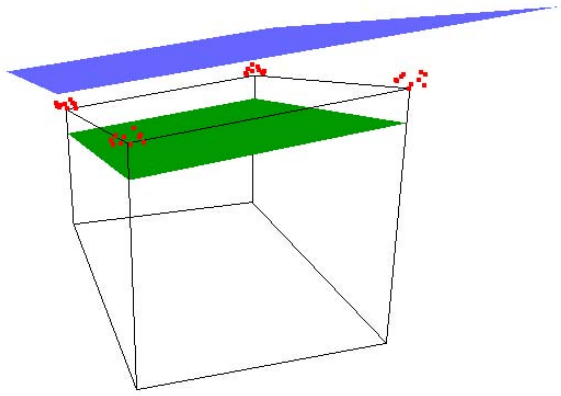

Fig. 9. By touching down on the surface of the 2DEG heterostructure one acquires a set of points *(red)*, on the surface of the sample. Using this points we calculate a plane parallel to the surface on which the AFM tip moves *(blue)*

Protecting the AFM tip

The Goldhaber-Gordon Lab uses a custom-built AFM for imaging the electron density of the sample 2DEGs.
To be able to perform these scans, the tip has to be very close to the surface. Since this surface is never perfectly flat, there is always the risk of unintentionally pushing the tip deep into or scratching along the surface, which results in blunting the tip. A blunt tip creates a lot more noise and therefore decreases the sensitivity and the resolution of the scans tremendously.
In order to assure protection of the tip it was necessary to work on the source code of the AFM-controlling software, originally engineered by Mark Topinka, Michael Jura, and Dennis Lo of the Goldhaber-Gordon Lab. Together with Dennis Lo, I added an algorithm, which is able to find the three-dimensional plane in which the sample surface is included. Now it is easy to calculate a parallel plane in which the tip can move in constant, safe distance from the sample surface.
For finding the so-called "z plane" of the surface, one touches down very softly with the tip at the four corners of a specified rectangular region – several times at each corner – covering the devices that one wants to scan. This gives us a set of points through which we can fit a 3D plane – using a least-squares algorithm – on which the surface of the sample should be found. Now we simply incorporate a safety value, which tells the AFM how far off that plane the tip should stay. This gives a plane in which the tip can move without risking of being blunted. Figure 9 shows, how the algorithm works.

Discussion

There are many exciting results to expect from electron spectroscopy of 2DEGs. Besides the fundamental research on the intrinsic properties of electrons in arbitrary potentials, the research done in the Goldhaber-Gordon Group is expected to have great influence on many other fields of quantum physics, nanotechnology, and computer science.

One possible application of quantum dots, for example, could be the storage and ma-

nipulation of data in the form of properties like the spin of the confined electrons. This could lead the way to quantum computers which could quickly solve very difficult, even non-deterministic-polynomial-time (NP)-hard problems, such as the factorisatzion of very large numbers. Another interesting field of experimentation is the use of floating gate electrodes which are not connected to an external voltage source but instead obtain a charge by means of a charged AFM tip touching them. This will enable the Goldhaber-Gordon Group to change not only the size but the entire geometry of the devices *in situ*.

Acknowledgements

I would like to thank all members of the Goldhaber-Gordon Lab, especially Dr. Mark Topinka, who did not only impress me by his vast knowledge and professional skills but also by his willingness and ability to outline and explain even the most difficult papers (from the point of view of a high-school graduate) and his 'Jedi-Like Speed-Scrabble-Playing-Skills' – Dennis Lo, who went with me through the hell of spending hours rethreading the wire bonder – and Michael Jura. Thank you for giving me the opportunity to join you in your exciting field of research and trusting me to work on your AFM. Special thanks goes to Yhani Zhai, who gave me the opportunity to join her on the talks and the excursions to SLAC and the Lick observatory with the Stanford Summer School. I am indebted to Michael Grobis, Hung-Tao Chou, Michael Jura, Lindsay Moore, John Cummings, Ron Potok, Ileana Rau, and Joseph Sulpizio for accepting and integrating me in their group and making me feel so welcome.

My sincere appreciation is expressed to Prof. David Goldhaber-Gordon for making this internship possible, for his trust and his kind support whenever I needed help.

All of these people showed me how wonderful and exciting research in the physical science can be, an invaluable experience for my choice to become a physicist.

