Cancer Genomics Centre

Report 2002 - 2005

Cancer Genomics Centre

Report 2002 - 2005

Colophon

Cancer Genomics Centre
Report 2002 - 2005

ISBN-10: 90-810122-2-3
ISBN-13: 9789-90810122-2-5 (as of 1/1/2007)

Editors
Annelies Speksnijder, managing director
Hans Bos, scientific director

Organization
Tekst *N* Redactiewerk,
Nicoline J. Ekama van Dorsten, Amsterdam

Design
I am, AnneMarike Pieterse, Amsterdam

Photography
Henk Rougoor, Amsterdam

Printer
De Boer Ecodrukkers, Nieuwkoop

This report is printed on Reviva silk fsc certified paper
with bio-ink on vegetable basis, www.ecodrukkers.nl

©Cancer Genomics Centre, 2006
Stratenum 3.223
Universiteitsweg 100
3584 CG Utrecht
www.cancergenomics.nl

Contents

Executive summary 4

The why and how of the CGC 8

CGC in names and numbers 10

Organization 12

Research strategy 13

Research themes 14

Research results 16-51

Scientists meet at Royal Tropical Institute 52

Patent applications 54

From scientific discovery to economic value 55

CGC collaborations with academia and industry 56

Societal communication 58

Genomics education 60

Societal research 62

CGC investigator awards 64

Research results

Bernards, Functional screens in cultured mammalian cells 16

Berns, Gain of function screens using insertional mutagenesis 20

Bos, Signal transduction by small GTPases of the Ras family 24

Clevers, TCF factors, mediators of Wnt signaling in development and cancer 28

Grosveld, Regulation of gene expression and development of transposon-based functional screens 32

Hoeijmakers, Molecular basis and biological impact of genome (in)stability 36

Klein, Molecular profiling of breast tumors 40

Plasterk, Protection against genomic instability 44

Van 't Veer & Rodenhuis, Molecular profiling for disease staging and therapy response in breast cancer 48

Cancer is a disease of the genes. Healthy cells can turn into tumor cells as a result of four to six mutations in a subset of cellular genes. From the mid-seventies onwards, we have started to acquire a basic understanding of the tumor cell: a number of genes that are mutated in cancer were discovered, signaling pathways in which these genes act were elucidated, and a series of studies showed promising correlations between cancer prognosis and the molecular characteristics of a tumor cell. Yet, in order to obtain a complete picture of the genetic changes that turn a healthy cell into a cancer cell, a substantial scale-up of research activities and methods was needed. This pertains to high-throughput data acquisition and analysis including the generation of a complete catalog of all cancer-related genes, a full description of the ways in which they interact and extensive studies to correlate molecular parameters to patient histories in order to mount a targeted tailor-made attack on each cancer. A number of leading groups in the Netherlands that had adopted these genomics approaches have joined forces in the Cancer Genomics Centre (CGC) following the establishment of the Netherlands Genomics Initiative (NGI). Since its start in October 2002, the CGC has made important contributions to the field of cancer genomics, resulting in a better understanding of the disease process, improved diagnosis and insight into novel treatment modalities.

About molecular profiles, genome instability, functional screens and cancer proteomics

Since its foundation, the CGC has focused on a better understanding of how a normal cell turns into a cancer cell and on translating this knowledge into clinical applications for the benefit of cancer patients. Currently, this benefit is twofold, i.e. the development of novel therapies based on detailed knowledge of the biology of the disease and the development of better diagnostic tools. The research of the CGC is executed in four research themes as described below.

Molecular profiling of cancer types

A major effort of the CGC is invested in developing genomic tools to classify different cancer types, in particular breast cancer (Klijn, Van ’t Veer). Using the microarray technology, a gene expression profile can be made for each tumor, which is then reduced to a ‘fingerprint’ that can predict the outcome of the disease or the response to treatment. Such a fingerprint profile is no more than a reflection of the various biological processes that occur in the tumor, therefore different profiling platforms and analysis strategies may well yield different profiles for one and the same type of tumor. Indeed, the two profiles that were developed independently in the Amsterdam and Rotterdam groups of the CGC are quite different, yet they have a similar predictive power. In this last year, both groups have collaborated extensively to validate and refine the profiles and to perform cross-validation studies. In addition, novel profiles were developed, for instance for cancers with a genetic background (BRCA1). The fidelity of prediction of these profiles currently is at such a level that doctors can use it in their decision how to treat breast cancer patients. Of great importance is the notion that profiles of a primary tumor are very similar to those of a subsequent metastasis, indicating that the majority of characteristic genetic changes occur during the development of the primary tumor, which underscores the predictive power of profiling primary tumors. Other tumors currently under investigation are lung tumors (Grosveld) and oligodendrogliomas (Hoeijmakers). Since gene expression profiles are only a reflection of the biological changes that occur in a tumor, the ultimate goal is to develop profiles that reflect the causative changes that turn a

Scientific highlights

- novel insights into the role of DNA-repair in cancer and aging
- novel cancer-related genes identified in functional screens using siRNA libraries
- novel dynamic protein complexes involved in gene regulation and cell polarity identified by proteomics
- largest database of oncogenic elements established using insertional mutagenesis screens
- 10 molecular expression and methylation profiles for the classification of different cancer types
- 22 PhD thesis
- more than 350 scientific publications
- more than 350 scientific lectures, mostly at research institutes and meetings abroad
- 2-day scientific meeting with 17 mostly international speakers, 26 posters and over 360 participants

healthy cell into a cancer cell. These may be gene mutations or changes in gene methylation. Methylation profiles are of great importance as they indicate which genes are specifically switched on or off in tumors. The development of such profiles is presently carried out in lung tumors (Grosveld) and breast tumors (Klijn). This novel approach is still in its infancy but may have a great predictive power. Most importantly, it directly addresses the cause of the disease.

Genome instability and DNA repair

For the understanding of cancer and the development of new therapies, mouse model systems are vital. In these models, the genetic make-up of human tumors is mimicked to get a better insight in tumor behavior and the possibilities for intervention. To this end, several new mouse models have been developed in the Berns lab. Another use of animal model systems is the identification of the genetic basis of the disease. Prominent in the CGC is the induction of tumors in mice by insertional mutagenesis using proviruses and transposons (Berns and Grosveld). In these tumors, at least one of the genetic changes is at - or near - the site of insertion and can therefore be readily identified using high-throughput sequencing technologies. Over 1,300 tumors have been sequenced for common integration sites in collaboration with the Sanger Centre Cambridge (UK) and software has been developed to analyse these data. Other important animal model systems in the CGC program are C. elegans and zebrafish (Plasterk). Both organisms are used to identify genes that function in genome stability, as instable genomes are one of the hallmarks of cancer. Indeed, several novel genes have already been found using this approach. The large panel of mouse-strains with DNA-repair defects developed in the Hoeijmakers lab turned out to be an excellent model for aging. Detailed gene expression analysis revealed that the dramatically accelerated aging observed in certain mutant mouse strains is very similar to normal aging. The expression profile further highlighted the heavy involvement of the IGF-1/growth hormone axis that for long has been thought to be tightly connected with aging. These findings point to DNA damage as the root cause of normal and accelerated aging driving the gradual decline of IGF-1/GH levels observed in human aging. Another important finding is that during the aging process, the entire energy metabolism is geared towards preservation of energy and that the anti-oxidant system is systematically upregulated. Important to note is that in these studies Fluorescence 2D Difference Gel Electrophoresis (DIGE), an emerging technology for the analysis of differences in the proteome of different cell types or tissues, was used.

Functional screens

To identify novel genes that play a role in various cellular processes related to cancer, a number of gain-of-function and loss-of-function screens were performed (Bernards). Particulary, the highly innovative loss-of-function screens using siRNA that were developed in the Bernards lab, have provided a wealth of novel information. Highlights are the finding of the HER2 gene as a critical factor in tamoxifen resistance and the PTEN gene in

Valorization highlights

- 1 diagnostic test for breast cancer patients in the clinic
- 9 other diagnostic tests for cancer patients in development
- several new targets for drug development
- 25 patent applications
- 13 collaborative projects with industry
- 2 spin-offs
- 2 valorization workshops for a total of 60 PhD students and postdocs

trastuzumab resistance in breast tumor cell lines. These findings demonstrate the importance of the signaling pathways in which these genes function for the occurrence of drug resistance.
To understand the mechanism of tumor formation in more detail, Clevers adopted the formation of adenomas in the gut of mice as a model system. These tumors are completely dependent on the β-catenin-Tcf signaling pathway. An essential role was uncovered for Notch regarding the maintenance of crypt progenitors. Moreover, it was found that inhibition of Notch through pharmacological inhibition of γ-secretase in adenomas induces terminal differentiation, indicating that γ-secretase inhibitors may be useful to treat colon cancer. Another unexpected observation was done in the follow-up study of the role of EphB genes in colorectal cancer. Expression of these Wnt target genes is lost during progression of this malignancy in patients. Moreover, in a mouse model it was found that EphB activity actively suppresses colorectal cancer progression (Clevers). This finding counteracts a popular model that inhibition of EphB would be a mechanism to treat cancer and shows the power of this system to (in)validate targets.

Cancer proteomics

A recurrent theme regarding the genes that are found to be important players in the cancer process is that most of them encode proteins that function in signal transduction. Several aspects of these signaling pathways are studied using proteomic approaches (Bos). Central in these studies are the spatial and temporal control of key processes such as gene regulation, cell proliferation and cell adhesion. The results of these studies have revealed novel dynamic protein complexes involved in gene regulation and cell polarity.

CGC research 2006-2007

It is anticipated that the research performed within the four themes of the CGC program will further unravel the behavior of tumor cells. This will require a continuous incorporation of novel technologies - such as high through-put genome-wide screening and DNA sequencing - and a higher demand for bioinformatic tools. It will also include systems biology, in which mathematical approaches are used to further understand the complex and dynamic behavior of tumors and the interactions between tumor and host.

The societal component of cancer genomics research

Apart from performing basic research within the four themes mentioned above, the CGC has also invested in the societal aspects of cancer genomics, i.e. valorization and communication of its research findings.

In addition to a large number of patent applications and collaborations with industry, two new start-up companies were generated by CGC researchers. The gene expression profiles developed by Laura van ’t Veer and René Bernards were the basis for the establishment of Agendia, a spin-off company that commercializes these diagnostic profiles. A second start-up company DNage was established based on technology and

Highlights communication and education

- 8,000 page views of the CGC website www.cancergenomics.nl
- more than 200 participants at the CGC symposium' Genes, erroneous DNA and cancer' for breast cancer patients
- 10,000 copies of the CGC publication ' Genes, erroneous DNA and cancer' distributed to cancer patients, hospitals and high school students
- more than 500 visitors in the monthly Doelen Science Café in Rotterdam
- 23 articles on cancer & genomics published on the public website www.watisgenomics.nl
- more than 130 publications and presentations for the general public via various media
- 3,800 pupils in 100 high schools experienced the mobile DNA-lab 'Read the language of the tumor'
- 60 pupils from 15 high schools participated in a 2-week Junior Science Molecular Medicine research project at the Erasmus MC
- 2 CGC/CSG co-funded research projects on the societal component of genomics research

knowledge developed by Jan Hoeijmakers in the field of DNA repair, aging and cancer. Very recently, DNage has been acquired by the Dutch biotech company Pharming.

To communicate the relevance of the CGC research activities for the patient, the CGC held a symposium for breast cancer patients in collaboration with the Netherlands Breast Cancer Organization (BorstkankerVereniging Nederland) and published a book describing the contents of the meeting that was distributed to all 7,000 members of this organization.

As there is still a large gap between modern genomics research and subjects such as DNA and genetics in the current high-school curriculum, the NGI Centres of Excellence developed a series of five genomics teaching modules, which include a mobile lab that provides the high school student an opportunity to obtain hands-on experience with modern genomics technniques. The CCG developed the 4-hour module 'Read the lanaguage of the tumor' (Lees de taal van de tumor), which is offered to all schools in the Netherlands and will have reached about 100 schools and 3,800 pupils in the school year 2005-2006.

Based on this 'tumor' module, a larger 40-hour module is now being developed in collaboration with the Junior College Utrecht and the Centre for Science Education of Utrecht University that will initially be offered to schools in the Utrecht area. Subsequently we aim for it to be integrated in the new biology and science curriculum that is currently being developed for high schools in the Netherlands. Incorporated in our plans are refresher courses in genomics for high school teachers to help them prepare for these new developments.

Furthermore, the CGC supports the Junior Science Molecular Medicine Program of partners at the Erasmus MC, in which high school students participate in a two-week research project at the laboratories of the Medical Genetics Cluster.

Finally, the CGC supports two research projects that are carried out in collaboration with the Centre for Society and Genomics, one of the other Centres of Excellence of the NGI. The first, entitled 'DNA labs for citizenship: Learning for understanding and valuing genomics in upper-secondary education' focuses on the mobile DNA-lab and is expected to provide a solid scientific basis for the development of the new teaching modules mentioned above. The second project entitled 'The benefits and risks of cancer genomics for society: How new genomics-related technologies affect the perception and handling of cancer risks and prognosis by patients, doctors, nurses and the public' deals with information and communication on cancer related to genetic and genomical aspects in medical practice, the media and public health policy.

The CGC has also invested in the societal aspects of cancer genomics

Netherlands Genomics Initiative

Cancer | GENOMICS | CENTRE
improving cure rates for cancer patients

The why and how of the CGC

Every year, 69,000 people in the Netherlands are affected by cancer and approximately 37,000 die from the disease. Multiply these numbers by 20 and you have a picture of those affected across Europe. The aging of the population will lead to a further rise in the incidence of cancer and will soon make the disease the primary cause of death for individuals at young, middle and old age. Furthermore, the disease has enormous mental and physical impact on patients and their relatives. Thus, improving diagnosis, therapy and cure rates for cancer patients is of great importance for all those affected as well as for our society as a whole. It is with that exact goal that the Cancer Genomics Centre has been established.

In 2001 the Dutch government decided, on basis of the Report of the Committee Wijffels, that a large-scale approach and investment in genomics research was needed to improve the quality of life and strengthen the economic structure. The NGI was established under the auspices of the Netherlands Organization for Scientific Research (NWO) to develop a strategic plan for the development of an internationally renowned genomics knowledge infrastructure that stimulates excellent innovative research, generates a continuous stream of economic activities and is firmly anchored in society.
As a first step in the realization of this plan, NGI published a call for proposals for genomics Centres of Excellence. After a two-step procedure, four out of eleven proposals were finally selected by an international peer review committee, among which the proposal for the Cancer Genomics Centre (CGC).
On 1 October 2002, the CGC was formally established with a 14.6 million euro grant from the NGI for the period 1 October 2002 - 31 December 2007.

The CGC is a collaboration of prominent cancer research groups from the Netherlands Cancer Institute in Amsterdam, the Hubrecht Laboratory/Netherlands Institute for Developmental Biology in Utrecht, the Erasmus Medical Centre Rotterdam and the University Medical Center Utrecht. Its participants contribute complementary scientific and technical expertise combined with a large collection of clinical material, patient

databases and clinical expertise in the Netherlands.
The primary objective of the CGC research program is to obtain a complete picture of the genetic changes that turn a cell into a tumor cell and a full understanding of how each of these changes contributes to the behavior of tumor cells. This enables the development of effective diagnosis and therapy strategies and the identification of novel and suitable targets for therapeutic intervention. Such targets are validated in the specific model systems available in the CGC. Validated targets are further developed in collaboration with existing or new biotech companies and tested at clinical sites using the cancer clinics associated with the centre.
Furthermore, the CGC aims at transparent communication and education regarding the results and impact of cancer genomics for society. For this purpose, a variety of interactive projects are developed for several societal groups, e.g. high school students, teachers and cancer patients.

Mission

The mission of the Cancer Genomics Centre is to improve diagnosis, therapy and cure rates for cancer patients. Genomics offers new promising opportunities for cancer research, with realistic expectations for therapy improvement in the coming decade. For instance, the genomics signature of the cancer and that of the patient may serve in the near future as a basis on which to choose the most effective therapy for the individual patient ('personalized medicine') to improve cancer patients chances of recovery and their quality of life.

Erasmus MC
Universitair Medisch Centrum Rotterdam

NKI-AVL
Het Nederlands Kanker Instituut
Antoni van Leeuwenhoek Ziekenhuis

Royal Netherlands Academy of Arts and Sciences
HUBRECHT LABORATORY
Netherlands Institute for Developmental Biology

UMC
Universitair Medisch Centrum
Utrecht

CGC in names and numbers

Board of Directors

- Prof.dr. J.L. (Hans) Bos, scientific director
- Dr. J.E. (Annelies) Speksnijder, managing director
- Dr. J.P. (John) de Koning, valorization officer

Advisory Board

- Prof.dr. D. (Dirk) Bootsma (Erasmus Medical Centre Rotterdam)
- Prof.dr. P. (Piet) Borst (Netherlands Cancer Institute)
- Dr. E. (Els) Borst-Eilers (Netherlands Federation for Cancer Patient Organizations: NKF)
- Dr. R. (Rian) de Jonge (Guide Management)
- Prof.dr. P. (Peter) van der Vliet (University Medical Center Utrecht)

Research institutes

- Netherlands Cancer Institute/Antoni van Leeuwenhoek Ziekenhuis (NKI), Amsterdam
- Erasmus Medical Centre Rotterdam (Erasmus MC)
- Hubrecht Laboratory/Netherlands Institute for Developmental Biology (NIOB), Utrecht
- University Medical Center Utrecht (UMCU)

Participants

- Prof.dr. R. (René) Bernards, Division of Molecular Carcinogenesis, NKI
- Prof.dr. A. (Ton) Berns, Division of Molecular Genetics, NKI
- Prof.dr. J.L. (Hans) Bos, Department of Physiological Chemistry, UMCU
- Prof.dr. H. (Hans) Clevers, NIOB
- Prof.dr. F.G. (Frank) Grosveld, Department of Cell Biology & Genetics, Erasmus MC
- Prof.dr. J. (Jan) Hoeijmakers, Department of Cell Biology & Genetics, Erasmus MC
- Prof.dr. J. (Jan) Klijn, Department of Medical Oncology, Erasmus MC
- Prof.dr. R. (Ronald) Plasterk, NIOB
- Prof.dr. S. (Sjoerd) Rodenhuis, Division of Medical Oncology, NKI
- Dr. L.J. (Laura) van 't Veer, Division of Diagnostic Oncology, NKI

Personnel

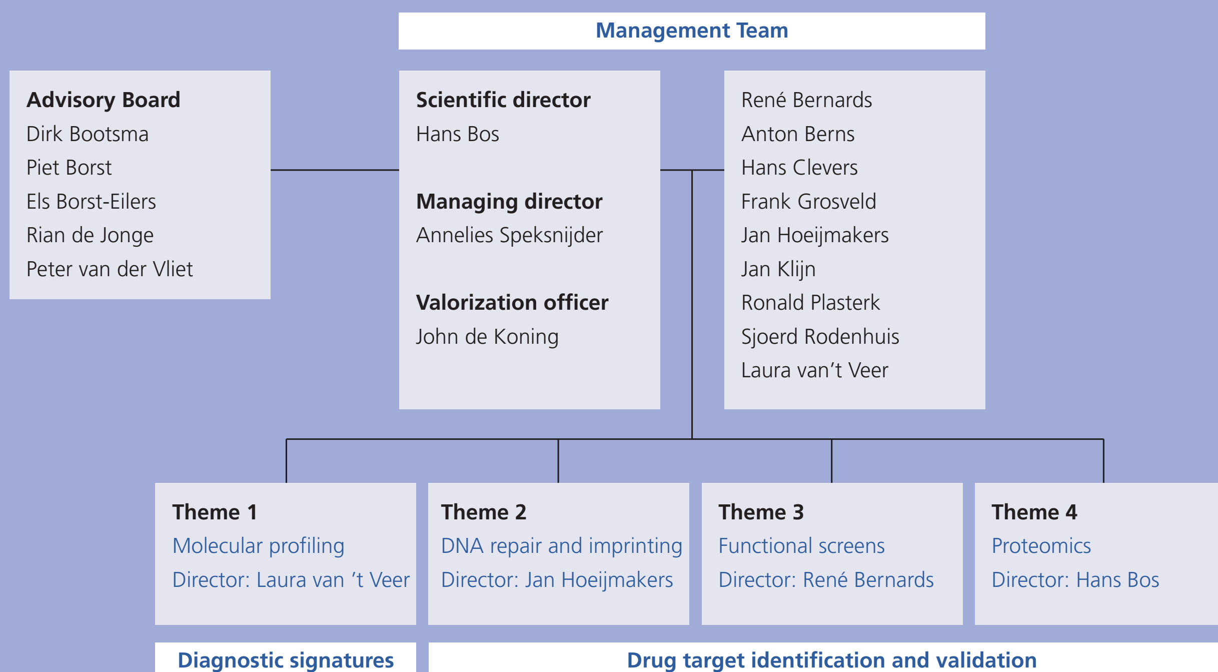
23 scientific staff
62 postdoctoral fellows
48 graduate students
8 bioinformaticians
6 database managers
4 research assistants
63 technicians
214 total, representing about 70 fte

Budget

The CGC receives a 14.6 million euro grant from the NGI for the period 1 October 2002 - 31 December 2007. Additional research funds have been obtained from the participating institutes and organizations such as the Netherlands Cancer Society (KWF Kankerbestrijding), the Netherlands Organization for Scientific Research (NWO), the EC and the Centre for Biomedical Genetics (CBG). The total budget of the centre is 44 million euro.

Contact

Annelies Speksnijder
University Medical Center Utrecht l Stratenum 3.223
Universiteitsweg 100 l 3584 CG Utrecht
PO Box 85060 l 3508 AB Utrecht l The Netherlands
T + 31 (0)30 2538988 / 2533992 l F + 31 (0)30 2539035
a.speksnijder@med.uu.nl l www.cancergenomics.nl

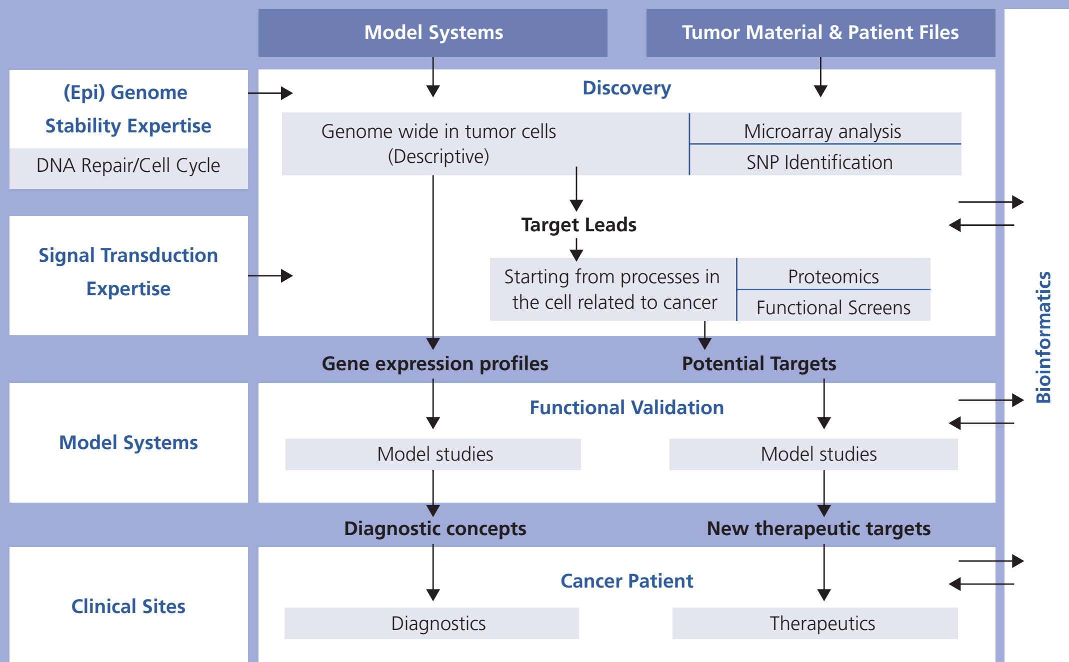


Organization

The program is managed by a Management Team consisting of the participants, the scientific director, the managing director and the valorization officer. The Management Team is responsible for strategic decisions and execution of the CGC program. In this first task, the team is aided by the Advisory Board. Execution of the research program is by four research teams, each coordinated by one of the participants acting as research theme director.

The scientific director acts as chairman of the management team and represents the CGC regarding scientific matters. Valorization of the research results is coordinated by the valorization officer. Daily coordination and representation of the CGC is in the hands of the managing director, who is also responsible for the budget and for preparing the half-yearly reports. The managing director is also in charge of the development and execution of the CGC education and communication programs. The final responsibility for all CGC activities lies in the hands of the scientific director.

The Management Team meets at regular intervals to discuss progress and the general strategy as well as budgetary matters and matters concerning investments in equipment. Many informal contacts already exist in the context of collaborative projects. Both directors are in regular contact with the other management team members on matters of daily interest for the group and to exchange scientific information. The members of the Advisory Board are consulted on a regular basis by the directors on matters regarding the general strategy.



Research strategy

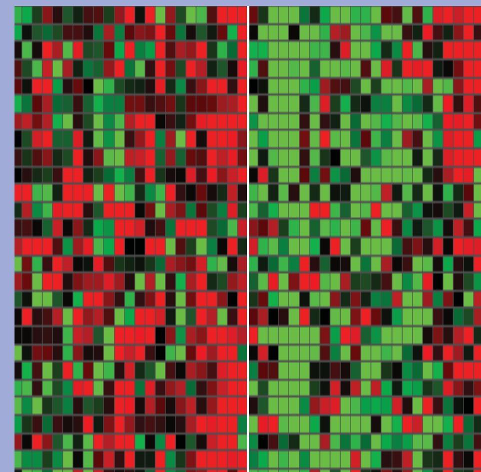
The CGC's research strategy is based on optimal utilization, expansion and integration of fundamental and applied research activities of the participants. In this way we can contribute to the development of improved diagnostic methods as well as better treatment methods and products for cancer patients.

Two critical resources accessible to the team make the research program internationally highly competitive and innovative:

- The tumor samples available to the CGC are utilized to establish molecular profiles of human tumors. This encompasses a major refinement of tumor classification and allows a more accurate prediction of outcome of disease. Furthermore, SNP analysis allows identification of (inherited) predisposing factors. This may lead to the uncovering of interactions with specific environmental/life-style factors putting individuals with a particular genetic profile at risk. The well-defined tumor banks with patient follow-up records form a unique resource that will be fully exploited.
- Model systems ranging from cultured mammalian cells to model organisms such as the nematode C. elegans, the zebrafish and genetically modified mice, are used to define and validate new potential targets for therapeutic intervention as well as high throughput genomic technologies and in silico approaches. Subsequently, validated targets are developed in collaboration with existing or new biotech companies and tested at clinical sites including the cancer clinics directly associated with the CGC.

To reach our goals, several integrated genomics platforms are used as in the schedule above.

ILLUSTRATIE: RENEE VAN AMERONGEN



Theme 1: Molecular profiling

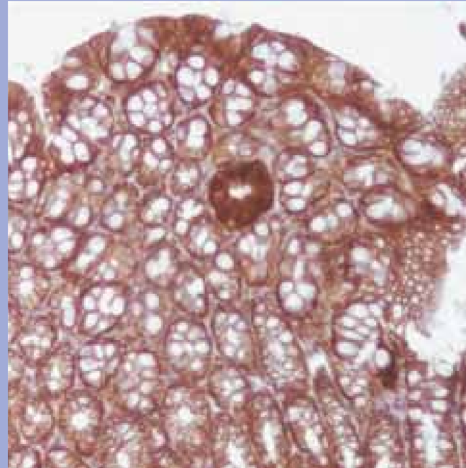
Research themes

Cancer is a disease of the genes. A limited number of genetic mutations can turn a healthy cell into a tumor cell. Over the past 30 years, researchers have acquired a basic understanding of how those genetic changes may contribute to the behavior of cancer cells. A number of cancer-causing genes (oncogenes) have been identified, and some pathways in which these genes act have been uncovered. However, to effectively attack each of the different cancers, a complete picture of the underlying mechanisms that can turn a normal cell into a cancer cell is needed. This requires large-scale data acquisition and analysis, extensive studies into the correlation of molecular parameters and patient histories, and studies in model systems that permit full exploitation of the power of experimental molecular genetics and genomic analysis. To realize such an approach, the CGC has developed an integrated research program that is divided into four themes.

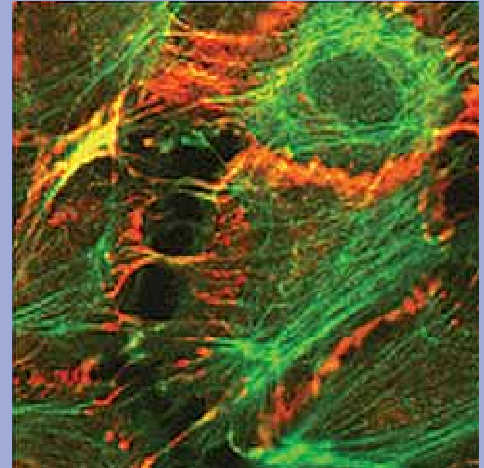
Theme 1: Molecular Profiling

The aim of this theme is to establish molecular profiles of human tumors that will improve diagnosis and clinical management of cancer patients. Combining molecular profiling of expressed genes in tumor tissue with advanced bioinformatics tools is beginning to show its power in revealing disease characteristics that are otherwise indistinguishable. This can be used to refine current tumor classification methods and allows for a more accurate prediction for patients of the disease outcome. The implementation of molecular profiles as diagnostic tools in daily clinical practice will lead to a more accurate and effective treatment that is tailored to the patient's needs. Research in this theme focuses on breast cancer, lung tumors and oligodendroglioma's.

Theme 2: DNA repair and imprinting



Theme 3: Functional screens



Theme 4: Functional proteomics

Theme 2: DNA repair and imprinting

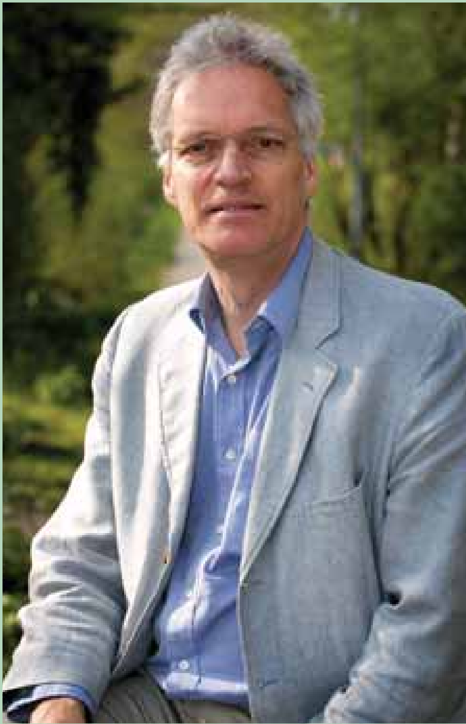
One of the hallmarks of cancer is the occurrence of genetic instability. It drives the initiation and progression of tumor-genesis (the emergence of a tumor) and frustrates effective therapy. Tumor cells display a wide array of derailments in genome control and repair systems, which allows tumors to rapidly acquire new properties. The aim of this theme is a comprehensive, systematic screening for genes that are implicated in genome stability. The mode of action of these genes at the molecular, cellular and organismal level and their involvement in carcinogenesis are examined. This will yield critical targets for improved diagnosis, reliable prognosis and development of new therapeutic medication and may identify novel etiological factors relevant for cancer prevention. In addition, various reporter systems for the detection of DNA damage, genetic stress and genome instability are developed. Finally, part of the genetic instability of tumors may be due to epigenetic mechanisms, which are addressed in this theme as well.

Theme 3: Functional screens

Research in this theme aims to identify novel genes that act in cancer-relevant pathways using functional genetic approaches. Such genes are prime targets for drug discovery programs. Two types of functional screens are used: gain-of-function and loss-of-function screens, both performed in cultured cells and animal model systems. Identification of genes is followed by validating them for therapeutic intervention, using RNAi in-hibition techniques. The next step, identifying suitable drugs for therapeutic interventions, requires a more industrial approach and is done in collaboration with new spin-offs or existing (bio)pharmaceutical companies.

Theme 4: Functional proteomics

The aim of this theme is to identify and classify differences in protein expression and modifications that underlie the cancer process. The focus is on the identification of protein modifications and protein-protein interactions using mass spectrometry of purified proteins or protein complexes and genome-wide inter-action screens using the yeast two-hybrid system. Both these technologies are applied on relevant proteins under study and are a major aspect of the functional characterization of proteins that are identified in microarray analyses and functional screens.



René Bernards

After René Bernards received his PhD in 1984 from the University of Leiden, he joined the group of Robert Weinberg at the Whitehead Institute in Cambridge, USA for his postdoctoral training. In 1988 he started working as an assistant professor at the Massachusetts General Hospital Cancer Center. Back in Holland in 1992, he was appointed staff scientist at the Netherlands Cancer Institute (NKI) where he studies cancer genetics. In 1994 he was appointed part time professor at Utrecht University. In the last three years, his laboratory has focused on the development of new tools for genome-wide loss of function genetic screens using RNA interference to identify novel genes that act in cancer relevant pathways. In July 2003, he co-founded 'Agendia', a genomics-based diagnostic company that started offering the first microarray-based diagnostic test for the clinical management of breast cancer in 2004. In 2005 he received the Spinoza award. René Bernards is a member of the European Molecular Biology Organization.

Goals

To identify novel genes that have a role in carcinogenesis using innovative functional genomics tools such as high-throughput gain-of-function and loss-of-function genetic screens.

Functional screens in cultured mammalian cells

Gain-of-function functional screens in cultured cells involve the introduction of high-complexity retroviral cDNA expression libraries into engineered cells, followed by selection of cells with an altered phenotype. In the past, we have successfully used this technology to isolate a number of genes that prevent the induction of cellular senescence. The loss-of-function screens in cultured cells take advantage of a new vector that was developed at the NKI and allows stable suppression of gene expression through RNA interference. We have successfully used this vector to stably suppress expression of large numbers of genes. As part of the CGC program, we use this vector system to generate a large collection of 10,000 siRNA vectors that each suppress the expression of a single gene. This collection of siRNA vectors has made it possible for the first time to perform large-scale screens for loss-of-function phenotypes in mammalian cells.

Gain of function genetic screens

To search for genes that control the sensitivity of cancer cells to a class of small molecules that inhibit Histone DeACetylases (HDACi), we have used high-complexity retroviral cDNA expression libraries. For that purpose we selected cDNAs whose elevated expression conferred resistance to one such compound, PXD-101. In 2003, two such cDNAs were found, encoding the Retinoic Acid Receptor α (RAR-α) and PReferential Antigen MElanoma (PRAME), respectively. Further investigation of the molecular mechanism of PRAME action showed that it acts as a dominant repressor of certain nuclear receptors, including RAR-α. Subsequently two major questions concerning the role of PRAME in cancer were addressed. First, we studied in which signaling pathways PRAME acts in the various types of cancer in which it is over-expressed. Our data indicate that PRAME can inhibit RAR signaling in a highly specific fashion through direct physical interaction with RAR-α. Second, we studied how PRAME mediates repression of transcription, as it does not appear to require HDAC. To address these issues, we investigated which proteins interact with PRAME in distinct cancer cells using a proteomics approach. In first instance, we expressed epitope-tagged PRAME and identified associated proteins. We

Group members

Postdoctoral fellows

Katrien Berns
Luis Borlado
Annette Dirac
Pieter Eichhorn
Sidong Huang
Rianne Oosterkamp
Benjamin Rowland
Miguel Rubio
Linda Smit
Avi Shvarts
Daniel Peeper
Blanca Scheijen
Reuven Agami
Liming Wang
Michael Edel

PhD students

Mirjam Epping
Ernst Geutjes
Roderik Kortlever
Jasper Mullenders
Hans Masselink,
Thijn Brummelkamp
Sebastian Nijman
Menno Creyghton

Technicians

Marielle Hijmans
Mandy Madiredjo
Miranda van Dongen

found that two components of the polycomb family interact with PRAME: EZH2 and EED. Knockdown of either of these two proteins interfered with PRAME-mediated suppression of transcription, showing that these proteins have a function in mediating PRAME suppression. We found that human melanoma cells (which often express high levels of PRAME) could be re-sensitized to Retinoic Acid by PRAME knockdown. Conversely, over-expression of PRAME in F9 teratocarcinoma cells conferred resistance to Retinoic Acid. Together these data indicate that PRAME is a major regulator of Retinoic Acid signaling (Epping et al. 2005).

shRNA library

The significant support of CGC in the first year for our shRNA library puts us in an excellent competitive position to perform large-scale loss-of-function genetic screens in mammalian cells. In the course of 2003, we completed a library of some 24,000 shRNA vectors, which together target 8,000 human genes for

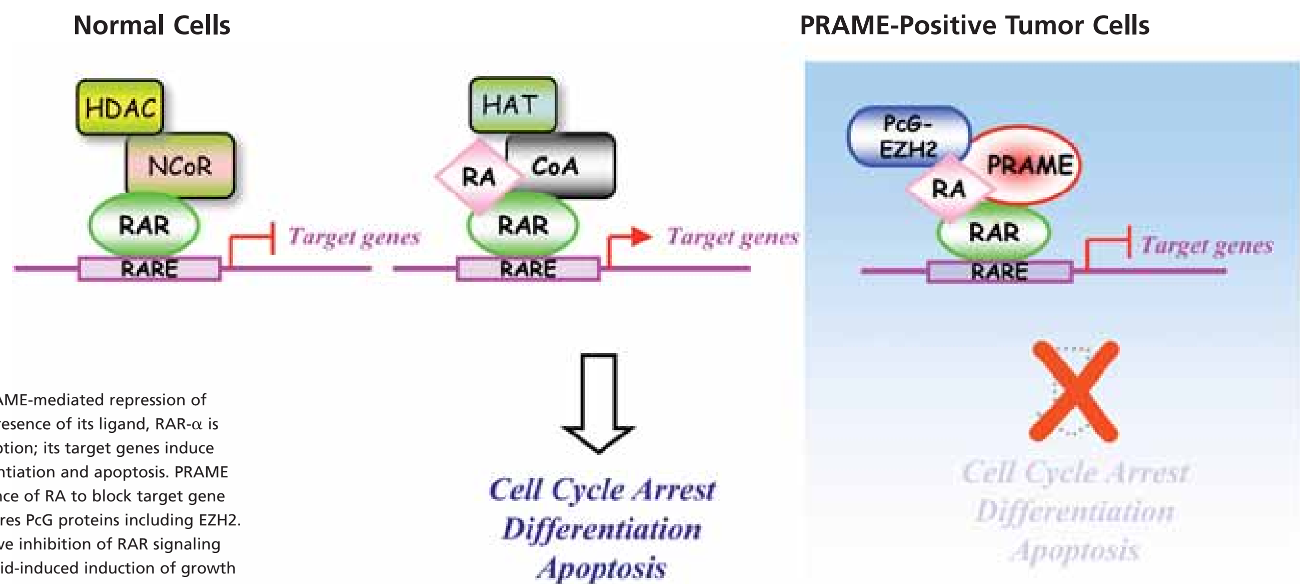


Figure 1. Model for PRAME-mediated repression of RAR signaling. In the presence of its ligand, RAR-α is an activator of transcription; its target genes induce cell cycle arrest, differentiation and apoptosis. PRAME binds RAR in the presence of RA to block target gene expression, which requires PcG proteins including EZH2. The resulting constitutive inhibition of RAR signaling prevents the retinoic acid-induced induction of growth arrest, differentiation and cell death. RARE: Retinoic Acid Responsive Element. RA: Retinoic Acid.

Selected publications 2002-2005

1. Bernards, R. and R. A. Weinberg (2002). 'A progression puzzle'. Nature 418(6900): 823.
2. Brummelkamp, T. R., R. Bernards et al. (2002). 'A system for stable expression of short interfering RNAs in mammalian cells'. Science 296(5567): 550-3.
3. Peeper, D. S., A. Shvarts et al. (2002). 'A functional screen identifies hDRIL1 as an oncogene that rescues RAS-induced senescence'. Nat Cell Biol 4(2): 148-53.
4. Van 't Veer, L. J., H. Dai et al. (2002). 'Gene expression profiling predicts clinical outcome of breast cancer'. Nature 415(6871): 530-6.
5. Van de Vijver, M. J., Y. D. He et al. (2002). 'A gene-expression signature as a predictor of survival in breast cancer'. N Engl J Med 347(25): 1999-2009.
6. Bernards, R. (2003). 'Cancer: cues for migration'. Nature 425(6955): 247-8.
7. Brummelkamp, T. R. and R. Bernards (2003). 'New tools for functional mammalian cancer genetics'. Nat Rev Cancer 3(10): 781-9.
8. Brummelkamp, T. R., S. M. Nijman et al. (2003). 'Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB'. Nature 424(6950): 797-801.
9. Bernards, R. (2004). 'Wiping out cancer'. Nat Genet 36(4): 319-20.
10. Berns, K., E. M. Hijmans et al. (2004). 'A large-scale RNAi screen in human cells identifies new components of the p53 pathway'. Nature 428(6981): 431-7.
11. Epping, M. T., L. Wang et al. (2005). 'The human tumor antigen PRAME is a dominant repressor of retinoic acid receptor signaling'. Cell 122(6): 835-47.
12. Kolfschoten, I. G., B. van Leeuwen et al. (2005). 'A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity'. Cell 121(6): 849-58.
13. Nijman, S. M., M. P. Luna-Vargas et al. (2005). 'A genomic and functional inventory of deubiquitinating enzymes'. Cell 123(5): 773-86.
14. Nijman, S. M., T. T. Huang et al. (2005). 'The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway'. Mol Cell 17(3): 331-9.
15. Brummelkamp, T.R., A. Fabius et al. (2006). 'An shRNA barcode screen provides insight into cancer cell vulnerability to MDM2 inhibitors'. Nature Chem. Biol. 2, 202-206.

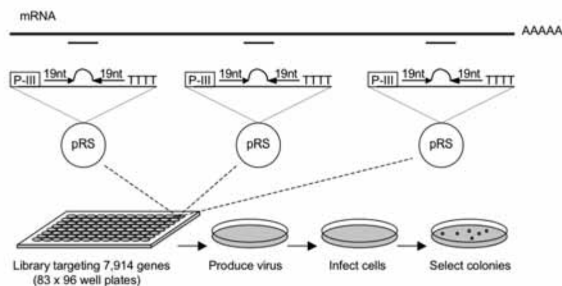


Figure 2. The NKI RNAi library. For each gene transcript, three 19 nucleotide (nt) sequences were designed. These were converted into pairs of complementary 59-mer hairpin oligonucleotides and cloned into pRETRO-SUPER. Three vectors targeting one gene were pooled in a single well of a 96 well plate. From each 96 well plate high titer polyclonal virus can be produced, which can subsequently be used to infect cells. Colonies of phenotypically distinct cells can be selected and the identity of the hairpin vector revealed by DNA sequence analysis.

suppression. As part of the first 'proof of concept', we used this shRNA vector library to identify five novel components of the cancer-relevant p53 tumor suppressor pathway. This was the first large-scale loss of function genetic screen in mammalian cells. The publication of this work attracted considerable attention from both the scientific press and the lay media (Berns et al, 2004). We then performed a saturating screen for genes that act in the p53 pathway, which identified an additional fifteen candidate p53 pathway components. These are currently being studied in the Bernards laboratory.

TGF-β and RB-pathways

We subsequently used our shRNA library to identify novel genes in a number of cancer relevant pathways and performed two screens for novel components of the TGF-β pathway and the RB pathway. Much time was spent to set up the screens to allow genome wide screens with the existing shRNA library. We performed a shRNA bar code screen to identify novel components of TGF-β signaling and searched for shRNA vectors that conferred resistance to the growth inhibitory effect of TGF-β in HaCaT human keratinocytes. Very gratifyingly, we identified several known components of the pathway in this screen, including SMAD4 and TGFBR2. In addition, this made it possible to identify over ten novel genes whose suppression confers resistance to TGF-β. These are currently being followed up in the laboratory.

For the screen in the RB pathway, we took advantage of a mutant form of RB in which fifteen of the phosphorylation sites were mutated into alanines, thereby conferring a constitutive growth inhibitory activity onto the protein. We searched for shRNAs that confer resistance to the growth arrest by this active RB protein and found that, apart from RB itself (which served as a positive control), suppression of several other genes conferred resistance to RB growth arrest, including an RB induced protein named RB1CC1. We are currently studying the functional interaction of these newly identified genes with the RB pathway.