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The Academy Participants

Markus Als

was born on April 6th, 1987. After graduating from high school (abitur) in 2006, I intend to spend my "Zivildienst" (social civilian service) at the "Nabu - Science and Biodiversity Research Center" near Hannover, Germany, where I will learn more about animals, which are my main interest – I presently keep and care for about 200 animals, from fishes to insects and reptiles.

Currently I am involved in the zoology and physics study groups of the *Heidelberg Life-Science Lab* and am interested in bionics and nanotechnology – I experiment with solar power and own a small solar panel station. Research in such fascinating innovative fields of science and technology may eventually lead to the creation of artificial life – my hobby is music: playing the accordion and the bass guitar. For sports and recreation I do capoeira, a Brazilian-African dance and fighting sport.

The ISA San Francisco was my first visit to the United States and I enjoyed it tremendously to have had the opportunity to do my own research at Kristin Scott's lab in Berkeley.

Anna Baum

born on July 3rd, 1987, in Heidelberg. This year is my last high school year at the Helmholtz-Gymnasium, Heidelberg. After my final exams, I will hopefully be able to study medicine in Heidelberg. I have been a member of the *Heidelberg Life-Science Lab* since September 2004 and have been participating in the medical sciences and molecular biology study groups.

The concept of the International Science Academy San Francisco has fascinated me from the very start. Together with Stefan, I was fortunate to be welcomed by Professor Chen and his very hospitable lab members at Stanford University. The experience and knowledge that I was allowed to share with the others during this Science Academy will be invaluable for my education and career. This significant experience, to work in a foreign country, in an international laboratory is so unique, and does not compare to anything alike. You may only get such an opportunity once in a lifetime, and so I can recommend it to everyone, to seize upon this special program.

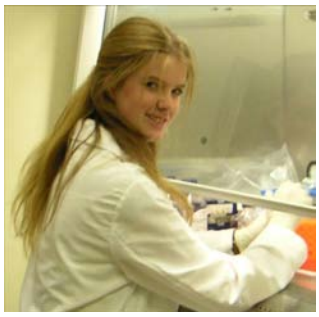
Tim Böltken

born on April 4th, 1985. After graduating from high school in Hockenheim in June 2004, I worked at a hospital in Speyer to fulfill my year of social civil services. Returning from San Francisco, I enrolled in biophysics at the University of Karlsruhe.

In 2003, I became a member of the *Heidelberg Life-Science Lab* and participated in the biochemistry study group and attended practical courses at the European Molecular Biology Laboratory (EMBL). To prepare myself for the ISA San Francisco I worked for one month at the Tumor Virology Department of the German Cancer Research Center (DKFZ). Together with Elisabeth Urban I founded the medical science study group of the *Heidelberg Life-Science Lab.*

I would like to thank Monika Gessat, Martin Schorb, and Christoph Fischer for arranging the ISA San Francisco 2005 – we had great fun with the two student coordinators and learned a lot. My intention is to become involved in planning the next Academy to San Francisco in 2007 and help making it as successful.

Sibylle von Bomhard



was born 27th May 1986 in Trier. After graduating from high school, I began my studies in biomedical chemistry at the Johannes Gutenberg University of Mainz.

During my high school years I participated in several national competitions: the "Landeswettbewerb Mathematik Rheinland-Pfalz 2001"; the "41. Mathematik-Olympiade 2002" in Hamburg; "Schüler experimentieren", in which I placed third in 2001 and second in 2002; "Jugend forscht" in 2003, where I received the First Prize on the regional level, resulting in a publication in "WLB – Wasser, Luft und Boden".

Within the *Heidelberg Life-Science Lab* I have been actively involved in the ecophysiology and ecotoxicology study group since June 2003 and also participated in the "Naturwissenschaftliche Sommerakademie der BASF 2004" as a member of the "Deutsche Schülerakademie 2004". For leisure I enjoy horseback riding, reading, visiting the theater, and meeting interesting people. My goal is to pursue a career in scientific research.

Christoph Fischer

was born on July 24, 1984 in Heidelberg. Having graduated from the St. Raphael Gymnasium in 2003, I performed my civilian service at a school for disabled children with speech disorders. After that I enrolled in law at the Ruprecht-Karls University of Heidelberg, right now studying in my second year. My special interest lies in the relation of science and law, especially environmental and bioethical issues.