Fanconi Anemia pathway

To search for DUBs that modulate the important Fanconi Anemia pathway, we used a subset of our shRNA library (targeting the family of de-ubiquitinating enzymes (DUBs) for suppression). Specifically, the search was for DUBs that control the mono-ubiquitination of Fanconi Anemia D2 protein (FANCD2),

Future projects

- Identification of synthetic lethal interactions in mammalian cells.
  We will search for genes whose inactivation confers additional sensitivity to cells having defined genetic lesions
- Study of mechanisms of drug resistance
  We will focus on the 'targeted therapeutics' such as EGFR and HER2 inhibitory drugs. By identifying genes whose over-expression or suppression can modulate cellular responses to these drugs, we will get insight into mechanisms of drug resistance Such studies may yield biomarkers that predict therapy responsiveness in the short term and suggest new therapeutic intervention strategies to prevent therapy resistance in the longer run

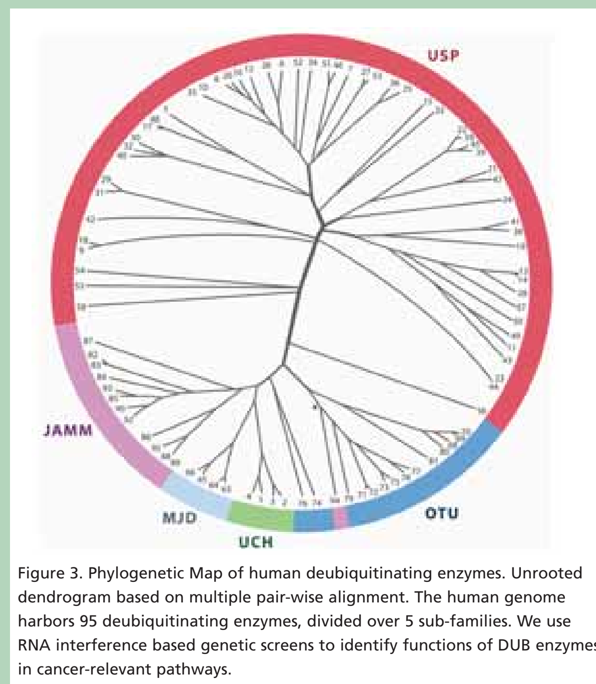


Figure 3. Phylogenetic Map of human deubiquitinating enzymes. Unrooted dendrogram based on multiple pair-wise alignment. The human genome harbors 95 deubiquitinating enzymes, divided over 5 sub-families. We use RNA interference based genetic screens to identify functions of DUB enzymes in cancer-relevant pathways.

a key component of the Fanconi Anemia pathway. In collaboration with Alan D'Andrea at the Dana Farber in Boston, we found that the DUB USP1 is a key regulator of FANCD2 (Nijman et al. 2005). More recently, we found that DNA damage signaling causes auto-cleavage of USP1, causing the protein to auto-inactivate after its activation by DNA damage.

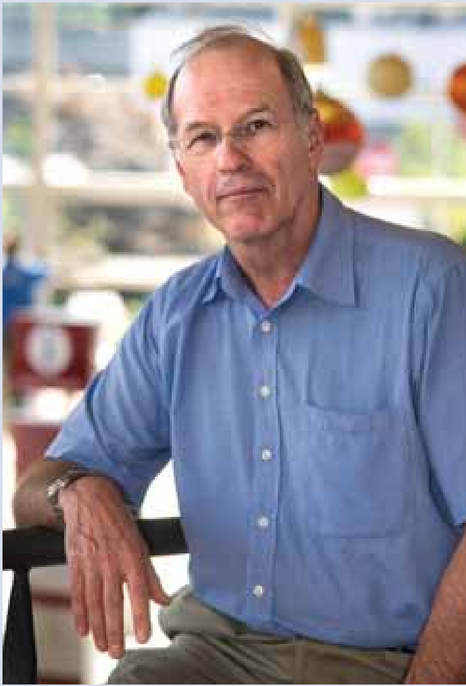
Tamoxifen resistance

Of more direct relevance to the clinic were our experiments that aimed to identify genes whose inactivation (by shRNA vectors) confers resistance to the anti-estrogen tamoxifen. This drug is frequently used in the clinic for the treatment of breast cancer, but resistance is often observed after prolonged treatment. We performed loss-of-function genetic screens to identify genes whose suppression confers resistance to the anti estrogen tamoxifen in breast cancer. We used the highly tamoxifen sensitive breast cancer cell line ZR-75-1 for these screens. After infection with the shRNA library, cells were exposed for two months to tamoxifen and resistant colonies were isolated. Several shRNAs have been isolated that confer resistance to tamoxifen. One of these has been validated in several additional functional assays. One of the hits in the screen is a regulator of HER2 expression, highlighting the functional interaction between HER2 signaling and Estrogen Receptor (ER) signaling. We are currently studying the functional interaction of these newly identified genes with the ER signaling pathway.

> Activation of signaling downstream of the HER2 receptor can bypass growth arrest induced by trastuzumab

Trastuzumab resistance

We also started a translational research project that aims to understand the mechanisms of resistance to the targeted therapeutic for breast cancer trastuzumab (Herceptin). This monoclonal antibody targets the HER2 growth factor receptor, which is over-expressed in some 15-25% of all breast cancers. However, for unknown reasons not all patients that over-express this receptor respond to trastuzumab therapy. By searching for genes whose inactivation confers resistance to trastuzumab in vitro, we hope to understand how resistance develops in patients. To this purpose we identified a breast cancer cell line, BT474, which has amplified copies of the HER2 gene and which is very sensitive to trastuzumab-induced growth arrest in cell culture. We performed an shRNA bar code screen in these cells in the presence of trastuzumab. Interestingly, we found only one gene whose suppression confers clear resistance to trastuzumab, the tumor suppressor PTEN. This suggests that activation of signaling downstream of the HER2 receptor can bypass growth arrest induced by trastuzumab. We are currently asking if mutations are found in patients that fail to respond to trastuzumab in the signaling pathway downstream of HER2. Of particular interest is the catalytic subunit of PI3 kinase, as this gene is mutated (activated) in some 30% of all human breast cancers.



Anton Berns

In 1972 Anton Berns received his PhD from the University of Nijmegen. He did his postdoctoral training in the group of Rudolf Jaenisch at the Salk Institute where he studied the role of retroviruses in causing lymphomas in mice. In 1976 he returned to the University of Nijmegen where his group explored proviral insertional mutagenesis as a means to identify new oncogenes. In 1985 he was appointed as staff scientist at the Netherlands Cancer Institute. Here his group did pioneering work to generate and utilize genetically modified mice as a tool to search for new cancer genes. In 1999, he was appointed as Director of Research and Chairman of the Board of Directors of the Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital. He is a member of the Scientific Advisory Board of several cancer institutes, of Xenogen corporation and Life Sciences Partners. Anton Berns is also a member of the Royal Netherlands Academy of Sciences and of the European Molecular Biology Organization.

Goals

To search for novel oncogenes, tumor suppressor genes, and regulatory elements in the mouse genome that contribute to tumorigenesis.

Gain of function screens using insertional mutagenesis

By high-throughput sequencing of proviral or transposon insertion sites in mice, we search for oncogenes, tumor suppressor genes, and regulatory elements in the mammalian genome that can contribute to tumorigenesis. Expectedly, 30-50% of these insertions have occurred near proto-oncogenes or in tumor suppressor genes and mark therefore genes that are potential candidates for therapeutic intervention. By determining the site of insertions of many thousands of proviruses and transposons we expect to perform saturation mutagenesis, thereby gaining access to several thousands of genes and regulatory elements relevant for cancer. We apply sensitized screens in transgenic or knockout (-/-) mice predisposed to particular tumors. In this way, we will be able to identify critical genes and control elements involved in various tumor types.

High-throughput analysis

Early in the project we have attempted to clone cells from the induced mouse tumors in order to be certain that the insertions found would be derived from a monoclonal cell population (allowing assignment of insertions to complementation groups in transformation). In spite of a substantial effort, we have been unable to achieve this goal for the lymphomas in our tumor panel. Subsequently, we have shown that in principle, a single-cell PCR amplification protocol can circumvent this problem. However, because of the additional effort required, we have decided to perform single cell analysis only on a selected subset of the tumors.

In the course of the project, we also realized that to make it really successful we would need access to high throughput sequencing capacity, especially when the project moves from proviral insertional mutagenesis to transposon-mediated mutagenesis. In the latter case a reservoir of several hundred transposons introduced in a single chromosomal site will be mobilized to randomly insert into the genome in a controlled fashion (Cre-mediated and Tet-inducible). This requires the sequencing

Group members

Co-supervisor

Prof. Maarten van Lohuizen

Paul Krimpenhof

Martijn Nawijn

Postdoctoral fellows

Anthony Uren

Jaap Kool

Anders Lund

PhD students

Konstantin Matentzoglu

Andrej Alendar

Technicians

Colin Pritchard

Jos de Moes

Danielle Hulsman

Wendy Lagcher

of large numbers of insertions per tumor in order to uncover the relevant insertions, so called common insertion sites (CIS). Therefore, we have established a collaboration with the Sanger Centre in Cambridge, which gives us access to high sophisticated automation permitting us to perform a much larger screen than originally planned.

Pipeline for handling sequence reads and datamining

Substantial emphasis has been put on expanding our tumor series and we have included most of the tumor sets that have been generated in the last ten years. PCR products from 1,300 tumors have been sequenced (over 200,000 reads). The first 45,000 served as a sample set used to design a suitable database and to develop algorithms that allow effective mining of the data. All sequences are analyzed for quality, vector clipped, mapped and oriented on the genome. Read information is then compiled between PCR samples derived from the same tumor and formatted into a file which contains insert mapping information as well as genotype/phenotype information for the mouse each insert came from. This file is then used by our statistics application CIMPL (common insertion site mapping platform) for identifying new common insertion sites and genotype/phenotype specificities. The database also has a web interface that allows it to be easily queried by non-informaticists wishing to analyze particular subsets of insertion sites from different screens. The data will be made publicly accessible. As it will constitute the largest database of oncogenic elements available today (oncogenes, tumor suppressor genes, and regulatory elements), the value of this dataset for the field is evident. It will be very suitable for cross-validating oncogenic element identified on other platforms (translocations, mutation analysis, expression array analysis and Comparative Genome Hybridization).

> This database will be very suitable for cross-validating oncogenic elements identified on other platforms

Screen of MuLV-induced tumors in p19ARF-/- and p53-/- mice

For the first screen we induced tumors in 167 wild-type, 220 p19-/- and 122 p53-/- mice. All tumors have been analyzed by FACS for their B/T cell content and DNA from all tumors has been prepped for analysis of proviral insertion sites. Each tumor is analyzed by PCR amplification of insertion sites at NKI, followed by shotgun subcloning and sequencing of PCR samples at the Wellcome Trust Sanger Institute. The set has been conducted with two restriction enzyme pairs as we determined that too many insertions were missed when only one restriction enzyme set was used (a matter of economy: extra information gained by doubling the sequence effort is substantial: increase of 70%). Per tumor we cloned and sequenced 192 fragments, yielding a total number of around 95,000

Selected publications 2002-2005

1. Jonkers, J. and A. Berns (2002). 'Conditional mouse models of sporadic cancer'. Nat Rev Cancer 2(4): 251-65.
2. Mikkers, H., J. Allen et al. (2002). 'High-throughput retroviral tagging to identify components of specific signaling pathways in cancer'. Nat Genet 32(1): 153-9.
3. Vooijs, M., J. Jonkers et al. (2002). 'Noninvasive imaging of spontaneous retinoblastoma pathway-dependent tumors in mice'. Cancer Res 62(6): 1862-7.
4. Berns, A. (2003). 'Tumour suppressors: timing will tell'. Nature 424(6945): 140-1.
5. Lyons, S. K., R. Meuwissen et al. (2003). 'The generation of a conditional reporter that enables bioluminescence imaging of Cre/loxP-dependent tumorigenesis in mice'. Cancer Res 63(21): 7042-6.
6. Meuwissen, R., S. C. Linn et al. (2003). 'Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model'. Cancer Cell 4(3): 181-9.
7. Mikkers, H. and A. Berns (2003). 'Retroviral insertional mutagenesis: tagging cancer pathways'. Adv Cancer Res 88: 53-99.
8. Auwerx, J., P. Avner et al. (2004). 'The European dimension for the mouse genome mutagenesis program'. Nat Genet 36(9): 925-7.
9. Berns, A. (2004). 'Good news for gene therapy'. N Engl J Med 350(16): 1679-80.
10. Jonkers, J. and A. Berns (2004). 'Oncogene addiction: sometimes a temporary slavery'. Cancer Cell 6(6): 535-8.
11. Berns, A. (2005). 'Cancer: two in one'. Nature 436(7052): 787-9.
12. Berns, A. (2005). 'Stem cells for lung cancer?'. Cell 121(6): 811-3.
13. Ma, X., A. C. Ziel-van der Made et al. (2005). 'Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis'. Cancer Res 65(13): 5730-9.
14. Meuwissen, R. and A. Berns (2005). 'Mouse models for human lung cancer'. Genes Dev 19(6): 643-64.
15. Uren, A. G., J. Kool et al. (2005). 'Retroviral insertional mutagenesis: past, present and future'. Oncogene 24(52): 7656-72.

sequences for this screen and constituting approximately 22,000 independent insertions. Of those, 4,300 were cloned at least twice and sequenced, suggesting that these likely represent clonal insertions. To provide insight into the preferential insertion sites of these retroviruses, we also analyzed the distribution of viral insertions shortly after infection. This serves both as a reference for insertions that occur in sites that promote tumorigenesis and provides insight in a possible correlation between chromatin structure and insertion events. Data mining of this first set of sequence reads showed, as expected, frequent insertions in already known oncogenes but also in many new oncogenes and tumor suppressor genes. Using stringent newly developed algorithms we identified 356 common insertion sites identifying oncogenes, tumor suppressor genes and several micro-RNAs (see below). More than 30 of the CIS were shown to be genotype specific.

Analysis of common insertion sites near micro-RNA genes

Furthermore we have investigated whether we find CISs of the p19-/-, p53-/- and wt screen near the currently known miRNA genes in the mouse genome and have identified a number of

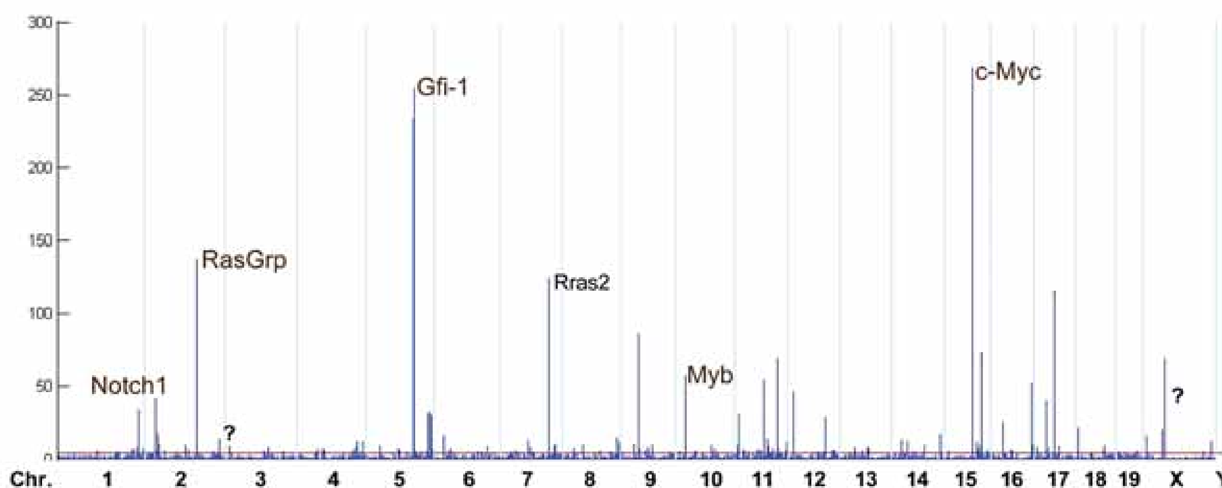


Fig. 1. Representation of a low sensitivity representation. Common insertion sites in the tumor panel. Vertical axis shows the number of tumors in which a particular insertion site was found (e.g. Gfi1 and Myc have been activated in over 250 of the tumors). Horizontal axis: the chromosomes are listed. The red horizontal line indicates the number above which the insertions are statistical significant using a kernel size of 30 Kbp. In total 356 common insertions sites reach statistical significance.

Future projects

- Characterization of oncogenes that act through new mechanisms. We expect to identify oncogenes that have biochemical functions allowing new intervention strategies
- Identification of obligatory haploinsufficient tumor suppressor genes. These fulfill essential functions and predispose to tumorigenesis when the gene dosage is reduced. The current screen is particular suited to identify such genes
- Deployment of transposon-based insertional mutagenesis to specifically search for tumor-type-specific oncogenes and tumor suppressor genes

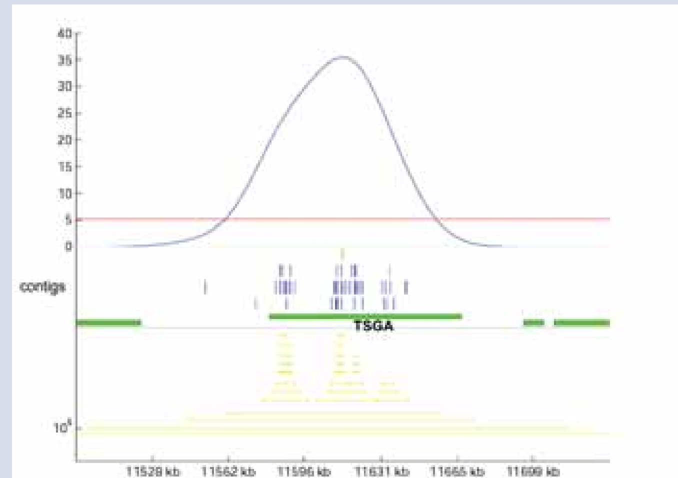


Fig.2. Identification of tumor suppressor genes. An insertion cluster in a putative tumor suppressor gene is shown. Tumor suppressor genes are scored by first identifying clustering of insertion sites between the transcriptional start and stop sites. Subsequently these clusters are scrutinized for the orientation and site of insertion of the proviruses. At least a subset of the insertions should lead to functional inactivation of the gene. Note the dispersed location of the insertions over the gene.

CISs near miRNA genes. One of the CISs is found near a polycistronic miRNA gene, which has already been demonstrated to be oncogenic by the group of S. Lowe, which shows that our approach is also suitable for identifying oncogenic miRNAs. At the moment, we are focusing on a locus containing a miRNA gene that contains insertions in a large number of tumors. We have already demonstrated that miRNA expression is affected by these insertions, and are now testing the oncogenicity of this miRNA using bone marrow stem cell transplantation assays.

Screen of MMTV-induced tumors in Pten+/- mice

A similar strategy is underway to identify common insertion sites in MMTV-induced mammary tumors. Originally, we had planned to induce tumors in conditional Pten and P53 knockout mice (inactivated by mammary-specific expression of Cre recombinase). In our pilot experiments, it appeared that in the genetic background of these conditional knockout/Cre transgenic mice, MMTV infection and thus tumor induction was very inefficient. Therefore, we have decided to perform these experiments in Pten heterozygotes that were backcrossed to the FVB strain (that can be infected efficiently with MMTV). Our aim is to generate at least 100 mammary tumors from Pten+/- and wt mice. We have designed a robust splinkerette amplification protocol for amplifying flanking sequences of MMTV proviruses. As soon as the tumors are collected, the DNA will be processed and analyzed using the platforms that have been developed for the MuLV insertional mutagenesis screens.

Proviral insertion sites from p21-/- p27-/-, p16p19-/-, p15-/- EmuMyc transgene Pim1Pim2-/- and wild type MuLV tumors

Another panel of 700 tumors generated in compound mutant mice lacking one or several knockout alleles of CDK inhibitors have been sequenced to a depth of 192 sequences per tumor. The data of this screen are currently analyzed. Preliminary analysis shows that they further extent the information found in the panels described above. This panel increases the number of statistically significant CIS to over 600.

Transposon strategy based on Sleeping Beauty

The DNA transposon Sleeping Beauty has recently been developed as an oncogenic insertional mutagen for non-hematopoietic/non-mammary tissues. We have placed several versions of Sleeping Beauty transposase into vectors where expression is activated by co-expression of Cre recombinase, and/or by a Tetracycline/Doxycyline regulatable promoter. We also generated new mutagenic transposons that alter the expression of nearby genes to differing extents and produced our first chimaeras that have been verified to transmit the transgene in their germline. Using different Cre strains we eventually plan to express the transposase to mutagenize cell types that give rise to clinically important tumor types, such as liver, lung, breast, prostate, colon, hematapoietic, brain and skin tumors.

Hans Bos

In 1980 Hans Bos received his PhD in molecular biology from the University of Amsterdam, where he studied mitochondrial RNA from yeast in the lab of Piet Borst. For his postdoctoral training he joined the group of Alex van der Eb at the University of Leiden to work on oncogenic transformation by adenoviruses. In 1985, he started his work on the analysis of Ras mutations in human tumors and the function of Ras in oncogenic transformation. Since 1991 he is professor of physiological chemistry at the Medical Faculty of Utrecht University (now University Medical Center Utrecht), where he continued to work on small GTPases of the Ras family. In 2000, he and Hans Clevers founded Semaia Pharmaceuticals, a company aiming to develop anti-cancer drugs by rational approaches. Hans Bos is member of the European Molecular Biology Organization and very recently, of the Royal Netherlands Academy of Sciences.

Goals

To unravel the signaling pathways that are controlled by small GTPases of the Ras family and to identify novel components that may serve as targets for the development of anti-cancer drugs.

Signal transduction by small GTPases of the Ras family

At the surface of cells, a variety of receptors is expressed that allow the cell to respond to signals provided by its environment. Activation of these receptors leads to cascades of biochemical events in which small GTPases and kinases play a crucial role. Small GTPases are molecular switches in the signaling networks that control cell proliferation, migration, survival and tumor formation. The paradigm of this family is Ras, a protein that is mutated in 30% of the metastatic cancers. Our lab aims to understand the signaling pathways that are controlled by Ras and Ras-like small GTPases. In the past the group has contributed to the identification of several pathways downstream from Ras, i.e. the Raf-ERK pathway, the RalGDS-Ral pathway and the PI-3K-PKB pathway. These pathways form a network that control cell survival, cell migration and cell proliferation. Recent additions to these studies are pathways that negatively regulate the Ras signaling pathway, particularly, the pathway mediated by the small GTPase Rap1 and by the second messenger cAMP. This has resulted in the unexpected discovery of a novel client protein for cAMP, Epac. This protein functions in many cAMP-mediated processes, including cell adhesion, cell junction formation and secretion. Importantly, activation of Epac reverts Ras-induced cell scattering. Cell scattering is a hallmark for tumor metastasis.

Molecular profiling of signaling pathways related to cancer

Cyclic AMP (cAMP) is a potent inhibitor of cell proliferation and oncogenic transformation. Previously we have shown that one of the effects of cAMP was the inhibition of the Ras-signaling pathway at the level of Raf. However, subsequent studies revealed that this is not the only point of interference. We therefore performed microarray analysis of cells stimulated with cAMP and identified a number of genes that are up-regulated. Some of these genes, like cyclinD1 and p27, were

Group members

Co-supervisor

Prof. Boudewijn Burgering

Staff member

Fried Zwartkruis

Postdoctoral fellows

Margarita Camachio Cavajal

Marion Blomenrohr

Leo Price

Arjan Brenkman

Holger Rehmann

Sanne Weijzen

PhD students

Mike Zhang

Ester Frische

Marta Roccio

Armando van der Horst

Jörgen Riedl

Diana Hoogeboom

Peter de Keizer

Jerome Korzelius

Judith Raaijmakers

Willem-Jan Pannekoek

Bea Kuiperij

Jun Zhao

Technicians

Niels van den Broek

Joost Das

Marjolein Vliem

Wendy van Berkel

Paulien Polderman

Ingrid Saarloos

IT support

Wim van Driel

previously found as targets of the transcription factor FoxO. Detailed biochemical analysis revealed that cAMP activates FoxO transcription factors by inhibiting the FoxO inhibitor PKB. cAMP activates two signaling networks, the protein kinase A signaling network and the Rap1 signaling network. To investigate the contribution of the Rap1 network in cAMP-induced gene expression, we performed microarray analysis of cells that were treated with a cAMP analogue that only activates the Rap1 network. However, in two cell types we did not observe any effect on gene expression. This indicates that cAMP-induced effects on gene regulation are predominantly mediated by PKA. An important result, considering the suggested role of the Rap1 signaling pathway in cell proliferation and differentiation.

Functional screens for novel components of the small GTPase signaling pathway

Very frequently the receptor tyrosine kinase (RTK) signaling network is mutated and current estimates are that this network is mutated in all solid tumors. These mutations are found in, among others, Ras, B-raf, PI-3-kinase and PTEN. Important effector pathways are the PKB-Tsc-Rheb-mTor-S6-kinase pathway, the PKB-FoxO pathway, the RalGDS-Ral pathway and the Rap1 pathway. These pathways are interconnected into a network of positive and negative feedback loops. To identify novel components of these signaling networks several functional screens were performed.

These pathways are interconnected into a network of positive and negative feedback loops

First, we performed a synthetic lethality screen in C. elegans. The screen is based in the observation that a deletion mutant of the small GTPase Rap1 has a mild phenotype, but that this mutant together with a mutant of the small GTPase Ral is lethal, whereas the deletion of Ral alone has no phenotype. Thus Ral is synthetic lethal for Rap1. In collaboration with the Plasterk lab we screened a 24,000 siRNAs-containing library for other synthetic lethal genes. Rap1-/- worms were fed with the siRNAs and a number of these siRNA resulted in a lethal phenotype of the Rap1-/- worm but not the wild-type worm. These si RNAs are for the GTPase Ral, for Sec5 and for Exo84. Sec5 and Exo84 are subunits of the exocyst complex and known effectors of Ral. This complex is involved in the exocytosis of cell adhesion molecules to cell junctions. Indeed, the lethality is caused by the inappropriate recruitment of cell adhesion molecules (e.g. Ce-Dlg) during the embryonic stage, resulting in the disruption of cell integrity. Interestingly, our cell biological studies have revealed previously that Rap1 is also involved in the regulation of cell junction formation. Apparently, Ral and Rap1 operate in parallel (redundant?) pathways to regulate cell junction formation. We hypothesize that whereas Ral is

Selected publications 2002-2005

1. Enserink, J. M., A. E. Christensen et al. (2002). 'A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK'. Nat Cell Biol 4(11): 901-6.
2. Kops, G. J., T. B. Dansen et al. (2002). 'Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress'. Nature 419(6904): 316-21.
3. Bos, J. L. (2003). 'Epac: a new cAMP target and new avenues in cAMP research'. Nat Rev Mol Cell Biol 4(9): 733-8.
4. Rangarajan, S., J. M. Enserink et al. (2003). 'Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor'. J Cell Biol 160(4): 487-93.
5. Rehmann, H., B. Prakash et al. (2003). 'Structure and regulation of the cAMP-binding domains of Epac2'. Nat Struct Biol 10(1): 26-32.
6. Enserink, J. M., L. S. Price et al. (2004). 'The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the alpha3beta1 integrin but not the alpha6beta4 integrin'. J Biol Chem 279(43): 44889-96.
7. Essers, M. A., S. Weijzen et al. (2004). 'FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK'. EMBO J 23(24): 4802-12.
8. Ponsioen, B., J. Zhao et al. (2004). 'Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator'. EMBO Rep 5(12): 1176-80.
9. Price, L. S., A. Hajdo-Milasinovic et al. (2004). 'Rap1 regulates E-cadherin-mediated cell-cell adhesion'. J Biol Chem 279(34): 35127-32.
10. Bos, J. L. (2005). 'Linking Rap to cell adhesion'. Curr Opin Cell Biol 17(2): 123-8.
11. Kooistra, M. R., M. Corada et al. (2005). 'Epac1 regulates integrity of endothelial cell junctions through VE-cadherin'. FEBS Lett 579(22): 4966-72.

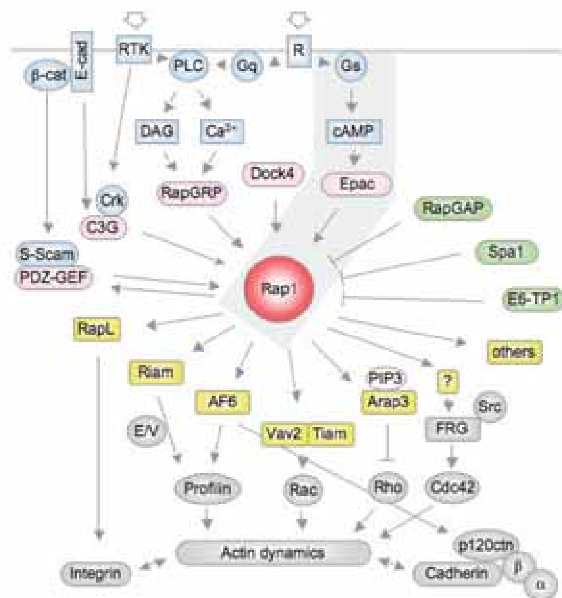


Figure 1. The Rap1 signaling pathway.

regulating the recruitment of cell adhesion molecules, Rap1 is keeping recruited molecules at the site of adhesion. We are in the process of performing further screens to find intermediates in the pathway.

Secondly, Boudewijn Burgering performed a functional screen in the PKB signaling pathway. This screen is based on the observation that FoxO expression induces apoptosis in superoxide dismutase two deficient cells. Using a retroviral cDNA expression library, we identified a number of genes that could rescue this defects. These genes are currently analyzed for their precise function.

Thirdly, in mammalian cells we planned to use the shRNA library from René Bernards to screen for genes involved in the signaling networks under study. However, we failed to develop a robust assay to screen for such genes, so this aspect is currently on hold. We will continue with the development of better assay systems.

Functional proteomics of small GTPases in cancer

To further characterize the interconnectivity of intermediates of the receptor tyrosine kinase (RTK) pathways and to identify novel intermediates we have performed extensive proteome analysis. First, a variety of yeast two-hybrid screens were performed for novel components of the signaling networks. In these screens, a number of novel interacting proteins were identified and a network of these interacting proteins is currently being built. An example is the identification of a molecular anchor for the guanine nucleotide exchange factor (GEF) Epac1. This GEF regulates the small GTPase Rap1, which plays an important role in the negative control of Ras-induced effects on cell adhesion. The molecular anchor is ezrin, a well-established cell polarity protein. In its active conformation ezrin links the cytoskeleton to the plasma membrane, particularly to the apical site of epithelial cells, where is serves as a scaffold protein for many signaling proteins. This interaction thus revealed a spatial regulation of Epac1 and consequently Rap1 signaling. We are currently trying to unravel the dynamics of this complex by mass spectrometry and microscopy. To understand the detailed regulation of Epac, we have determined the crystal structure. Importantly, this was the first structure of a cAMP domain in

12. Kuiperij, H. B., A. van der Horst et al. (2005). 'Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP'. Oncogene 24(12): 2087-95.
13. Pellis-van Berkel, W., M. H. Verheijen et al. (2005). 'Requirement of the Caenorhabditis elegans RapGEF pxf-1 and rap-1 for epithelial integrity'. Mol Biol Cell 16(1): 106-16.
14. Rehmann, H. and J. L. Bos (2004). 'Signal transduction: thumbs up for inactivation'. Nature 429(6988): 138-9.
15. Zhang, Z., H. Rehmann et al. (2005). 'AF6 negatively regulates Rap1-induced cell adhesion'. J Biol Chem 280(39): 33200-5.

Future projects

Future research will be focused on the further unraveling of the Ras and Rap1 signaling networks using genomics approaches like mass spectrometry and functional screens and to validate the findings by single cell biochemistry and microscopy. We will include systems biological approaches (mathematical modeling) in our analyses.

Huvec

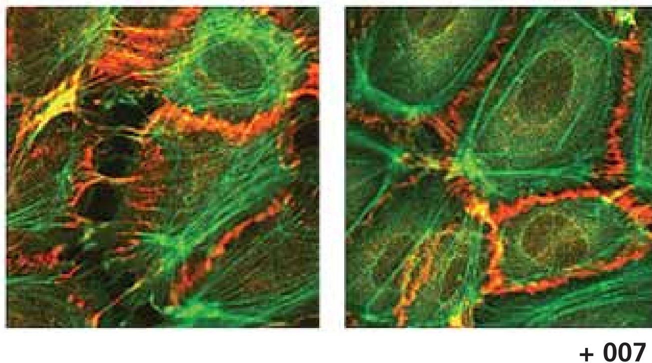


Figure 2. Epac in endothelial cells. Huvec cells were treated for 15 min with 8CPT-2'OMe-cAMP (007) and stained for actin (green) and VE-cadherin (red). Note the strong effect of 007 on the tightness of the junctions.

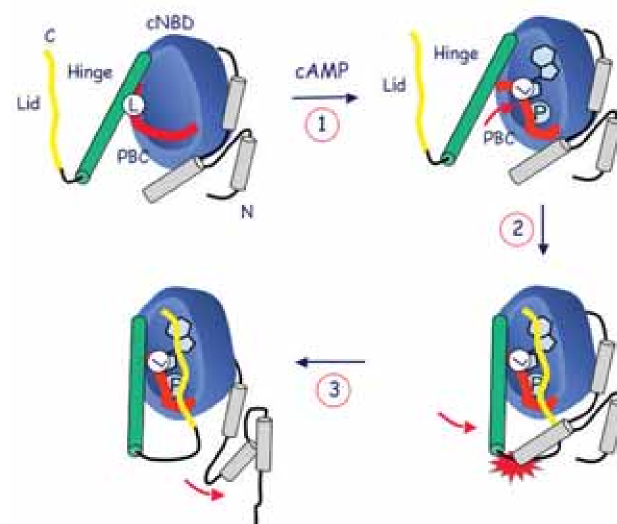


Figure 3. Universal model for cAMP action. cAMP binds to the cAMP binding pocket (NCB) (1), thereby displacing the phosphate binding cassette (PBC), which removes a leucine (L) that keeps the C-terminal helix away from the cAMP-binding site. The helix is now able to move and forms a lid over the cAMP-binding pocket (2). Consequently, the N-terminal helix is also displaced (3). These structural changes result in the release of auto-inhibition of Epac, Protein Kinase A and cAMP-regulated ion channels.

the absence of cAMP, allowing us to predict the molecular mechanism of cAMP action. This model is now confirmed for other cAMP client proteins. Furthermore, it revealed the molecular mechanism of how Epac is converted from the auto-inhibited into the activated state. (Rehmann et al, Nature 439(2006)625). This structure is currently the guide for identifying specific compounds for Epac as well as for understanding the topology of the Epac complex.
Secondly, we performed a number of interaction screens and investigated post-translational modifications (PTM) by mass spectrometry. A variety of different PTMs were identified for the transcription factor FoxO, including acetylation, phosphorylation and ubiquitination. The functions of these modifications are currently being tested.
Finally, we established a novel signaling complex involving both Ras and Ral. This complex includes the scaffold protein Jip3 and the kinases Ask, MEK4/7 and JNK and mediates Ras and oxidative stress-induced phosphorylation and activation of FoxO. This transcription factor is a direct target of PKB and plays a crucial role in the control of the cell cycle.

Hans Clevers

Hans Clevers received his MD in 1984 and his PhD one year later from the University of Utrecht where he studied signal transduction by antigen receptors on lymphocytes. For his postdoctoral training he joined the group of Cox Terhorst at the Dana-Farber Cancer Institute, Harvard, where he was involved in the cloning of genes encoding components of the T cell receptor complex. In 1989 he returned to Utrecht to establish an independent research group focusing on the identification of HMG box transcription factors. From 1991-2002, Hans Clevers was Professor in Immunology at the University of Utrecht and, since 2002, Professor in Molecular Genetics. Since 2002, he is Director of the Netherlands Institute for Developmental Biology, Utrecht. In 1996, he was scientific co-founder of of Ubisys, now Crucell, and in 2000, he co-founded the small biotech company Semaia. In 2001 he received the Spinoza award and in 2004 the Louis-Jeantet-Prize for Medicine. Hans Clevers is a member of the Royal Netherlands Academy of Sciences and of the European Molecular Biology Organization.

Goals

To further investigate the role of Wnt signaling in stem cells, and of the Tcf4-driven target gene program in colorectal cancer.

TCF factors, mediators of Wnt signaling in development and cancer

In 1991, we reported the cloning of a T cell specific transcription factor that we termed TCF1. We have shown that, upon Wingless/Wnt signaling, ß-catenin associates with nuclear TCFs and contributes a trans-activation domain to the resulting bipartite transcription factor. In the absence of Wnt signaling, we found that Tcf factors bind to Groucho transcriptional repressors. The tumor suppressor protein APC forms the core of a cytoplasmic complex which binds ß-catenin and targets it for degradation in the proteasome. In APC-deficient colon carcinoma cells, we demonstrated that ß-catenin accumulates and is constitutively complexed with the TCF family member TCF4. TCF4 gene disruption leads to the abolition of progenitor cells in the crypts of the small intestine, while TCF1 gene knockout severely disables the stem cell compartment of the thymus.

Cell signaling in normal gut homeostasis and intestinal cancer

Following up on previous work (Van de Wetering et al, 2002; Battle et al, 2002), we extensively studied the gene program that is activated by Wnt/Tcf4 in normal epithelium and in colorectal cancer. As a result we now have a 'complete' view on the genes that are regulated in normal and malignant epithelial cells and estimate that there are about 250 of such genes. The expression of a large majority of these has been studied by immunohistochemistry. This has led to the realization that the genes can be subclassified into a) genes associated with rapidly proliferating progenitor cells in crypts, b) genes that are associated with terminal differentiation of Paneth cells (Van Es et al, 2005) and c) genes that are expressed in putative stem cells, rare cells that reside near the crypt bottom.

To identify intermediates in the Wnt signaling pathway, we performed two loss-of-function screens in cultured cells, using the shRNA sublibrary of René Bernards targeting the family of deubiquitinating enzymes (DUBs). Three DUB genes were identified that affect the strength of Wnt signaling. In secondary cell-based assays, one of these DUBs, CYLD was validated.

Group members

Staff member

Marc van de Wetering

Postdoctoral fellows

Anna Pavlina Haramis

Pantelis Hatzis

Irma Oving

Andrea Thiele

Madelon Maurice

Adam Hurlstone

Markus Gerhard

Linda Smit

Edu Battle

Elena Sancho

PhD students

Vanessa Muncan

Alex Gregorieff

Annette Baas

Laurens van der Flier

Joost Hopman

Technicians

Menno Tjon-pon-fong

Harry Begthel

Jeroen Kuipers

Mark Reijenen

Suzanne Jonkheer

Maaike van der Born

Miranda Cozijnsen

Although this was unexpected, it opened new avenues to think about pharmacological manipulation of the WNT cascade in cancers. CYLD is a tumor-suppressor gene involved in the cylindromatosis syndrome that was previously identified by Bernards as a crucial regulator of the NFκB pathway. Follow-up studies have been initiated including the analysis of a knockout mouse for the Cyld gene. Homozygous knockout mice live and are fertile. Initial analyses indicate that epidermal progenitor cells hyperproliferate upon skin abrasion. The mice are also crossed into the APCmin strain to study effects on adenoma incidence. Initial observations indicate a strong increase in these Wnt-induced tumors.

To interfere in β-catenin-Tcf signaling we established two assays for testing chemical compounds. These assays we validated by a test run performed by us at the high-throughput screening facility at the Institute of Chemistry and Cell Biology (ICCB), Harvard, USA. Currently, the full library of 50,000 compounds has been screened. We have identified around 80 primary hits. These were retrieved from the ICCB library and retested manually. Thus, five to six hits have been reproduced. The hits are now further evaluated in a wide array of secondary assays. A large effort was put in the generation of (conditional) mouse knock-outs for genes in β-catenin-Tcf signaling. These genes are identified by extensive microarray analysis of cells in which Tcf is either induced or inhibited. A very exciting and unexpected finding was the identification of at least three genes that are uniquely expressed by stem cells in the crypt (these include Sox4 and GPR49). We have now isolated zebrafish null mutants in several of the genes relevant to our program: APC and APC2, Tcf4 and LKB1. An initial characterization of the APC mutant has been published (Hurlstone et al, 2003). We have since found that heterozygous fish develop adenomas in various tissues (Haramis et al, in press). The Tcf4 mutants phenocopy the mouse mutant in that they fail to maintain a proliferative crypt compartment (Muncan et al, in preparation). Phenotyping of Lkb1 has just commenced.

> An exciting finding was the identification of at least three genes that are uniquely expressed by stem cells in the crypt

Earlier we found the tumor suppressor kinase Lkb1 is exquisitely dependent on a partner molecule that we termed Strad, a conserved pseudo-kinase (Baas et al, 2003). By constructing LKB-STRAD inducible cell lines, we made the very interesting observation that complete polarization of single intestinal epithelial cells occurs upon activation of the intestinal tumor suppressor kinase LKB1 by STRAD (Baas et al, 2004). We have obtained a knockout mouse for STRAD-α and chimeric mice for an inducible allele of STRAD-β.

Selected publications 2002-2005

1. Van de Wetering, M., E. Sancho et al. (2002). 'The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells'. Cell 111(2): 241-50.
2. Batlle, E., J. T. Henderson et al. (2002). 'Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB'. Cell 111(2): 251-63.
3. Baas, A. F., J. Boudeau et al. (2003). 'Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD'. Embo J 22(12): 3062-72.
4. Boudeau, J., A. F. Baas et al. (2003). 'MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm'. Embo J 22(19): 5102-14.
5. Hurlstone, A. F., A. P. Haramis et al. (2003). 'The Wnt/beta-catenin pathway regulates cardiac valve formation'. Nature 425(6958): 633-7.
6. Baas, A. F., J. Kuipers et al. (2004). 'Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD'. Cell 116(3): 457-66.
7. Clevers, H. (2004). 'At the crossroads of inflammation and cancer'. Cell 118(6): 671-4.
8. Gregorieff, A., R. Grosschedl et al. (2004). 'Hindgut defects and transformation of the gastro-intestinal tract in Tcf4(-/-)/Tcf1(-/-) embryos'. Embo J 23(8): 1825-33.
9. Haramis, A. P., H. Begthel et al. (2004). 'De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine'. Science 303(5664): 1684-6.
10. Batlle, E., J. Bacani et al. (2005). 'EphB receptor activity suppresses colorectal cancer progression'. Nature 435(7045): 1126-30.
11. Clevers, H. (2005). 'Stem cells, asymmetric division and cancer'. Nat Genet 37(10): 1027-8.

An essential role was uncovered for Notch in maintenance of crypt progenitors. Moreover, it was found that inhibition of Notch through pharmacological inhibition of γ-secretase in ademas induces terminal differentiation (Van Es et al, 2005). An unexpected observation was done in the follow-up study of the role of EphB genes in colorectal cancer. Expression of these Wnt target genes is lost during progression of this malignancy in patients. Moreover, in a mouse model we could show that EphB activity actively suppresses colorectal cancer progression (Battle et al, 2005).

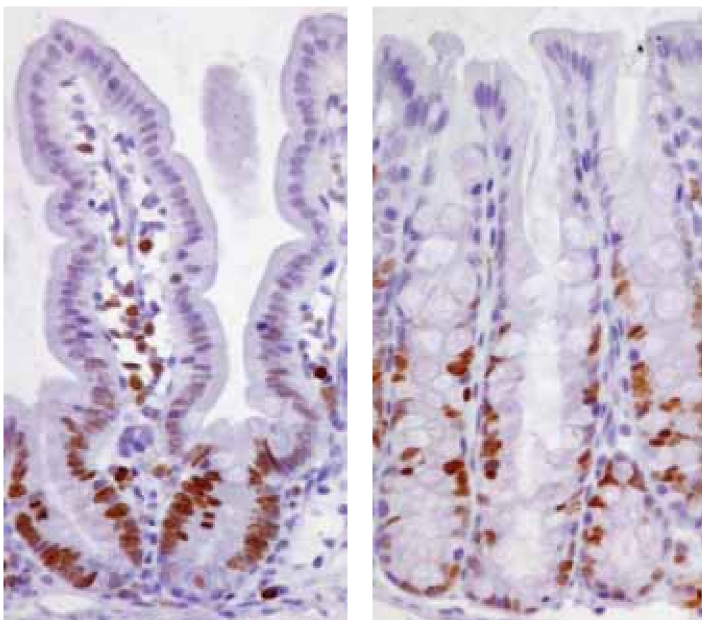


Figure 1. Proliferative compartments in the crypts of intestinal epithelium (brown nuclei). Small intestine (left) and colon (right).

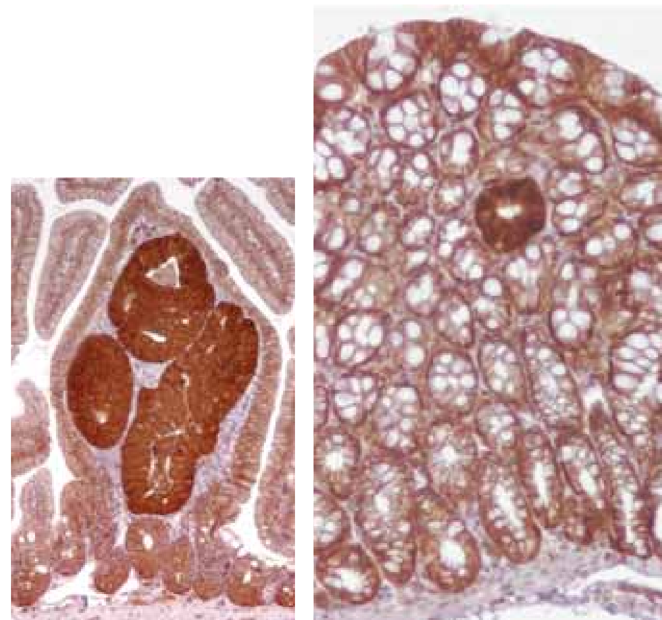


Figure 2. Adenomas in APCmin mice and man are high in ß-catenin protein in dark brown. Adenoma in small intestine (left) and in colon (right).