After participating in the first International Science Academy San Francisco 2003, I decided to stay involved in the *Heidelberg Life-Science Lab* and promote the successful San Francisco program. Also, I have initiated a new International Science Academy to Estonia that will take place in 2007 for the first time. I believe there is no experience as precious for young people as to getting in touch with other cultures. Having lived in Belgium for seven years, I experienced such enriching exchange myself and try my best to pass on this appreciation to the academy participants.

Being involved with the San Francisco Academy for nearly 4 years now, I would like to thank everyone who put their efforts into making this project so successful, and I am looking forward to another San Francisco Academy in 2007!

Clarissa Fischer

born on December 13th, 1986, in Heidelberg. In 1993 my family moved to Antwerp, Belgium, where we lived for seven years. Having returned to Heidelberg, I joined the *Heidelberg Life-Science Lab* in 2004 and graduated from high school in 2005. Currently, I am completing a social internship, volunteering at the University Clinics of Heidelberg (Dept. of Neurology) for one year. Afterwards, I am going to enroll in medical studies at the University of Gießen. My hobbies are sports, books, movies, photography and arts.

In the *Heidelberg Life-Science Lab* I participated in the study groups of neuropsychology, molecular biology, philosophy, and medical science – the most prominent highlight being the San Francisco Academy 2005.

Elisabeth Fischer

was born on August 16th, 1986. After graduating from secondary school at the Kurfürst-Ruprecht-Gymnasium in Neustadt/Weinstraße, I enrolled in the bachelor's program in molecular biotechnology at the University of Heidelberg. Within the *Heidelberg Life-Science Lab* I have been a member for the molecular biology study group which I am tutoring since 2005. During the ISA San Francisco 2005 I spent one month in the lab of Mark Kay at Stanford University together with Melanie Weißer and enjoyed working in such a friendly atmosphere! This academy has become a milestone in my studies for becoming a scientist.

Stefan Gerhardy

was born on March 4th, 1986 in Heidelberg. In high school I focused on science, particularly biology and chemistry – and graduated in 2005. To meet my interest in science, I joined the *Heidelberg Life-Science Lab* in 2002 as a member of the biochemical study group, in the context of which we attended several practical lab training courses at the German Cancer Research Center (DKFZ) and the European Molecular Biology Laboratory (EMBL). This special program offers the excellent opportunity for exchanging ideas on scientific issues with other equally interested students. I have become a tutor of the biochemical study group and the scientific English study group.

As a participant of the second International Science Academy to San Francisco I'm looking back in pride to a memorable experience, which will certainly have an enormous influence on my career. I was really impressed about the Californian "easy-going" mentality and the positive attitudes of people at the lab. The whole trip can never be described in words – it was wonderful, interesting, and very instructive. After the academy I have decided to volunteer at a charitable institution for the elderly and disabled. Thereafter, I intend to enroll at the University of Heidelberg in the curriculum for Cellular and Molecular Biology. Next to science, my hobbies are sports, including basketball, biking, and jogging.

Monika Gessat

Mentor of the *Heidelberg Life-Science Lab's* San Francisco Academies in 2003 and 2005. Senior high school instructor for biology, chemistry, and physics at the Edith-Stein-Gymnasium in Bretten, Germany. There my main focus is to encourage my students to ask questions, to explore nature, and to help them appreciate the spirit of science.

My hobbies are traveling, hiking, skiing, listening to music, and reading contemporary literature – some of my favorite authors are Americans, as Siri Hustvedt, Paul Auster, John Irving, Philipp Roth, and Jeffrey Eugenides.

Alexandra Kahn

born on June 11, 1986 in Heidelberg. I graduated (Abitur) from the Bunsen Gymnasium in Heidelberg in 2005 with profile courses in French, English, and biology. My intention is to study medicine. As my mother is from Argentina, I speak fluent Spanish and German. In 2002, I joined the *Heidelberg Life-Science Lab*. Over the years, I attended the neurobiology, philosophy, and medical science study groups where my interest for the neurosciences developed. Because of my involvement in sciences and languages I participated in the "Fête de la Science" in 2003. Several work placements and weekend workshops with the *Life-Science Lab* contributed to help finding my way. My hobbies are playing the piano, reading, and dancing.

While working in the laboratory at UC Berkeley, I learned new aspects of scientific study and could pursue my interest in neuroscience. The experiences I made in the lab of Prof. Gerald Westheimer, the daily life in San Francisco, and the journey itself have molded me deeply and will significantly influence my further career. I want to thank all who have worked hard for this San Francisco Academy, who made it possible, and especially my professor who gave me the opportunity to have such a good experience in his lab.