12. Radtke, F. and H. Clevers (2005). 'Self-renewal and cancer of the gut: two sides of a coin'. Science 307(5717): 1904-9.
13. Reya, T. and H. Clevers (2005). 'Wnt signaling in stem cells and cancer'. Nature 434(7035): 843-50.
14. Van Es, J. H., M. E. van Gijn et al. (2005). 'Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells'. Nature 435(7044): 959-63.
15. Van Es, J. H., P. Jay et al. (2005). 'Wnt signaling induces maturation of Paneth cells in intestinal crypts'. Nat Cell Biol 7(4): 381-6.

Future projects

We initiated an extensive gene-targeting project aimed to generate tools and insights into the biology of the normal stem cell in the intestine as well as in the stem cells of intestinal cancers. The genes of choice are the Wnt target genes Sox4, GPR49, sFrp5, FoxD3, and Mash2, as they are specifically expressed in these cells. We have obtained floxed alleles as well as knock-in of GFP and/or GFP-IRES-cre-ER, which will allow us to delete these genes at will in adult stem cells (loxP), to visualize individual stem cells (GFP) and to mark stem cells (cre-ER) genetically in vivo to study their offspring.

Independently, we study the role of Notch signaling in (pre)neoplastic diseases of the intestinal tract. Specifically, we seek to utilize small-molecule Notch pathway inhibitors (γ-secretase inhibitors) for the treatment of Barrett's esophagus and of sporadic and hereditary adenomas.

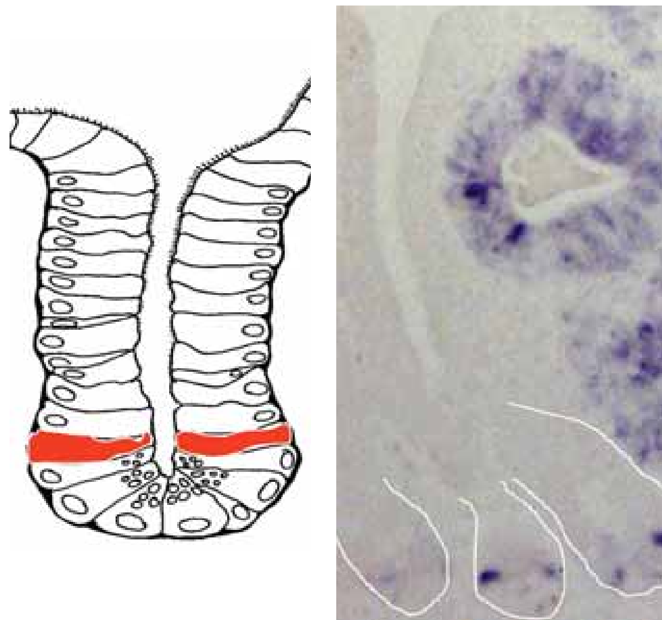


Figure 3. Expression of the Wnt target gene Sox4 marks the stem cells at position +4. Left: schematic, stem cells in orange. Right: Sox4-positive cells in four crypts are blue, as is the entire adenoma on the right.

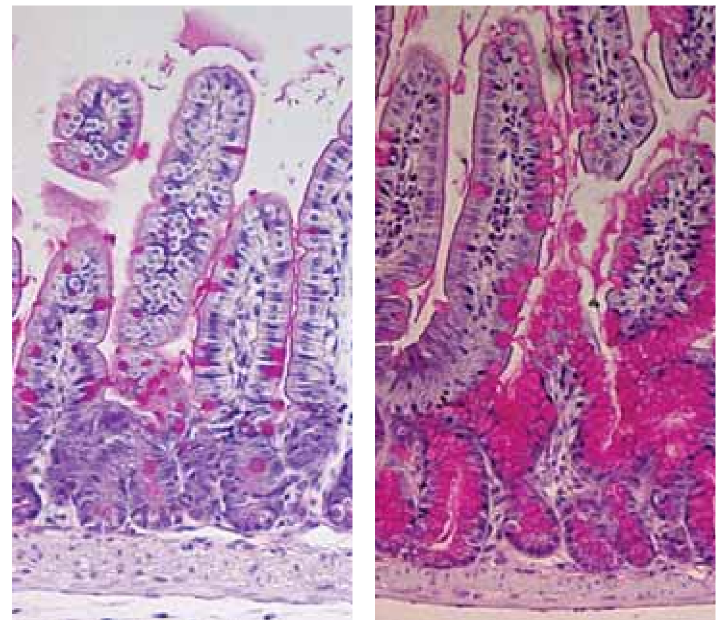
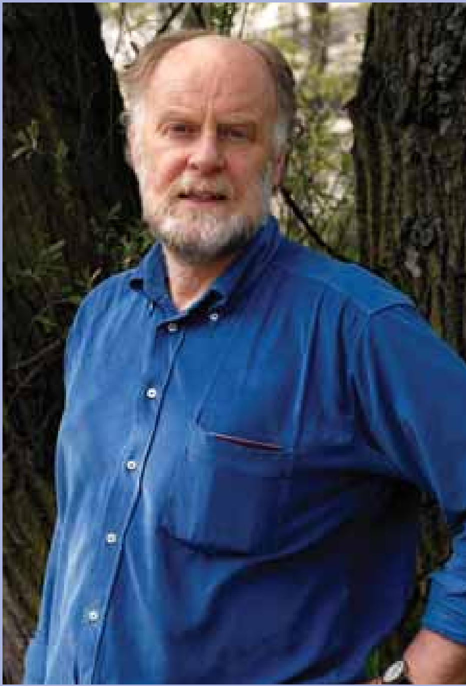


Figure 4. Inhibition of Notch signaling by γ-secretase blockers. Normal crypts (left) convert into PAS-positive (red) goblet cells upon 5 day treatment (right).

Fifteen yeast 2-hybrid screens were performed in collaboration with Hybrigenics on many components of the Wnt and LKB1 cascades (the TCF proteins, β-catenin, the BCL9 proteins, the pygopus proteins). A large variety of interacting proteins were identified. Importantly, known interactions were found, indicating the high quality of the screen. At least one pygopus-interactor, a novel E3 ubiquitin ligase, has been validated in secondary assays and appears to be a very promising new component of the Wnt cascade.



Frank Grosveld

Frank Grosveld studied biochemistry at the University of Amsterdam and obtained a PhD from McGill University in 1976. After two postdoctoral periods, he became head of the Division of Gene Structure and Expression at the National Institute for Medical Research, Mill Hill, UK in 1981. In 1993, he accepted the Chair of Cell Biology at the Erasmus University in Rotterdam. Mechanisms of gene regulation and epigenetic phenomena have been the primary focus of his research. His group has carried out pioneering work in many aspects of gene regulation, from concluding that DNA methylation inhibits gene expression via an indirect mechanism to the first description of a locus control region (LCR) or visualizing the primary transcription process in the nucleus. His group has filed a considerable number of patents and has closely collaborated with industrial partners. He is and has been on the advisory board of a number of companies and is co-founder of four start-ups, including a gene therapy based company (Therexsys; now Cobra Therapeutics), a genomics based company (Minos Biosystems) and an antibody based company (Harbour Antibodies). In 1991 he received the Louis-Jeantet-Prize for Medicine and in 1995 the Spinoza award. Frank Grosveld is a member of the Royal Netherlands Academy of Sciences and of the European Molecular Biology Organization.

Goals

The study of gene expression and gene regulation in relation to cancer and to develop novel (tools for) functional genomic screens.

Regulation of gene expression and development of transposon-based functional screens

Lung tumors are among the most prevalent cancers in the human population, with very poor prognosis for the patients. Lung tumors can be broadly subdivided in two different types: large cell lung cancer (LCLC) and small cell lung cancer (SCLC). LCLC is the most common form of lung cancer, accounting for approximately 75% of new cases. It is thought that more than 90% of lung tumors arises as a direct consequence of smoking. In addition, it is known that other environmental factors, such as high concentrations of nickel, arsenate and radon, increase the risk of developing lung cancer. The combination of smoking and exposure to such environmental factors appears to have a strong synergistic effect on carcinogenesis of the lung. A major problem with lung tumors is that they are usually first observed when the disease is already in an advanced stage when treatments such as surgical removal and chemotherapy have little or no therapeutic effect. It would therefore be highly desirable to develop a set of molecular markers that enable early detection of lung tumors and premalignant lesions in the groups at risk. This is a challenging question, because smoking per se has a major impact on gene expression profiles in the lung. However, the large majority of smokers will never develop lung cancer. We have therefore started a systematic study of gene expression and gene regulation profiles in the lungs of patients. Furthermore, we wish to develop transposon-mediated mutagenesis in mice to produce tumor models and perform genome-wide functional screens in mice.

Gene Expression Profiling of Lung Tumors

The study involves patients with full clinical description and

Group members

Staff

Sjaak Philipsen

John Strouboulis

Wilfred van IJcken

PhD students

Patrick Rodriguez

Jun Hou

Technicians

Nynke Gillemans

Bianca den Hamer

Ernie de Boer

Christel Cockx

Edgar Bonte

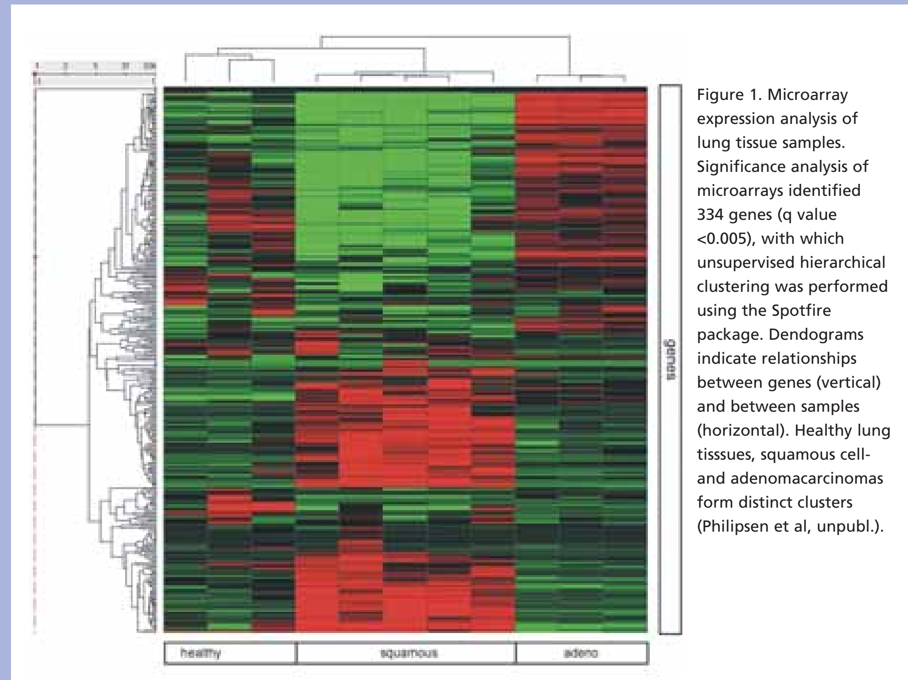


Figure 1. Microarray expression analysis of lung tissue samples. Significance analysis of microarrays identified 334 genes (q value <0.005), with which unsupervised hierarchical clustering was performed using the Spotfire package. Dendograms indicate relationships between genes (vertical) and between samples (horizontal). Healthy lung tisssues, squamous cell- and adenomacarcinomas form distinct clusters (Philipsen et al, unpubl.).

follow-up. To serve as a reference, we are collecting samples from ~fifteen healthy volunteers. Of each patient, a sample of healthy, dysplastic and tumor tissue is collected. A part of the biopsies is snap-frozen in liquid nitrogen, while the remainder is used for routine pathological examination. Sputum and blood are also collected and stored frozen. Premalignant tissue is identified by fluorescence bronchyoscopy. With this technique, premalignant tissue, which fluoresces for as yet unknown reasons, can be distinguished from normal tissue in situ. It is however not possible to predict the progression of premalignant tissue to a full-blown tumor by fluorescence alone, and this technology therefore has little predictive value. For that reason we decided to use these samples to perform a genome-wide gene expression analysis on oligonucleotide microarrays representing ~19,000 human genes (Sigma/Compugen set). To allow multi-dimensional analysis of the vast amount of microarray data generated in this study, the results are stored in an Oracle database integrated in the Rosetta Resolver analysis software suite. In this way, we are building up a data set that we will use to define a set of predictor genes.

From the results obtained until the end of 2004, we have found a set of ~140 differentially regulated genes, of which many are down-regulated in the tumor samples. Because validation is a problem with homemade microarrays, we switched to Affymetrix U133 plus 2.0 gene chips in the last quarter of 2004. With these chips we are currently analyzing a unique collection of ~80 paired tumor samples representing mainly early stages of non-small cell lung carcinomas (NSCLC) and healthy samples. To obtain sufficient statistical power and for validation purposes, we will extend this dataset with surgical samples systematically collected since 1998 by the Erasmus MC tissue bank. Because the potential extension of our dataset stretches well beyond the financial limits of an academic setting, we have started looking for a commercial partner. Such an extension is required if the gene signatures are to be used as a diagnostic tool. It will also be essential to develop diagnostic DNA methylation profiles (see below).

> The promoters of genes whose expression is reduced in tumors are often aberrantly hypermethylated

Methylation profile as a prognostic marker

Having identified sets of predictor genes for lung cancers, the next challenge would be to develop a robust assay for the signatures of these genes in early detection. This is particularly difficult because in the early stages of tumorigenesis the (pre)malignant cells cannot be isolated as a pure population, but are hidden between a large number of healthy cells. Therefore, changes in gene expression determined in RNA isolated from populations of cells will fail to reveal the presence of (pre)malignant cells. An alternative approach stems from the

Selected publications 2002-2005

1. Chow, C. M., A. Athanassiadou et al. (2002). 'LCR-mediated, long-term tissue-specific gene expression within replicating episomal plasmid and cosmid vectors'. Gene Ther 9(5): 327-36.
2. De Krom, M., M. van de Corput et al. (2002). 'Stochastic patterns in globin gene expression are established prior to transcriptional activation and are clonally inherited'. Mol Cell 9(6): 1319-26.
3. Tolhuis, B., R. J. Palstra et al. (2002). 'Looping and interaction between hyper-sensitive sites in the active beta-globin locus'. Mol Cell 10(6): 1453-65.
4. Whyatt, D. and F. Grosveld (2002). 'Cell-nonautonomous function of the retinoblastoma tumour suppressor protein: new interpretations of old phenotypes'. EMBO Rep 3(2): 130-5.
5. Bottardi, S., A. Aumont et al. (2003). 'Developmental stage-specific epigenetic control of human beta-globin gene expression is potentiated in hematopoietic progenitor cells prior to their transcriptional activation'. Blood 102(12): 3989-97.
6. De Boer, E., P. Rodriguez et al. (2003). 'Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice'. Proc Natl Acad Sci U S A 100(13): 7480-5.
7. De Laat, W. and F. Grosveld (2003). 'Spatial organization of gene expression: the active chromatin hub'. Chromosome Res 11(5): 447-59.
8. Drabek, D., L. Zagoraiou et al. (2003). 'Transposition of the Drosophila hydei Minos transposon in the mouse germ line'. Genomics 81(2): 108-11.
9. Gillemans, N., T. McMorrow et al. (2003). 'Functional and comparative analysis of globin loci in pufferfish and humans'. Blood 101(7): 2842-9.
10. Palstra, R. J., B. Tolhuis et al. (2003). 'The beta-globin nuclear compartment in development and erythroid differentiation'. Nat Genet 35(2): 190-4.
11. Drissen R., R.J. Palstra et al. (2004) 'The active spatial organisation of the ß-globin locus requires the transcription factor EKLF'. Genes & Dev, 18(20):2485-90, 2004.

observation that the promoters of genes whose expression is reduced in tumors are often aberrantly hypermethylated. Thus, detection of hypermethylated promoter sequences in a back-ground of unmethy-lated promoter sequences from healthy cells is a sensitive and positive assay for the presence of (pre)malignant cells. We have therefore started to determine which of the potential predictor genes are subject to promoter methylation in lung cancers. Initially we aimed to determine methylation profiles using FT-MS analysis as a novel and sensitive technique for the analysis of DNA methylation in a medium-throughput fashion. However, this approach was not feasible due to technical problems with the FT-MS equipment. For the time being, we will therefore switch to classical molecular biological analysis of DNA methylation.

Transposon-mediated mutagenesis and screens in mice

To produce tumor models in mice, we have also been develop-ing transposon-mediated mutagenesis in mice. In order to successfully set up such models it is essential to obtain very efficient transposition. Using the Minos transposon from Drosophila hydeii in the female germline, our original data (Drabek et al 2003) showed that we could obtain transposition throughout the genome, but that the efficiency was rather low for efficient functional screens. We therefore redesigned the system and made a number of changes: we changed the codon usage of the transposase enzyme, changed to transposition in the male germline and increased the number of copies of the transposon to about 30 in single arrays. These changes have resulted in very efficient transposition; every offspring has undergone three to four transpositions which appear to take place at random positions across the genome. Thus, efficient genome wide screens have now become possible.
To this end, we started a collaboration with the group of Anton Berns, generated a double trap transposon and transferred all of the technology to a BL6 mouse background. We will now generate sufficient starter lines (five are already established) to obtain a genome wide coverage of transposition. A number of transpositions will be bred to homozygosity for phenotypic testing and a pilot high-throughput screen will be carried out.

Diagnostic screens of genome rearrangements

We are in the process of modifying the 3C (Chromosome Conformation Capture) methodology we first used to measure interactions between different DNA elements in a specific genomic locus (Palstra et al, 2003; Splinter et al, 2004), to an array based method called 4C. Proof of principle experiments (with Nimblegen) show that this method has the potential to determine the position of chromosomal rearrangements. We will therefore validate the method on a number of well-characterized cancers and leukemias and test the detection of chromosomal deletions, inversions, duplications and rearrangements. A patent application has been filed.

Novel tools in proteomics

We developed a novel protein purification/identification system

12. Patrinos, G. P., M. de Krom et al. (2004). ‘Multiple interactions between regulatory regions are required to stabilize an active chromatin hub’. Genes Dev 18(12): 1495-509.
13. Splinter, E., F. Grosveld et al. (2004). ’3C technology: analyzing the spatial organization of genomic loci in vivo’. Methods Enzymol 375: 493-507.
14. Driegen, S., R. Ferreira et al. (2005). ’A generic tool for biotinylation of tagged proteins in transgenic mice’. Transgenic Res 14(4): 477-82.
15. Rodriguez et al. (2005). ‘GATA-1 forms distinct activating and repressive complexes in erythroid cells’. EMBO J. 24(13): 2354-66.

Future projects

We will continue analyzing lung tumor samples to develop and validate expression profiles for diagnostic and prognostic purposes. Furthermore we will develop the 4C technology into a diagnostic tool for genome rearrangements, initiate large-scale transposon mutagenesis screens and continue the analysis of transcription factor complexes in particular the complexes formed by factors that appear to be involved in the maintenance of stem cell characteristics and factors involved in cell proliferation.

using biotinylation tagging in vivo. Briefly, a short peptide sequence containing the recognition site for the bacterial biotinylation enzyme BirA is cloned onto a protein of interest and expressed in the relevant cell type together with the BirA enzyme (De Boer et al, 2003; Splinter et al, 2004; Grosveld et al, 2005). This results in efficient biotinylation of the tagged protein and allows its purification plus the proteins with which it interacts in the nucleus by binding to streptavidin beads. The proteins in the complex are subsequently identified by mass spectrometry analysis. Using this method, we were able to characterize several transcription factor complexes and to show that this approach is very robust and fast to identify interacting proteins in large or distinct multiple protein complexes.

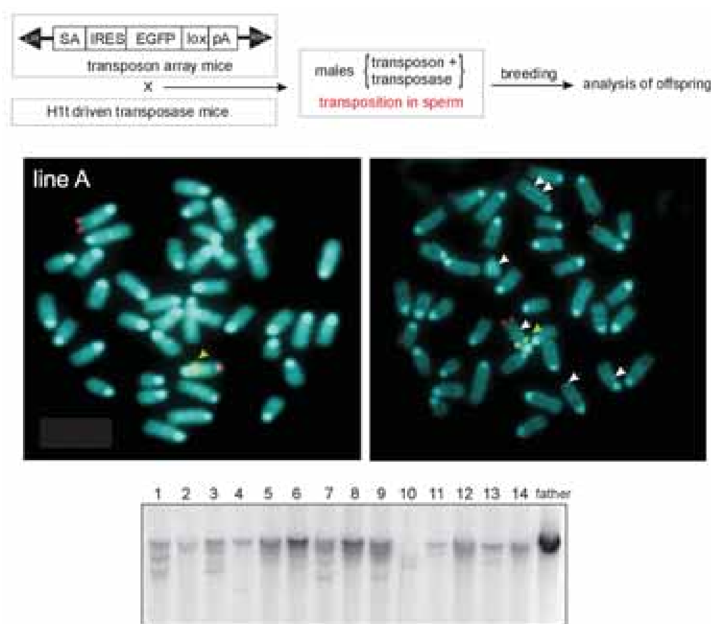


Figure 2. Transposition technology. Top: Transposon construct and breeding scheme to generate transpositions. Second row: FISH analysis of the starter line A (left) and of offspring with high number of transpositions (right). Transposon probe in green and chromosome 4 specific probe in red. Green arrows, parental array of transposons; white arrows, novel transpositions. Bottom row: Southern blot of genomic DNA from the progeny of a transposon/transposase positive male probed with the transposon (EGFP) probe. Each band represents a novel transposition event (De Wit et al., unpubl.).

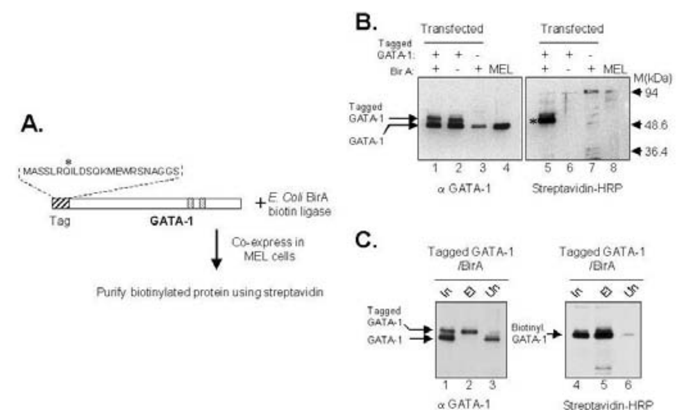


Figure 3. In vivo biotinylation technology. (A) Scheme for specific biotinylation of tagged GATA-1 by BirA biotin ligase in mouse erythroleukemic (MEL) cells showing the lysine residue (asterisk) in the peptide tag that becomes biotinylated. (B) Biotinylation of tagged GATA-1. Left: Western blot of nuclear extracts with an anti-GATA-1antibody to detect endogenous and tagged GATA-1 proteins. Right: Western blot of the same extracts with streptavidin-HRP conjugate to detect biotinylated GATA-1. Biotinylated GATA-1 (asterisk) is clearly visible in the right panel only in the lane of the double transfected cells. (C) Efficiency of GATA-1 biotinylation and binding to streptavidin beads. Left: Western blot using anti-GATA-1 antibody to detect binding of tagged GATA-1 to streptavidin beads (lane 2). Right: the same filter stripped and re-probed with streptavidin-HRP to detect the binding of biotinylated GATA-1 to streptavidin beads (lane 5). In: Input (nuclear extract); El: Eluted material; Un: Unbound material. Reproduced from De Boer et al (2003) PNAS 100(13):7480-5, copyright ©National Academy of Sciences of the USA, with permission.

Jan Hoeijmakers

Jan Hoeijmakers studied biology at Nijmegen University. His PhD work at the University of Amsterdam (supervisor P. Borst) resolved the molecular basis for antigenic variation by which trypanosomes escape from immune surveillance. In 1981, he joined the Institute of Genetics of the Erasmus University (head D. Bootsma) to work on DNA-repair in mammals. His team cloned the first of many subsequent human DNA-repair systems, elucidated the basis of several human repair and basal transcription syndromes, generated a large number of DNA-repair mouse mutants that provided insight into the etiology of human repair syndromes and discovered a link between repair, transcription and aging. In 1993, he was appointed Professor of Molecular Genetics and since 1999 he has been head of the Institute of Genetics at the Erasmus University. His team owns several patents in genome instability. In 2004 he founded the new start-up DNage, which in early 2006 was acquired by the Dutch biotech company Pharming. In 1995 he received the Louis-Jeantet-Prize for Medicine and in 1999 the Spinoza award. Jan Hoeijmakers is a member of the Royal Netherlands Academy of Sciences and the European Molecular Biology Organization.

Goals

To obtain a comprehensive understanding of the efficiency, fidelity and specificity of DNA damage repair systems, to extend our knowledge on the biological impact of these pathways in relation to carcinogenesis and aging and to utilize this understanding to prevent cancer and aging-related diseases.

Molecular basis and biological impact of genome (in)stability

Preserving the genome is of prime importance to all living organisms. However, the integrity of DNA, the carrier of genetic information, is continuously threatened by endogenous and exogenous agents and by intrinsic instability of chemical bonds in DNA itself. Oxidative stress e.g. produced by oxidative respiration, UV- and X-rays and numerous chemicals lead to cell death, cancer, and overall functional decline (senescence) contributing to aging. To counteract the gradual erosion of the vital genetic information and prevent its detrimental consequences, an intricate network of genome care-taking and protection systems has evolved. DNA repair pathways and cell cycle control mechanisms constitute an important component of this genome protection network. The overall objectives of our research are to understand the molecular mechanism and the biological impact of the systems designed to preserve the precious genetic material and to translate this knowledge into strategies for prevention, early detection, diagnosis and therapy of cancer and aging-related pathology.

CPDs and carcinogenic potential of UV

Ultraviolet (UV) radiation delivers different types of lesions to DNA, including cyclobutane pyrimidine dimers (CPDs). We applied functional genomic analysis using a combination of microarray technology and biological assays to CPD photolyase-transgenic mouse fibroblasts in a UV-dose and time-dependent fashion. We found that CPDs are responsible for the major part of the carcinogenic potential of UV. Concomitantly, the great majority of transcriptional changes elicited upon UV irradiation require the continuing presence of CPDs. Using a novel interactive database developed in our institution to link gene expression information to underlying biological processes and gene networks, we demonstrate CPDs to impinge on a wide range of biological processes. Upon the presence of CPD lesions, we identified a novel transcriptional regulation of DNA repair and damage signaling factors associated with the response to DNA double strand breaks. This is unexpected, as UV does not induce double strand breaks per sé. We attribute this to processing of

Group members

Postdoctoral fellows

George Garinis
Koos Jaspers
Wim Vermeulen
Alex Maas
Mauro Modesti
Bobby Florea
Mitja vandePutte
Gosia Ocklejewiec
Henk Roest
Sheba Agarwal
Roald van der Laan

PhD students

Ingrid van der Pluijm
Magda Budzowska

Technicians

Yvette Verhoeven
Esther van der Kamp
Renata Brandt
Yurda Sert
Iris Jasper
Esther Appelboom
Roel Janssens
Hannie Odijk
Anja Raams

Figure 1. Striking parallels between TTD mouse and patient. Trichothiodystrophy (TTD) is a rare inherited NER-transcription disorder due to mutations in subunits of TFIIH. The clinical features involve UV sensitivity, severe progressive neurological and developmental abnormalities often leading to death in childhood. The most characteristic symptom is the presence of brittle hair.

replication intermediates of CPD-containing DNA templates requiring the recombination pathway for lesion bypass. The following main conclusions can be derived from these studies: 1) CPD lesions are responsible for all major UV-induced effects including sunburn, oedema, immune suppression as well as skin cancer. Genetic introduction of the CPD-specific photolyase provides a very efficient protection against all of these deleterious consequences. 2) In proliferating cells a significant proportion of the cytotoxic and in part mutagenic effect of UV exposure appears to follow from double strand breaks generated during replication of a UV-damaged template (Garinis et al, 2005). We are now investigating the consequences of the presence or absence of CPD- or 6-4PP-specific photolyase transgenes when either global genome nucleotide excision repair (GG-NER) operating genome-wide, transcription-coupled NER that removes DNA damage which blocks transcription, or both are absent at the level of the intact organism.

> The in part mutagenic effect of UV exposure appears to follow from double strand breaks

Genes with longer primary transcripts at greater risk to transcription-blocking lesions

In a second study, we investigated the idea that expression of genes with longer primary transcripts may be at a greater risk to transcription-blocking lesions than shorter ones. If so, selection mechanisms should act against long introns in favor of short transcription units in genes essential to acute stress responses. For both hypotheses, we provided strong evidence by using expression data from the UV-irradiated photolyase-transgenic mouse fibroblasts that are able to completely remove cyclobutane pyrimidine dimers (CPDs) from their genome in a light-dependent manner. We observed a stochastic time- and dose-dependent correlation between gene length and expression in the presence of UV-induced CPDs that was markedly rescued upon removal of these lesions through the potent repair process of photoreactivation. Genes involved in acute stress-related responses maintain a high ratio of primary transcript to intronic length suggesting that natural selection reduces the risk by acting against a long total intron length in favor of short transcription units.

Exploration of the link between XPC and ubiquitin

Analysis of the XPC-HR23B damage sensor complex involved in lesion detection in global genome NER has resulted in the discovery of a high and fast (within minutes) accumulation of ubiquitin in UV-damaged regions in the nucleus in an XPC-dependent manner (in collaboration with Dr. N. Dantuma, Karolinska Institutet, Sweden). This ubiquitination is strongly dependent on intact NER and appears to be triggered after the incision stage of the NER reaction. The main target was found to

Selected publications 2002-2005

1. De Boer, J., J.O. Andressoo et al. (2002). 'Premature aging in mice deficient in DNA repair and transcription'. Science 296(5571): 1276-9.
2. Kamiuchi, S., M. Saijo et al. (2002). 'Translocation of Cockayne syndrome group A protein to the nuclear matrix: possible relevance to transcription-coupled DNA repair'. Proc Natl Acad Sci U S A 99(1): 201-6.
3. Schul, W., J. Jans et al. (2002). 'Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice'. Embo J 21(17): 4719-29.
4. Takao, M., S. Kanno et al. (2002). 'Novel nuclear and mitochondrial glycosylases revealed by disruption of the mouse Nth1 gene encoding an endonuclease III homolog for repair of thymine glycols'. Embo J 21(13): 3486-93.
5. Yagita, K., F. Tamanini et al. (2002). 'Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein'. Embo J 21(6): 1301-14.
6. Hasty, P., J. Campisi et al. (2003). 'Aging and genome maintenance: lessons from the mouse?'. Science 299(5611): 1355-9.
7. Mitchell, J. R., J. H. Hoeijmakers et al. (2003). 'Divide and conquer: nucleotide excision repair battles cancer and aging'. Curr Opin Cell Biol 15(2): 232-40.
8. Budzowska, M., I. Jaspers et al. (2004). 'Mutation of the mouse Rad17 gene leads to embryonic lethality and reveals a role in DNA damage-dependent recombination'. Embo J 23(17): 3548-58.
9. Giglia-Mari, G., F. Coin et al. (2004). 'A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A'. Nat Genet 36(7): 714-9.
10. Mone, M. J., T. Berna et al. (2004). 'In vivo dynamics of chromatin-associated complex formation in mammalian nucleotide excision repair'. Proc Natl Acad Sci U S A 101(45): 15933-7.

be the histone H2A protein. Using sophisticated confocal laser microscopy combined with photobleaching of GFP-tagged biquitin we found that this histone modification involved a UV-dose dependent significant quantity of the free ubiquitin pool within the nucleus and a region of minimally 30kb of chromatin around the damage.

Discovery of a new NER factor

An unexpected finding was the discovery of the gene responsible for the last NER-deficient complementation group associated with the severe clinical symptoms of the neuro-developmental repair-transcription disorder trichothiodystrophy (TTD), designated TTD-A. This gene turned out to encode for the human homolog of a very small ~ 8 kDa protein, that in yeast comprised an -as yet unknown- 10th subunit of multifunctional repair-transcription complex TFIIH. TTD-A patients were found to carry deleterious mutations in this gene. The protein appears to stabilize the complex and is indispensable for its NER function, but seems to be dispensable for its role in basal transcription initiation. Future research will be aimed at understanding the molecular function of this previously overlooked, small subunit as well as the clinical and medical impact. Finally, a proteomics approach for DNA repair protein complexes using the 2-DIGE technique in combination with mass spec analysis has revealed a number of differentially expressed proteins in cells defective in the tenth subunit of the multifunctional transcription initiation/NER/TCR complex TFIIH, when compared with the same cells corrected by introduction of the TTD-A gene. These proteins are currently analyzed for their functional link with the genetic defect in the cells.

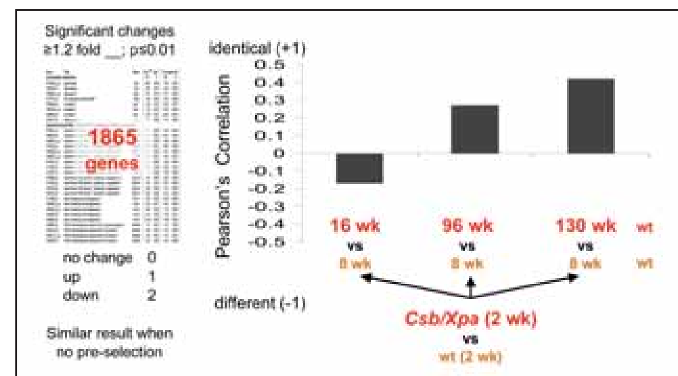


Figure 2. Comparison of full mouse transcriptomes of liver RNA from 15 day-old CSB/XPA double mutant mice and from 2.5 year old wildtype mice using Affymetrix full mouse genome arrays. The significant differences were compared with the differences between liver expression profiles of old (2.5 year) normal mice when aligned with adult (8 week) mice. The Pearson correlation indicates the degree of similarity between both differential expression profiles. A highly significant positive correlation is found between the 15-day-old mouse mutant liver expression profile and that of 2.5-year-old naturally aged mice.

DNA repair deficient mice

We have established a large battery of DNA repair deficient mice, some of which carry mutations that meticulously mimic human NER-deficient syndromes (see e.g. figure 1 depicting the mouse model for the human neuro-developmental brittle hair disorder Trichothiodystrophy, TTD). These mutants show either a cancer susceptibility phenotype (when the global genome NER subpathway is affected), or segmental premature aging (when transcription-coupled repair is compromised, enhanced

11. Van den Boom, V., E. Citterio et al. (2004). 'DNA damage stabilizes interaction of CSB with the transcription elongation machinery'. J Cell Biol 166(1): 27-36.
12. Garinis, G. A., J. R. Mitchell et al. (2005). 'Transcriptome analysis reveals cyclobutane pyrimidine dimers as a major source of UV-induced DNA breaks'. Embo J 24(22): 3952-62.
13. Jans, J., W. Schul et al. (2005). 'Powerful skin cancer protection by a CPD-photolyase transgene'. Curr Biol 15(2): 105-15.
14. Niedernhofer, L. J., A. S. Lalai et al. (2005). 'Fanconi anemia (cross)linked to DNA repair'. Cell 123(7): 1191-8.
15. Prasher, J. M., A. S. Lalai et al. (2005). 'Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1-/- mice'. Embo J 24(4): 861-71.

Future projects

Future research will -among others- be focused on unraveling the connection between DNA damage and the GH/IGF1 regulation, the consequences for the entire metabolism and on exploration of possibilities for intervention to prevent the onset of cancer and aging-related diseases in the DNA repair syndromes as well as in the normal polulation.

when in addition global genome repair is completely abrogated) or a combination (when both NER subpathways are partially deficient). To assess to which extent the accelerated aging features - as opposed to enhanced cancer susceptibility - that are observed in some of the repair-deficient mice represent the natural process of aging, full-mouse genome microarray analysis was performed of the liver of fifteen-day old single and double repair mutants and compared to age-matched and naturally aged wt mice. The mutants studied were the single XPA (GG-NER defective, highly cancer-prone), the single CSB (TCR-deficient, mild aging phenotype) and the double repair mutant (XPA/CSB exhibiting dramatic accelerated aging, limiting life span to ~three weeks).

The microarray analysis, which was confirmed at the RT-PCR and protein level, yielded the following very interesting results:
1) The dramatically accelerated segmental aging in the double mutants showed an impressive resemblance with normal aging (see figure 2).
2) The expression profile highlighted the heavy involvement of the IGF-1/growth hormone axis that for a long time has been thought to be tightly connected with aging. Interestingly, genetic down-regulation of this pathway is uniformly linked with life span extension and up-regulation usually favors growth and development but at the expense of a shorter life span. These findings point to DNA damage as the root cause of normal and accelerated aging driving the gradual decline of IGF-1/GH levels observed in human aging.

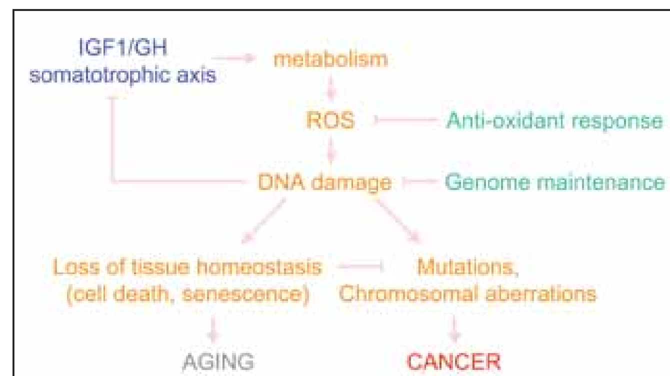


Figure 3. Scenario for the link between DNA damage, metabolism, GH/IGF1 somatotrophic axis, cancer and aging. Oxidative respiration and metabolism generate reactive (oxygen) species (ROS) that damage biomolecules in the cell including DNA. DNA damage will in surviving cells increase mutagenesis and eventually cancer. Alternatively, DNA damage may facilitate apoptosis or induce cellular senescence which will prevent carcinogenesis, however at the expense of contributing to aging. The induction of DNA damage is counteracted by the anti-oxidant defense system and DNA repair processes attempt to prevent the consequences of DNA damage. Persisting DNA damage triggers down-regulation of the growth hormone/insulin-growth factor 1 (GH/IGF1) somatotrophic axis that will reduce metabolism (as well as growth) to limit the generation of ROS and release the pressure on the DNA.

3) The entire energy metabolism is geared towards preservation of energy: low glucose, prominent glycogen storage, absence of subcutaneous and visceral fat, instead storage of triglycerides in the liver and in various other tissues takes place.
4) The anti-oxidant system is systematically upregulated. These features are initiated soon after birth, are progressive and systemic, explaining the severe growth attenuation of the double mutants. The above findings support a scenario of aging that is depicted in figure 3.

Jan Klijn

Jan Klijn received his MD degree in 1972 from the University of Amsterdam, graduated in Internal Medicine in 1978 and received his PhD in 1987 from the Erasmus University in Rotterdam. Since 1981 he has been Head of the Division of Endocrine Oncology and Genetics (Department of Medical Oncology) consisting of both a clinical and laboratory section at the Daniel den Hoed Cancer Center and the Erasmus University Medical Center. After merging of both institutes, he was appointed Professor in endocrine oncology (1998). Also in Rotterdam, he was instrumental in the establishment of the multidisciplinary Family Cancer Clinic in 1991. Furthermore he established a research group that is internationally active especially in the fields of oncogenetics, hereditary cancer, prognostic and predictive molecular biological factors, early diagnosis and prevention of breast cancer, and clinical trials. Both the Rotterdam Tumor Bank and the Family Cancer Clinic belong to the largest ones in the world. Jan Klijn is one of the founders and the first (and current) chairman of the National Dutch Breast Cancer Trialists' Group (BOOG) and is board-registered in the Netherlands for both Medical Oncology and Endocrinology.

Goals

To come to individually designed treatments based on individual tumor phenotypes of (subgroups) of breast cancer patients, to identify predictive markers for therapy resistance and uncover novel targets for treatment.

Molecular profiling of breast tumors

Multiple genetic alterations are responsible for the transformation of normal cells into cancer cells. Subsequently, a variety of cell biological systems, often linked together, are involved in the regulation of tumor growth, the processes of angiogenesis and metastasis, and the development of drug resistance. In patients with breast cancer, we study a variety of both hereditary and non-hereditary cell biological factors that play a role in prognosis and/or can predict response to systemic therapy in patients with advanced disease. The development of specific drugs such as trastuzumab, which targets HER2/neu, has opened a new era of therapeutic possibilities. Unfortunately, therapy failure is a major drawback in systemic treatment of tumor patients. Patients are either intrinsically resistant or acquire resistance during treatment. Therefore, it is important to identify predictive markers for resistance and to uncover novel targets for treatment. To identify potential predictive biomarkers, expression profiling using human cDNA microarrays and protein expression arrays coupled with Seldi-ToF-MS are conducted in different sets of patients with breast and ovarian cancer treated with endocrine therapy and/or chemotherapy. The identification of new genes will also allow for mechanistic studies aimed at the development of new effective individualized treatment strategies.

Tissue samples and clinical data

For our studies, we have the availability of a large collection of cancer tissues and sera from patients with known follow-up, so we could isolate RNA from more than 2,000 breast tissue samples (malignant, benign and normal). From over 1,700 patients, good quality RNA of the primary tumor is available for various microarray studies as well as complete follow-up information, including the type of response to systemic treatment for recurrent disease. From the same samples, DNA has been isolated allowing SNP and DNA-methylation analysis.

Prognostic profiles in lymph-node negative patients

Using a 22,000 oligoarray, we identified a 76-gene expression profile for lymph-node negative patients who did not receive adjuvant systemic hormonal or chemotherapy. In collaboration with Veridex LLC in San Diego, USA, the profile was validated

Group members

Scientific staff
John Foekens
Els Berns
Mieke Schutte
Hanne Meijers-Heijboer
Marion Meijer van Gelder
Cécile Brekelmans

Postdoctoral fellows
Maurice Jansen
John Martens
Buddy Setyono Han
Rian Rijnsburger

PhD students
Jozien Helleman
Mieke Kriege
Antoinette Hellestelle
Marijke Wasielewski

Technicians
Kirsten Ritstier
Annette Heemskerk
Mieke Timmermans
Joan Bolt-de Vries
Anita Trapman-Jansen
Anieta Sieuwerts
Harry Peters
Iris van Staveren

Bioinformatician
Marcel Smid

Database managers
Anneke Goedheer
Maxime Look
Ellen Crepin
Henk Portengen

in an independent group of patients and proved strongly prognostic for all lymph-node negative patients, including the subgroups of pre- and postmenopausal patients, and patients with small primary tumors (Wang et al, 2005). Using a dedicated Affymetrix gene chip containing the 76 genes and controls, our 76-gene signature has recently been validated in 180 untreated lymph node-negative patients in a multi-centric study involving four European institutions (Foekens et al, J Clin Oncol 24(2006)1665). Finally, a completely independent multicentre validation study was performed in collaboration with the TransBig group. In this study, RNA from frozen breast tumors of untreated lymph node-negative patients from five European institutions was isolated by van Laura van 't Veer of the NKI and hybridized at the Jules Bordet Institute in Brussels to dedicated Affymetrix gene chips containing the 76 genes and controls. The raw expression data were analyzed at Veridex; for statistical analysis the calculated relapse score was sent to an independent statistician at the International Drug Development Institute at Brussels. Again, the results of this study showed validation of the Rotterdam 76-gene expression profile.
Finally, in collaboration with seven European institutions a multicentre validation study involving lymph node-negative patients who did receive adjuvant tamoxifen is ongoing.

> Patients with tumors carrying this 31-gene signature could be candidates for adjuvant therapy including biphosphonates

Tamoxifen profile of recurrent disease

Using a 20,000 gene microarray, we further obtained an RNA expression profile of 81 genes that predicts which patients with recurrent disease will respond to first-line treatment with the anti-estrogen drug tamoxifen. From those 81 genes, a predictive signature of 44 genes was extracted and validated on an independent set of 66 tumors. In silico analysis showed that the HOXB13-to-IL17BR ratio is predictive for favorable response to tamoxifen therapy (Jansen et al, 2005). Currently, the tamoxifen profile is being validated on an additional set of tumors in collaboration with the NKI and Agendia BV (Van 't Veer).

Profile predicting bone metastasis

In addition to the 76-gene prognostic profile and the 81-gene tamoxifen profile, we developed a 31-gene signature that upon validation was shown to accurately predict, with a sensitivity of 100% and a specificity of 50%, the occurrence of a relapse to the bone in patients with breast cancer (Smid et al, J Clin Oncol 24(2006)2261). Patients with tumors carrying this gene-signature could be candidates for adjuvant therapies including biphosphonates. To validate the performance of the 31-gene bone metastasis signature, additional studies have been planned.