Alexander Koch

born in October 1986, living in Heidelberg, and currently attending the last grade of high school at the Hölderlin Gymnasium Heidelberg. My interests are in understanding the processes of life at the molecular level, particularly from the perspectives of biochemistry and molecular biology. An additional passion of mine is information technology and modelling of structures and systems on a mathematical level within an interdisciplinary context. My hobbies are judo and singing in a choir.

I have been a member of the *Heidelberg Life-Science Lab (LSL)* since 2003 and have participated in the chemistry, biochemistry, molecular biology, math, computer science, philosophy, and bioinformatics study groups. Last year I became a tutor of the biochemistry study group and, also being very much interested in languages, founded a scientific English study group at the *LSL*.

As our society becomes increasingly information/communication based, and knowledge as well as education more and more important, I want to help other interested high school students to get a better understanding of the life sciences and to return to the *LSL* some of those things that it provided to me during these last three years. I have become an assistant lecturer for computer science at our high school and tutor of a school study group for talented students in which we won an award for didactical school software in 2005 with a project that teaches the function of neuronal networks.

The International Science Academy to San Francisco and my internship in the lab of Mina Bissell was a marvelous experience and confirmed my decision to pursue my studies in the life sciences. I have become very confident in laboratory work and got a more vivid view on these topics than by merely reading textbooks. Thanks to Mina Bissel and Paraic Kenny for all their guidance! ...and then, the nice and open-minded people at the lab and our student group who provided a pleasant working atmosphere – thanks, to all! For my civil service I will work for a youth organization and thereafter am intending to study biochemistry and molecular biology along with computer science. My goal is to become engaged in research in fields that are beneficial to the well-being of others, especially AIDS and cancer research.

Annemarie Lüdecke

was born on May 12th, 1986 in Heidelberg. I graduated at the "Englisches Institut Heidelberg" in 2005. Currently, I am enrolled in physics at the Freie Universität Berlin. As I always was very interested in the human brain as the origin of consciousness, I joined the neurobiology and neuropyschology study groups after being accepted to the *Heidelberg Life-Science Lab* and completed an internship at the 'Zentralinstitut für Seelische Gesundheit' (a mental health clinic and research institution) in Mannheim. As I participated in several exchange programs (Australia/USA) I knew that visiting another country always is an exiting experience, which is even more true when not only travelling as a tourist. This together with the opportunity for carrying out own scientific work were the reasons why I was anxious to join the International Science Academy San Francisco 2005. The San Francisco Academy was a great opportunity to broaden my horizon scientifically as well as interpersonally. In my free time I enjoy playing the piano.

Philipp Rauch

was born in Heidelberg on June 4th, 1986. After graduating with honors from the St.-Raphael-Gymnasium in Heidelberg in June 2005 (Abitur), I was accepted at the medical school of the University of Heidelberg. Besides acting, reading, and playing the cello, science has always been my foremost passion ever since I attended primary school.

My participation in the *Heidelberg Life-Science Lab (LSL)* started in 10th grade (2002) and included working in the biochemistry study group at EMBL – among the theoretical and practical projects we conducted a study on the possible link between tyrosine kinase and the human estrogen receptor. I also contributed to the physics study group of the *LSL* and attended its CERN study tour in 2003. In 2004, I became student tutor for the biochemistry study group; the responsibilities linked to this task are highly motivating.

The ISA San Francisco 2005 was a most significant experience, especially since I discovered so many different and fascinating facets of authentic lab research. Hopefully, the next generation of high school students will have the same opportunity to take part in this meaningful program. My goal for the future is to help transfer recent results of basic research into clinical practice, among others in the field of oncology.

Carolin Reif

was born on January 5, 1987 in Freiberg. My high school years have been spent at the Heinrich-Böll-Gymnasium in Ludwigshafen, where I will be graduating (Abitur) early in 2006. Within the *Heidelberg Life-Science Lab* I have been participating in the study groups on neurobiology and medical science. As a hobby I enjoy exploring the underwater-world, especially wrecks, by scuba-diving. After finishing high school I am intending to enroll in geosciences.