Selected publications 2002-2005

1. Meijers-Heijboer, H., A. van den Ouweland et al. (2002). ''Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations'. Nat Genet 31(1): 55-9.
2. Baum, M., A.U. Buzdar et al. (writing committee) on behalf of The ATAC Trialists' Group (2002). 'The first results of the ATAC trial (Arimidex, Tamoxifen Alone or in Combination): an adjuvant trial in postmenopausal women with early breast cancer'. The Lancet, 359: 2131-39.
3. Look, M.P., W.L.J. van Putten et al. (2002). 'Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients'. J Natl Cancer Inst. 94: 116-28.
4. Narod, S.A., M-P. Dubé et al. (2002). 'Oral contraceptives and the risk of breast cancer in BRCA1 and BRCA2 mutation carriers'. J Natl Cancer Inst 94: 1773-79.
5. Meijers-Heijboer, H., J. Wijnen et al. (2003). 'The CHEK2*1100delC mutation identifies families with a hereditary breast and colorectal cancer phenotype'. Am J Hum Genet 72: 1308-1314 (2003).
6. Schutte, M., S. Seal et al. (2003). 'Variants in CHEK2 other than 1100delC do not make a major contribution to breast cancer susceptibility'. Am J Hum Genet 72: 1023-1028 (2003).
7. Meijers-Heijboer, H., C.T.M. Brekelmans et al. (2003). 'Use of genetic testing and prophylactic mastectomy and oophorectomy in women with breast and/or ovarian cancer from families with a BRCA1 or BRCA2 mutation'. J Clin Oncol 21: 1675-1681.
8. Kriege, M., C.T.M. Brekelmans et al. for the MRISC Study Group (2004). 'Efficacy of MRI and mammography for breast-cancer screening in women with a familial or genetic predisposition'. New Engl J Med 351: 427-37.
9. Wang, Y., J.G.M. Klijn et al. (2005). 'Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer'. Lancet 365(9460): 671-9.
10. Martens, J.W., I. Nimmrich et al. (2005). 'Association of DNA methylation of phosphoserine aminotransferase with response to endocrine therapy in patients with recurrent breast cancer'. Cancer Res 65(10): 4101-17.

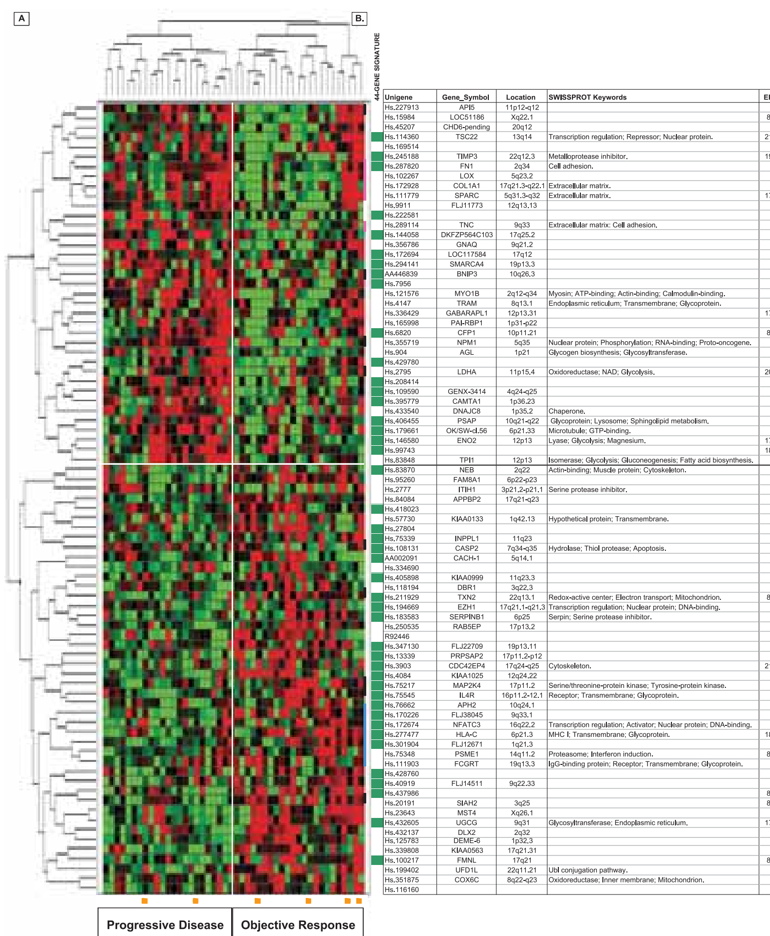


Figure 1. Supervised hierarchical clustering of 46 tumors using the 81-gene signature. (A) Expression plot showing clusters of tumors with progressive disease and objective response. Orange bars below indicate misclassified tumors. Red indicates upregulated and green indicates downregulated genes. (B) Bars next to plot indicate genes of predictive signature (green), apopotosis (black), extracellular matrix (purple), and immune system (blue). Information includes cytoband location and references of estrogen function. Reproduced from Jansen et al (2005) J Clin Oncol 23(4):732-40, copyright ©American Society of Clinical Oncology, with permission.

Profiles for other breast cancer groups and ovarian cancer

A start has been made with microarray analyses of three new subgroups of breast cancer patients. The first two include patients who received either chemotherapy combination CMF (cyclophosphamide, methotrexate, fluorouracil) or anthracyclin-containing polychemotherapy (FEC/FAC) for recurrent disease. A total of 150 RNA samples from primary tumors were hybridized to Affymetrix chips. The bioinformatic analysis for these two subgroups of patients is ongoing. Similarly, bioinformatic analysis is ongoing on the Affymetrix gene-expression data of 150 RNA samples from tumors of the third subgroup, i.e. hereditary breast cancer patients (BRCA1, BRCA2 and CHEK2 carriers, and separately for non-BRCA1/BRCA2/CHEK2 carriers).

Finally, we constructed an algorithm of nine genes for cisplatin-resistant ovarian cancer. This was validated in collaboration with C.G.J. Sweep in Nijmegen, with a sensitivity of 89% and a specificity of 57% (Helleman et al, (2006), Int J Cancer 118(8): 1963-71).

DNA methylation

In the same group of patients from which we derived the 81-gene tamoxifen profile, we studied the DNA-methylation status of 499 CpG sites in 117 candidate genes and obtained a DNA methylation profile existing of no more than five genes. The predictive value of this profile was confirmed in an independent group of patients. The results of this study have been

11. Jansen, M.P., J. A. Foekens et al. (2005). 'Limits of predictive models using microarray data for breast cancer clinical treatment outcome'. J Natl Cancer Inst 97(24): 1851-2; author reply 1852-3.
12. Jansen, M.P., J. A. Foekens et al. (2005). 'Molecular classification of tamoxifen-resistant breast carcinomas by gene expression profiling'. J Clin Oncol 23(4): 732-40 (2005).
13. Eisen, A., J., Lubinski et al. (2005). 'Breast cancer risk following bilateral oophorectomy in BRCA1 and BRCA2 mutation carriers: an international case-control study'. J Clin Oncol 23: 7491-7496 (2005).
14. Sieuwerts, A. M., M. E. Meijer-van Gelder et al. (2005). 'How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen treatment in patients with recurrent breast cancer: a retrospective study'. Clin Cancer Res 11(20): 7311-21.
15. Umar, A., J. C. Dalebout et al. (2005). 'Method optimisation for peptide profiling of microdissected breast carcinoma tissue by matrix-assisted laser desorption/ionisation-time of flight and matrix-assisted laser desorption/ionisation-time of flight/time of flight-mass spectrometry'. Proteomics 5(10): 2680-8.

Future projects

- Further validation of our prognostic 76-gene expression profile
- Development of a gene expression profile predictive for locoregional recurrence of breast cancer
- Validation of our gene expression profile predictive for bone metastasis
- Development of a gene expression profile predictive for visceral disease
- Validation of our gene expression profile predictive for response to tamoxifen in collaboration with the NKI
- Development of gene expression profiles for resistance to other endocrine and chemotherapeutic treatment modalities in breast cancer
- Continuation of our studies on the role of DNA-methylation
- Further microarray studies in ovarian cancer
- Development of gene expression profiles specific for several types of hereditary breast cancer

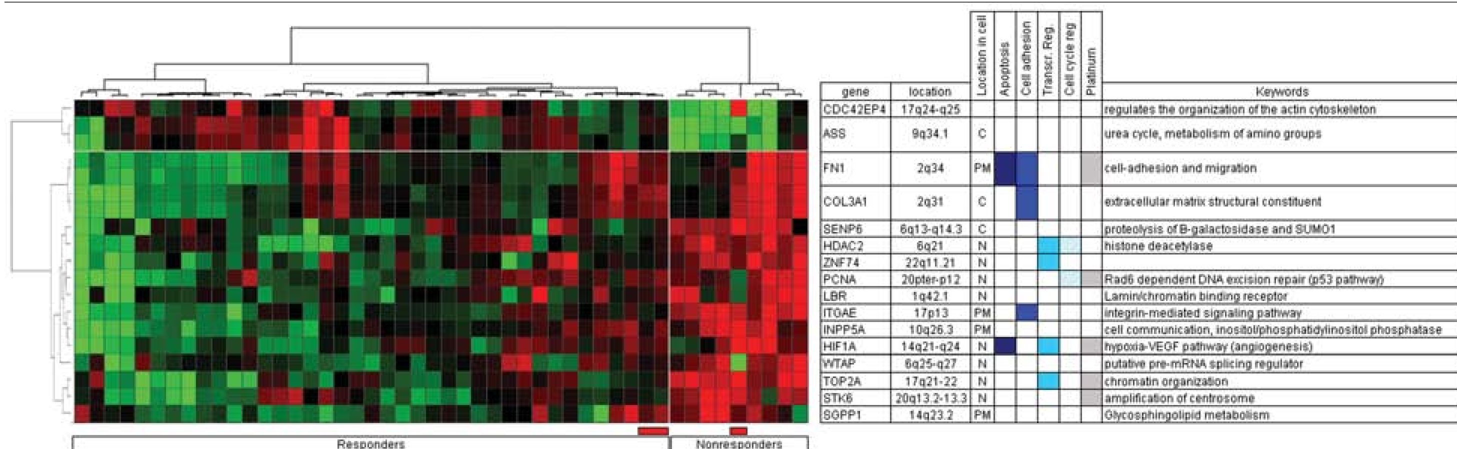
published (Martens et al, 2005). Efforts to raise antibodies against the most promising clinically relevant DNA-methylation targets are in progress.

RT-Q-PCR validation of genes

For a variety of genes that we identified in both the prognostic and the predictive studies described above, large-scale validation studies with RT-Q-PCR on over 100 genes were performed on over 1,000 RNA preparations. Data analysis for specific sub-groups of patients has been completed (e.g., Sieuwerts et al, 2005) yet analyses of several relevant clinical subgroups are still ongoing. These include samples derived from paraffin-embedded tissues.

SNP-analysis and proteomics

In addition to the gene-expression, DNA-methylation, and RT-Q-PCR studies mentioned above, we initiated large-scale SNP analyses of DNA isolated from primary breast tumors as well as proteomic profiling by mass spectrometry for the subgroups of patients who received tamoxifen (Umar et al, 2005) or FAC/CMF poly-chemotherapy for recurrent disease.



gene	location	Location in cell	Apoptosis	Cell adhesion	Transcr. Reg.	Cell cycle reg	Platinum	Keywords
CDC42EP4	17q24-q25							regulates the organization of the actin cytoskeleton
ASS	9q34.1	C						urea cycle, metabolism of amino groups
FN1	2q34	PM						cell-adhesion and migration
COL3A1	2q31	C						extracellular matrix structural constituent
SENP6	6q13-q14.3	C						proteolysis of B-galactosidase and SUMO1
HDAC2	6q21	N						histone deacetylase
ZNF74	22q11.21	N						
PCNA	20pter-p12	N						Rad6 dependent DNA excision repair (p53 pathway)
LBR	1q42.1	N						Lamin/chromatin binding receptor
ITGAE	17p13	PM						integrin-mediated signaling pathway
INPP5A	10q26.3	PM						cell communication, inositol/phosphatidylinositol phosphatase
HIF1A	14q21-q24	N						hypoxia-VEGF pathway (angiogenesis)
WTAP	6q25-q27	N						putative pre-mRNA splicing regulator
TOP2A	17q21-22	N						chromatin organization
STK6	20q13.2-13.3	N						amplification of centrosome
SGPP1	14q23.2	PM						Glycosphingolipid metabolism

Figure 2. The 16-gene signature: supervised hierarchical clustering. The heat map shows clusters of ovarian tumors with identified response to cisplatin-based chemotherapy. Genes that are upregulated are shown in red, those downregulated are shown in green. The intensities relate to the level of expression. Incorrect classified tumors are indicated in red. Lines next to heat map point to genes with defined function, for example platin resistance (blue) and apoptosis (green). Additional information includes cytoband position and cellular localization.

Ronald Plasterk

Ronald Plasterk received his PhD from the University of Leiden in 1984 on a thesis in molecular genetics of transposon Mu. He was a postdoc at the California Institute of Technology with Melvin Simon, and at the MRC-LMB in Cambridge, England with John Sulston, where he started his work on the nematode C. elegans. His focus has been on DNA transposition, genome stability, functional genomics and the mechanism of RNA interference and miRNA. Presently, he is director of the Hubrecht Laboratory/Netherlands Institute for Developmental Biology and professor of Developmental Genetics at the University of Utrecht. In 1999 he received the Spinoza award. Ronald Plasterk is a member of the EMBO, the Royal Netherlands Academy of Arts and Sciences, and the Board of Governors of The Wellcome Trust; he interacts with the public and policymakers via a weekly column in a newspaper and a bi-weekly television column.

Goals

To understand how genomes are protected against mutations and transposon activity and to develop new tools for functional genomics, and more recently, to investigate the potential role of microRNAs in cancer development.

Protection against genomic instability

Apart from genomic instability that results from transposon jumping, our lab is interested in genes that protect the genome against endogenous spontaneous damage, such as replication errors that occur during development. Genes that protect the genome's integrity are named mutator genes, because their loss frequently results in a so-called mutator phenotype: an enhanced level of spontaneous genetic mutations. Mutator genes, such as the mismatch repair genes, have been found to be causally involved in cancer development in humans: a failure to correct mismatches during replication increases the mutation frequency and thus the chance that specific combinations of mutations in tumor suppressor genes and proto-oncogenes occur within the same cell (or clonally expanded cells), thereby transforming a normal cell into a tumor cell. Although many mutator genes have been described, still many cancers exist in which the underlying defect that results in genomic instability is unknown. We use RNA interference and C. elegans and zebrafish genetics to identify novel mutator genes. Any mammalian holomologs are analyzed in human cells and tumors.

Microsatellite instability in C. elegans

Using a genome-wide RNAi screen, we identified thirteen genes that caused elevated levels of frame-shift mutations (microsatellite instability (MSI)) in somatic cells of C. elegans. Of those, nine genes have a single human homolog, two have several strongly-related orthologs and for two no human homolog was found. The set of thirteen conserved genes contains all known C. elegans mismatch repair genes (MLH1, PMS2, MSH2, MSH6) of which the human homologs have been demonstrated to be associated with MSI-positive tumors.
We developed oligo sets for resequencing the remaining genes (REV1L, MPPE1, CYCLIN K, CUL5, and CL25022) for further analysis of familial MSI-positive samples. In collaboration with Laura van 't Veer of the NKI, we selected blood or tumor DNA-samples from 64 patients or relatives with a familial history of MSI-positive tumors in which mutations in the known mismatch repair genes were not yet found. Apart from known single nucleotide polymorphisms (SNPs), we found new SNPs that have no effect on the coding capacity of the genes. However, we also found mutations in four of five genes that result in a change of an amino acid, but these mutations were

Group members

Staff member

Edwin Cuppen

Postdoctoral fellows

Marcel Tijsterman

Ellen Nollen

Zhenya Berezikov

Celine Moorman

PhD students

Karin Brouwer

Wigard Kloosterman

Josien van Wolfswinkel

Harma Feitsma

Nadine Vastenhouw

Bastiaan Tops

Sander van der Linden

Technicians

José van der Belt

Evelien Kruisselbrink

Mark Reijnen

Kristy Okihara

Esther Hazendonk

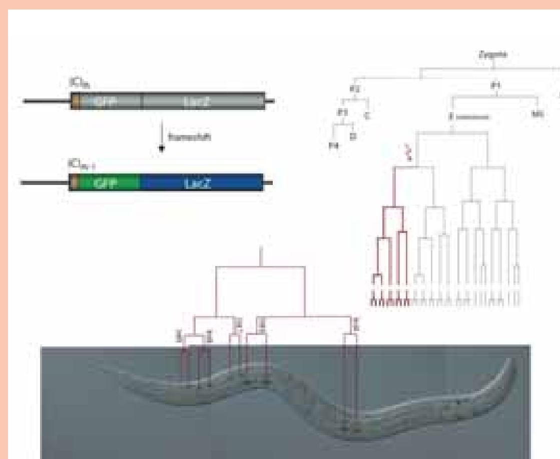


Figure 1. C. elegans DNA instability reporter. A) Schematic representation of the construct that allows detection of frameshifting errors and small DNA insertion or deletion events. The ORFs of the reporters GFP and LacZ are put out of frame by a microsatellite repeat (in brown). Frameshifts that restore the GFP/LacZ ORF can be visualized by fluorescence (GFP; not shown here) or X-gal staining for (-galactosidase (LacZ) expression. B) The lineage tree for the cells that form the C. elegans intestine (E-lineage). The intestinal sub-lineage that shows (-galactosidase expression in panel C is marked in red, thus pinpointing the mutagenic event to one founder cell (Ep) that divided approximately 2 hours post-fertilization. C) A mismatch repair defective (msh-6) transgenic animal carrying one or few copies of the construct displayed in panel A. Only (-galactosidase expressing intestinal cells are marked.

found in only a few patients.

In order to study the role of these mutator genes in genomic stability in more detail, we wished to make use of strains that are mutated in the corresponding genes. To that purpose we first screened the existing C. elegans libraries for mutations in the genes we previously identified and secondly isolated null alleles for the genes msh-2, msh-6, rev-1 and Y76A2B and obtained a reduction-of-function allele of mlh-1.

Improved MSI-indicator strains in forward genetic screens

In parallel, we made improved MSI-indicator strains and used these in forward genetic screens. These strains differ from the first generation indicator strains in three aspects: a) the reporter has a less stable microsatellite, a (C)23 instead of an (A)17 repeat, making the assay more sensitive, b) the reporter ORF now contains a nuclear localization signal which allows lineage tracking and better quantification, and c) the reporter is made by biolistic transformation which results in single to low copy integrated transgenes (figure 1). The latter feature made the reporter more sensitive and specific. The advantage of using a forward approach is threefold: First, we will quickly isolate genetic alleles of the known (previously identified) MSI genes and the causal mutation could point towards functional amino acid domains. Second, we anticipate isolating hypomorphic alleles of genes that are both essential for viability and genomic integrity: for these it would be almost impossible to obtain mutants using reverse genetic approaches. Third, genes will be identified that were missed in the genome-wide RNAi approach; this however requires further positional cloning. We thus far isolated 21 alleles (mutant strains) that have elevated levels of somatic repeat instability. Sequencing known mismatch repair (MMR) genes in these strains revealed likely null alleles of all classical C. elegans MMR genes: msh-2, msh-6, pms-2 and mlh-1. Mutant strains that do not have a mutation in these genes are currently being studied and the mutations are being mapped. However, as mapping this population-based phenotype by classical means turns out to be too laborious, we are currently exploring novel mapping technology to increase pace and throughput.

Most of the newly identified proteins function later in animal development to determine cell fate

Role of novel LIN-61-protein complexes in genome protection and cell fate determination

The genome-wide RNAi screen also yielded factors that protect the genome against mutations by other, yet unknown, means. We focused our efforts on one of them, C. elegans LIN-61, which when mutated in flies leads to brain tumors. We first characterized the molecular defects in C. elegans mutants, and placed the protein in the damage response pathway relative to known components. We then identified and phenotypically

Selected publications 2002-2005

1. Plasterk, R. H. (2002). 'RNA silencing: the genome's immune system'. Science 296(5571): 1263-5.
2. Tijsterman, M., R. F. Ketting et al. (2002). 'RNA helicase MUT-14-dependent gene silencing triggered in C. elegans by short antisense RNAs'. Science 295(5555): 694-7.
3. Wienholds, E., S. Schulte-Merker et al. (2002). 'Target-selected inactivation of the zebrafish rag1 gene'. Science 297(5578): 99-102.
4. Caudy, A. A., R. F. Ketting et al. (2003). 'A micrococcal nuclease homologue in RNAi effector complexes'. Nature 425(6956): 411-4.
5. Pothof, J., G. van Haaften et al. (2003). 'Identification of genes that protect the C. elegans genome against mutations by genome-wide RNAi'. Genes Dev 17(4): 443-8.
6. Sijen, T. and R. H. Plasterk (2003). 'Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi'. Nature 426(6964): 310-4.
7. Wienholds, E., M. J. Koudijs et al. (2003). 'The microRNA-producing enzyme Dicer1 is essential for zebrafish development'. Nat Genet 35(3): 217-8.
8. Denli, A. M., B. B. Tops et al. (2004). 'Processing of primary microRNAs by the Microprocessor complex'. Nature 432(7014): 231-5.
9. Nollen, E. A., S. M. Garcia et al. (2004). 'Genome-wide RNA interference screen identifies previously undescribed regulators of polyglutamine aggregation'. Proc Natl Acad Sci U S A 101(17): 6403-8.
10. Tijsterman, M. and R. H. Plasterk (2004). 'Dicers at RISC; the mechanism of RNAi'. Cell 117(1): 1-3.
11. Van Haaften, G., R. H. Plasterk et al. (2004). 'Genomic instability and cancer: scanning the Caenorhabditis elegans genome for tumor suppressors'. Oncogene 23(51): 8366-75.
12. Van Haaften, G., N. L. Vastenhouw et al. (2004). 'Gene interactions in the DNA damage-response pathway identified by genome-wide RNA-interference analysis of synthetic lethality'. Proc Natl Acad Sci U S A 101(35): 12992-6.

characterized biologically relevant interaction partners. The molecular identity indicates that these proteins, most likely as part of a chromatin remodeling complex, could act in response to genotoxic stress to repress transcription of genes that are required for cell proliferation. Strikingly, apart from their roles in genome protection in proliferating germ cells, most of the newly identified proteins function later in animal development to determine cell fate in somatic cells, by antagonizing the RAS signaling pathway. The marked overlap in genetic determinants suggests that this effector complex acts in two completely different signaling pathways that have non-overlapping inputs: DNA stress and developmental cues.