The San Francisco Academy has been an immensely impressive experience for me. I learned a lot, not only in reference to science. Discussions, both with the students of our group and the people I met at the lab of Kenneth Downing, were inspiring and revealed new perspectives. It was interesting to learn about the conditions under which scientific research is conducted in the United States. I am grateful for this experience and want to thank everyone contributing to the academy.

Janina Schneider

was born on the 12th of June 1987. As one of the youngest members of the San Francisco Academy 2005, I will be graduating in the summer of 2006 from the Burghardt-Gymnasium in Buchen. My interests are in sciences, theater, and cultural studies. After graduation, I would like to study medicine either in Germany or in England. Since the summer of 2003 I have been a member of the *Heidelberg Life-Science Lab* joining the study groups of biochemistry and chemistry as well as attending several internships at the European Molecular Biology Laboratory (EMBL) and the Heidelberg Exploratorium.

My great passion is theater, no matter whether as a member of the audience, as an actor, as a costume designer, as a director, or even as an author. Music is another part of my life, piano as well as oboe, in the Symphonic Orchestra of the Music School of Buchen.

In Gregory Barsh's lab at Stanford University, I was able to learn basic aspects of coat color patterns and mutations as well as basic laboratory techniques. Those three weeks were a wonderful experience and a unique chance to gain an insight into the various aspects scientific research at its best. I am convinced that this wonderful experience will accompany me throughout my entire life!

Martin Schorb

in the year 2000, I joined the *Heidelberg Life-Science Lab* while I was in 11th grade and was part of the International Science Academy Portugal 2002 and the first San Francisco Academy in 2003. After graduating from high school in Eppelheim in 2003, I enrolled in physics at the University of Heidelberg – now in my third year. In 2004, I was accepted as a fellow of the German National Academic Foundation.

My particular scientific interests are in the biological and medical aspects of physics. Besides, I enjoy playing the saxophone and learning Portuguese and Russian, and riding my bicycle.

As coordinators and mentors, Christoph and I are proud that the continuation of the San Francisco Academy has proven to be as successful as our first pioneering academy in 2003 – another scientific success story of the *Heidelberg Life-Science Lab.* Working with our fellow students and the American professors and their staff has meant an enormous cultural, scientific, and personal enrichment.

Christian Stoy

was born on December 2nd, 1983. I became a member of the *Heidelberg Life-Science Lab* in its founding year, 2000, participating in several study groups – molecular biology, internet, biochemistry, and chemistry; since spring 2003 I have become a tutor for the molecular biology group. After graduating from high school in July 2003, I completed my ten-month social civilian service at the Department for Viral Transformation Mechanisms of the German Cancer Research Center (DKFZ) in Heidelberg. In October 2004, I began studying biology at the University of Heidelberg. Recently, I have taken up work in the office of the *Heidelberg Life-Science Lab* where I am responsible for managing the annual application procedure and typesetting the annual report.

My stay at Stanford University was a unique and challenging experience. It was great to work relatively independently on an aspect of an ongoing research project in a lab of a renown and leading university, where I learned a great lot. Also, exploring San Francisco and the Bay Area was a very enriching experience. If I ever again had the opportunity to return to Stanford, I surely would grab it.

Kai Ueltzhöffer

was born on September 9, 1985, in Heidelberg. In 10th grade I founded and tutored a programming and computer graphics study group at my secondary school, Carl-Friedrich Gauß Gymnasium, Hockenheim. In the same year I joined the *Heidelberg Life-Science Lab*, namely the study groups on mathematics, bioinformatics, and neuroscience. Not only interested in performing independent research, such as constructing an electro-encephalographic device, developing neuronal networks to analyze gene-expression-data or solving mathematical puzzles, but also in teaching and lecturing, I became tutor of the mathematics group which focuses on mathematical problem solving, optimization, and scientific computing. During my time at school I also attended the "Schülerzirkel Mathematik" (a mathematics college prep course) of the University of Mannheim. After having graduated (Abitur) with honors, I enrolled in physics at the University of Heidelberg.

Next to science, my hobbies are hiking, mountaineering, and travelling, which I preferably enjoy with the boy scout group of my hometown. Mountain-biking and road race cycling are the two passions which suffer most from my current lack of spare time due to university and my involvement at the *Heidelberg Life-Science Lab* and the boy scouts.

The San Francisco Academy 2005 was a key event in my life and encouraged my decision to pursue a career in the natural sciences. Not only the challenging work in the laboratory, but also the atmosphere, the charme, creativity, and enthusiasm of the academy participants and the scientists at Stanford University left me deeply impressed.

Elisabeth Urban

was born on April 23, 1985. Having attended the St. Raphael Gymnasium in Heidelberg, and graduating (Abitur) in 2004, I started working at the EXPLO Learning Laboratory. Soon I will enroll at the Medical School of Nursing in Heidelberg. In 2001, I joined the *Heidelberg Life-Science Lab*, and there developed a strong interest in science through workshops in molecular biology and neurobiology and different internships at the German Cancer Research Center (DKFZ) and Max Planck Institute for Medical Research. Presently, I am a tutor for the medical science study group, a member of the philosophy study group, and also trying to establish a theater group.

I would like to thank all the people who made the ISA San Francisco possible, especially Christoph, Martin, and Monika who thoroughly planned and arranged everything. The academy was an important experience for me, and I appreciate having had the opportunity to work in the lab of Bruce MacIver and the unforgettable time at Stanford University with people I now call friends. Thank you, all!

Melanie Weißer

was born on June 23rd in 1986. I attended high school at the Werner-Heisenberg-Gymnasium in Bad Dürkheim, graduating in 2005. Afterwards I worked at a biotechnology laboratory in Neustadt/Weinstraße. In 2004, I joined the *Heidelberg Life-Science Lab* as a member of the molecular biology study group and became one of the student tutors for molecular biology in 2005. Recently, I enrolled in Molecular Cell Biology at the University of Heidelberg.

Together with Elisabeth Fischer I have been fortunate to having spent an entire month at the laboratory of Mark Kay at the Stanford Medical School and very much enjoyed the ISA San Francisco 2005.

Life Sciences Division
Mina J. Bissell, Ph.D.
Distinguished Scientist
Senior Advisor to the Laboratory Director on Biology

August 19, 2005

RE: Life Sciences Academy, 2005

To Whom It May Concern:

I would like to express my sincere admiration for this program which provides senior high school students with an outstanding introduction to the life sciences at such an early stage in their academic career. Each year, the students themselves organize an international internship program, arrange placements in laboratories and travel to a foreign city for a four week stay. Most impressively, they produce a very professional scholarly review, in which each student completes a short literature review and reports the results of their research activities during the program.

I was pleased to host two excellent students from this Academy in my laboratory in August 2005, and was greatly impressed by their enthusiasm and abilities. I have no hesitation in giving this program my unqualified endorsement.

Yours sincerely,

M. J. Bissell

Mina J. Bissell, Ph.D.
Distinguished Scientist
Life Sciences Division

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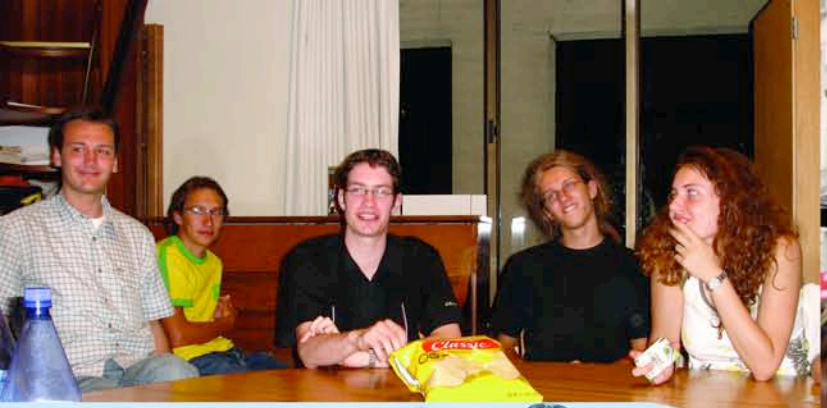
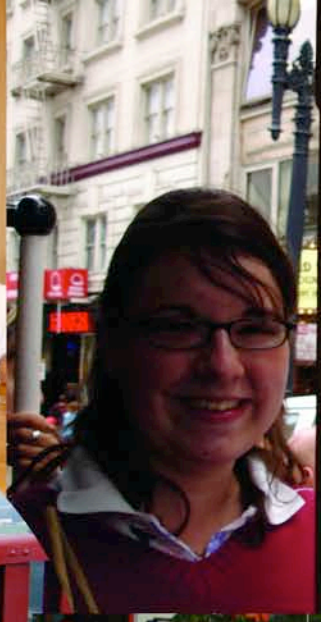


Stanford