Other types of genomic instability

In addition, we have established reporter assays for various other types of genomic instability, i.e. G-tract (telomere) instability (likely the result of replication fork collapse), DNA transposition and single base-pair substitutions. To identify G-tract instability various constructs were designed according to the following rationale: a reporter ORF is interrupted by specific sequences (e.g. G-tracts) that cause DNA instability. Their loss (by e.g. DNA deletions) could restore reporter expression. Transgenic nematode strains were created and crossed to dog-1, the indicator mutant background (the only currently known mutant that displays loss of endogenous G-tracts), to test whether the reporters recapitulates this specific genomic instability phenotype. Reporter constructs that were designed in keeping with possible molecular mechanisms were efficient detectors of G-tract loss: within such cultures, numerous animals were detected that showed large blue patches, indicative of LacZ expression. Sequencing PCR products obtained from single animals revealed that the reporter construct suffered from deletion events; a pilot forward mutagenesis screen using this assay subsequently identified several new alleles of dog-1 including a premature stop, a splice site mutation and amino acid substitutions in conserved residues. We have also carried out RNAi screens to identify the complement of genes required to protect cells against other types of DNA damage. These include double-strand breaks that result from exposure to ionizing radiation (figure 2), alkylating DNA adducts induced by MMS, and intrastrand cross-links by exposing worms to the medically relevant agent Cis-platin. To increase throughput we developed the technology to perform genome-wide RNAi screens in liquid cultures on a 96-well platform.

Loss of function screens in mice

Our initial aim was to perform loss-of-function screens in mice by chemically mutagenising parental mice and applying our robotized high-throughput procedures to screen for mutations in the F1 progeny. 80 male founder mice were mutagenised with ENU and used in an extensive breeding program to set up a library of mutant F1 animals. Unfortunately, most of the founder animals turned out to be permanently sterile and those that became fertile had very poor breeding properties. In total only 400 F1 progeny could be derived by breeding for almost half a year. Therefore, we decided to switch to another model organism, the zebrafish, while keeping the initial goals, making knockouts in potential novel tumor suppressors.

13. Berezikov, E., V. Guryev et al. (2005). ‘Phylogenetic shadowing and computational identification of human microRNA genes’. Cell 120(1): 21-4.
14. Berghmans, S., R. D. Murphey et al. (2005). ‘tp53 mutant zebrafish develop malignant peripheral nerve sheath tumors’. Proc Natl Acad Sci U S A 102(2): 407-12.
15. Wienholds, E., W. P. Kloosterman et al. (2005). ‘MicroRNA expression in zebrafish embryonic development’. Science 309(5732): 310-1.

Future projects

- Continuation of the analysis of factors that stabilize the C. elegans genome, with special focus on small deletions as they occur in Fanconi anemia
- Analysis of the mechanism of RNAi in C. elegans, with special emphasis on mechanism of inheritable stable gene silencing
- A new research line is the role of microRNAs in cell differentiation, and in carcinogenesis. Our lab is currently leading the field of microRNA discovery by deep-sequencing

Knockout zebrafish

Over the last few years, we developed technology and obtained extensive experience, making knockouts in the zebrafish using target-selected mutagenesis. The Hubrecht Laboratory continuously maintains a large library of more than 10,000 mutant F1 fish, which in most cases is large enough for the successful identification of a knockout for any gene of interest. This library was already screened for mutations in several genes of interest for this program and knockouts have been found for the known mismatch repair genes MSH6, MSH2, and MLH1. Homozygous knockout fish are normally viable, and in contrast to mouse models, no obvious tumors have yet been observed in a limited number of animals that are up to 1 year old. However, a systematic histological analysis of a larger group of animals is needed to come to any firm conclusions. Evidence for a loss-of-function phenotype was obtained by studying germline microsatellite instability. In about 3% of progeny from homozygous Msh2 and Msh6 knockout fish, a single microsatellite was found to be instable. Transgenic reporter fish carrying the phage shuttle vector BigBlue (Stratagene) are currently generated to characterize the mutation spectrum and to study somatic mutation frequencies. In addition, we will screen for spontaneous and induced mutations by high-throughput resequencing.
In line with mouse Mlh1 knockouts, male zebrafish knockouts for this gene are sterile, due to a crucial role for MLH1 in meiotic cell division. Histological analysis showed that spermatogonial development is blocked in the first meiotic division and that no late spermatogonial developmental phases or mature sperm can be detected. In contrast, for female animals, which are also completely sterile in mouse, zebrafish produce eggs that can be fertilized. However, early development is severely compromised and only 1% of the embryos develop up to seven days. Quantitave SNP analysis in early and late progeny indicates aneuploidy: both monosomic as well as trisomic states are observed for single chromosomes, up to the complete genome.

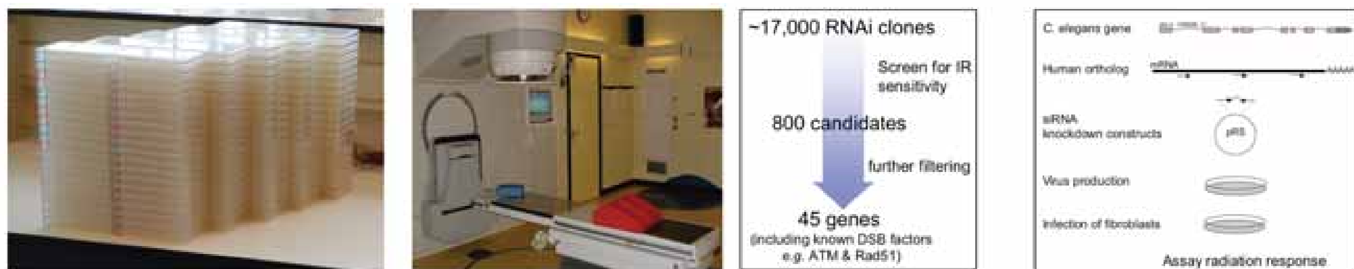


Figure 2. Identification of genes involved in the DSB response pathways of C. elegans and man. A) Animals were cultured in a 96-well liquid culture platform. Each well contains a different genetically engineered E. coli culture that expresses dsRNA homologous to only one C. elegans gene. Because of the systemic nature of C. elegans RNAi, feeding animals these cultures results in the knockdown phenotype of the cognate gene. B) Plates were irradiated with 140 Gy and after 5 days, cultures were inspected for the presence of progeny animals. Cultures that failed to produce progeny or had a marked reduction in brood size compared to the non-irradiated controls, were retested in three-fold and a dose-response curve was determined. C) This scheme yielded 45 genes that protect the C. elegans germline against irradiation. D) Schematic drawing of the protocol to test the orthologs human genes for a role in the DSB response.

Laura van ’t Veer

In 1989 Laura van ’t Veer received her PhD from the University of Leiden, and subsequently did her postdoctoral training at the Cancer Center of Harvard Medical School/Massachusetts General Hospital, Boston, USA and at the Netherlands Cancer Institute (NKI), Amsterdam. In 1993, she became group leader at the NKI where she initiated the Molecular Pathology Laboratory and the Family Cancer Clinic for genetic counseling and DNA diagnostics. Her research activities include molecular markers of breast cancer and several studies on breast cancer susceptibility. In 2003, Laura van ’t Veer was one of the founders of the NKI spin-off company for molecular profiling, Agendia.

Goals

To identify molecular profiles of different of disease outcome and response to

Molecular profiling for disease staging and therapy response in breast cancer

Molecular profiling of expressed genes in tumor tissue combined with advanced bio-informatics is beginning to show its power in delineating disease entities that are otherwise indistinguishable. This refinement in tumor classification allows a more accurate prediction of outcome of disease for patients than at present with the same stage of disease based on conventional clinical and histopathological criteria. Gene activities determining the biological behavior of the tumor are more likely to reflect the aggressiveness of the tumor than general parameters like tumor size, age of the patient, or even tumor grade. Therefore, the immediate clinical consequences of molecular profiling are that treatment schemes can be tailored based on the gene activity patterns of the tumor. Thus, we plan to determine molecular profiles that are predictive for disease outcome. Another important question to answer is the prediction of treatment response. In general, only a subset of the patients will respond to particular therapies, yet current clinical and histopathological criteria are not able to distinguish these subgroups. For specific therapies, non-responding groups are of considerable size. Since they do not benefit from those therapies there is a great need to identify these patients. To this end, we plan to determine molecular profiles for short-term clinical response in ongoing prospective clinical trials.

Tumor samples and bioinformatics tools

Since 1983, the NKI has collected and frozen about 20,000 tumor samples of which complete patient follow-up information is available, including the response to systemic treatment for recurrent disease. The registration system of these samples was

Sjoerd Rodenhuis

Sjoerd Rodenhuis received his MD in 1978 and his PhD in Medicine in 1983, both from the University of Groningen. He was licensed as an internist in 1984. Following his clinical training, he completed two years of laboratory training in molecular biology (NKI) and in experimental pathology (Yale University, New Haven). In 1987 he was appointed as a consultant Medical Oncologist at the NKI where he became Head of Medical Oncology and Chair of Medicine in 1994. In 1996 he was appointed Professor of Medical Oncology at the University of Amsterdam and in 1998 Medical Director of the NKI. Sjoerd Rodenhuis is board-registered in the Netherlands for both Medical Oncology (1991) and Clinical Pharmacology (1997).

breast cancer types for the prognosis
therapy.

adapted to the current codes of the national PALGA system (Pathologisch Anatomisch Landelijk Geautomatiseerd Archief). The samples form the basis of the various microarray studies performed at the NKI. Furthermore, using the available datasets, we developed and improved bioinformatics tools to analyze and validate tumor signatures. This concerns both the establishment of classification systems for tumor types as well as the unraveling of the underlying biology through 'pathway' analysis. These analytical tools were standardized, implemented in existing analysis software and were instrumental to all output of the profiling studies (Wessels et al, 2005; Van Houwelingen et al, 2005).

Prognostic profile for premenopausal breast cancer patients

We were successful in establishing a molecular profile of 70 marker genes predictive for a short interval to distant metastases in premenopausal lymph node negative breast cancer patients. The presence of a so-called 'good-prognosis' profile can predict with 90% certainty that a patient will remain disease-free for five years after her primary diagnosis (Van 't Veer et al, 2002; Van de Vijver et al, 2002). In collaboration with the NKI spin-off company Agendia, the 70 gene profile was developed into a diagnostic test suitable for diagnosis in patients. The prognostic profile was further validated for individual genes using independent techniques such as RT-PCR and tissue FISH, extended and improved to allow more detailed subgrouping for disease outcome (Chang et al, 2005; Van 't Veer et al, 2005; Wessels et al, 2005). Furthermore, the profile is now used prospectively in a patient study sponsored by the 'College voor Zorgverzekeringen' and validated in the international randomized 6,000 patient trial MINDACT (partly subsidized by EC FP6). The protocol for the latter study was approved by EORTC in December 2005; the coordinator of this trial is Emiel Rutgers (NKI) with Laura van 't Veer as coordinator biotechnology.

> The prognostic profile is now used prospectively in a patient study and validated in an international randomized 6,000 patient trial

Genetic make-up of primary tumor and metastases match

Analysis of matched pairs of primary breast tumors and of lymph nodes and distant metastases of the same patient has demonstrated a 97% similarity in the expression profiles (Weigelt et al, 2005). This finding supports the idea that the genetic make-up of the tumor is determined in an early stage and has important consequences for the choice of adjuvant therapy as this can now be based on the characteristics of the primary tumor.

Laura van ’t Veer & Sjoerd Rodenhuis
Molecular profiling for disease staging and therapy response

Group members

Postdoctoral fellows
Ron Kerkhoven
Annuska Glas
Erik van Beers
Annegien Broeks
Bas Kreike
Dimitry Nuyten

PhD students
Britta Weigelt
Juliane Hanneman
Marleen Kok
Roelien Olivier

Bioinformaticians
Carmen Lai
Lodewyk Wessels
Arno Velds
Daoud Sie
Guus Hart
Arne Waalkens
Yi Li

Database managers
Bart Maertzdorf
Ronald Zwaan

Research assistants
Marja Nieuwland
Renate Udo
Anke Nooyen

Technicians
Miranda van Dongen
Mike HeimerikxMark
Daemen
Wim Brugman
Sanne de Kemp
Arenda Schuurman
Anke Witteveen
Hans Halfwerk
Cathy Bosch
Nashet Nasr
Tibor van Welsem
Simon Joosse
Geert van Haalem
Linde Braaf
Angelina Huseinovic

Selected publications 2002-2005

1. Van ’t Veer, L. J., H. Dai et al. (2002). ‘Gene expression profiling predicts clinical outcome of breast cancer’. Nature 415(6871): 530-6.
2. Van ’t Veer, L. J. and D. De Jong (2002). ‘The microarray way to tailored cancer treatment’. Nat Med 8(1): 13-4.
3. Van de Vijver, M. J., Y. D. He et al. (2002). ‘A gene-expression signature as a predictor of survival in breast cancer’. N Engl J Med 347(25): 1999-2009.
4. Weigelt, B., A.M. Glas et al (2003). ‘Gene expression profiles of primary breast tumors maintained in distant metastases’. Proc Natl Acad Sci USA 100: 15901-05.
5. Broeks, A., L. de Witte et al (2004). ‘Excess risk for bilateral breast cancer in CHEK2*1100delC germline mutation carriers’. Br Cancer Res Treat 83: 91-93.
6. Voskuil, D. W., A. Bosma et al. (2004). ‘Insulin-like growth factor (IGF)-system mRNA quantities in normal and tumor breast tissue of women with sporadic and familial breast cancer risk’. Br Cancer Res Treat 84(3): 225-33.
7. Dai, H., L.J. van ’t Veer et al. (2005). ‘A cell proliferation signature is a marker

Response to therapy

Having established molecular profiles for prognosis, another important question to answer is the prediction of treatment response. Thereto profiling for tamoxifen resistance and local recurrence was initiated in a large-scale collaboration between the Amsterdam (Laura van ’t Veer, Sabine Linn, Marc van de Vijver) and Rotterdam (Jan Klijn, Els Berns, John Foekens) profiling groups. The collaboration has resulted in an inter-institutional validation of putative profiles that greatly strengthen the value of these findings, but has also resulted in a more extensive collaboration to be expanded in the coming years.

Cancer susceptibility genes

Particular low/moderate and high risk inferring germline alterations in genes (SNPs and known breast cancer susceptibility genes) have been implicated in different biology of the tumor and of disease outcome. SNPs/gene mutations in BRCA1, BRCA2, ATM, MDM2 are being evaluated in case-control studies as well as consecutive breast cancer cohorts. A genomic profile for BRCA1 breast cancer was recently developed (Van Beers et al, 2005).

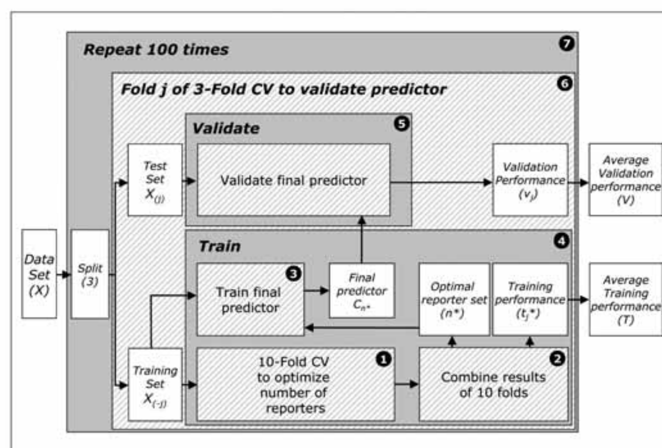


Figure 1. The train-validation protocol in simplified schematic format. The most important steps in the protocol are the training step, consisting of the optimization of the number of reporters and training the final predictor, and the validation step, in which this final predictor is validated on a completely independent validation set. These steps are performed in a 3-fold cross-validation procedure which is repeated 100 times, each time for a different split of the dataset. Reproduced from Wessels et al (2005) Bioinformatics 21(19):7355-62, copyright ©Oxford University Press, with permission.

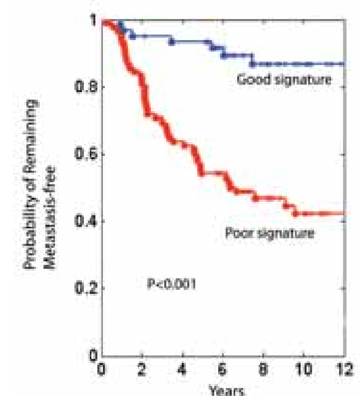


Fig. 2. Kaplan-Meier analysis of the probability that lymph-node-negative patients would remain free of distant metastases. Reproduced from Van de Vijver et al (2002) N Eng J Med 347(25):1999-2009, copyright ©Massachusetts Medical Society, with permission.

of extremely poor outcome in a subpopulation of breast cancer patients'. Cancer Res 65(10): 4059-66.

8. Weigelt, B., J. L. Peterse et al. (2005). 'Breast cancer metastasis: markers and models'. Nat Rev Cancer 5(8): 591-602.
9. Van Beers, E., T. van Welsem et al. (2005). 'CGH profiles for BRCA1 and BRCA2 breast tumors highlight differential sets of genomic aberrations'. Cancer Res 65, 822-827.
10. Chang, H.Y., D.S.A. Nuyten et al. (2005). 'Robustness, scalability and integration of a wound-like expression signature in predicting breast cancer survival'. Proc Natl Acad Sci USA 102, 3738-3743.
11. Van 't Veer, L.J., Paik, S. et al. (2005). 'Gene expression profiling of breast cancer: A New Tumor Marker'. J Clin Oncol 23, 1631-1635.
12. Wessels, L.F.A., M.J.T. Reinders et al. (2005). 'A protocol for building and evaluating predictors of disease state based on microarray data'. Bioinformatics 21: 3755-3762.
13. Weigelt, B., Z. Hu et al. (2005). 'Molecular portraits and 70-gene prognosis signature are preserved throughout the metastatic process of breast cancer'. Cancer Res 65: 9155-9158.
14. Van Houwelingen, H.C., T. Bruinsma et al. (2005). 'Cross-validated Cox-regression on microarray gene expression data'. Stat Med, Epub Sep5.

Future projects

Current knowledge of genetic make-up of patients and molecular characteristics of the tumor of a patient is revealing that these biomarkers can be used for tailored, individualized treatment. We showed the clinical usefulness of molecular tumor profiling and determination of genetic susceptibility. Our working group will, in collaboration with the Erasmus Medical Center,remain focusedon therapy response prediction for patients subgroups by use of genomics on our archival tissue banks and current clinical trial patients. Ultimately, this will lead to increasing life-expectancy for cancer patients.

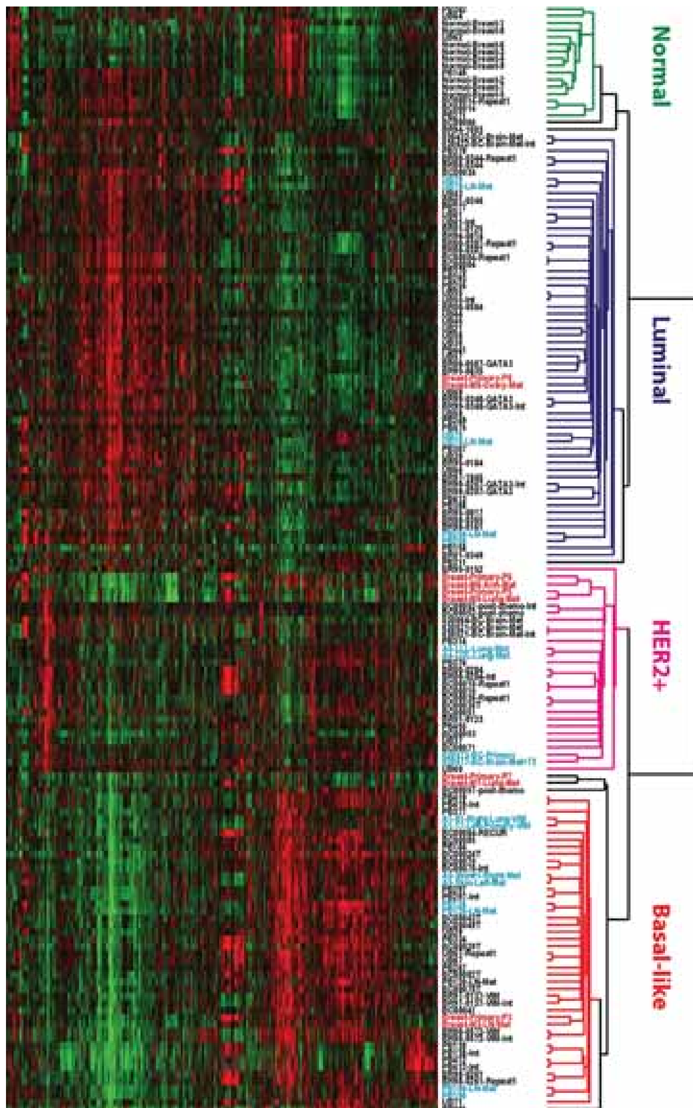


Figure 3. Determination of molecular subtypes of primary tumors and their metastatic counterparts. A hierarchical clustering analysis of the 156 arrays representing 107 patients was performed using a breast tumor classification gene list. Scaled-down version of the complete cluster diagram: genes were arranged in horizontal and samples in vertical. Sample-associated dendrogram: Sample names in red were from the NKI and sample name in light blue from UNC at Chapel Hill. Reproduced from Weigelt et al (2005) Cancer Res 65(20):9155-8, copyright ©American Society for Cancer Research, with permission.

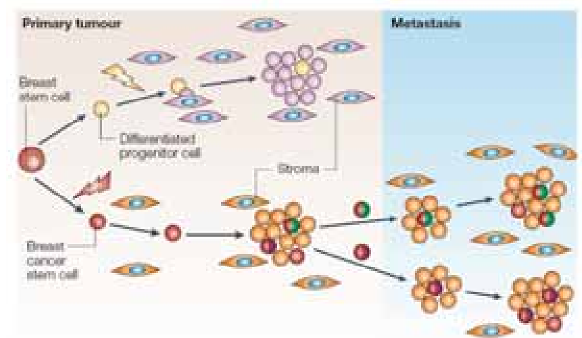


Fig. 4. An integrative model of breast cancer metastasis. Oncogenic mutations occurring in a breast stem cell (red) can cause the transformation to a breast cancer stem cell, generating 'poor prognosis tumors' (orange). Mutations occurring in differentiated progenitor cells (yellow) might form a non-metastatic 'good prognosis' breast carcinoma (pink). In the metastatic poor-prognosis tumors, under the influence of stromal fibrobasts, only the population of breast cancer stem cells has the ability to metastasize. There might be variant cancer stem cells that differ in their tissue selectivity for metastasis, expressing an additional tissue-specific profile (e.g.: green, bone; purple, lung). At the site of metastasis, the disseminated cancer stem cells would again induce a similar stromal response as in the primary breast tumor. Reproduced from Weigelt et al (2005) Nature Rev Cancer 5(8):591-602, copyright ©Nature Publishing Group, with permission.

Nobel prize recipient Harold Varmus on an Amsterdam canal boat during the CGC/CBG meeting, 27 October 2005

Scientists meet at Royal Tropical Institute

In collaboration with the Centre for Biomedical Genetics, on October 27th and 28th the CGC presented a two-day scientific symposium entitled 'Genomic Approaches and Molecular Mechanisms in Cancer'. With an attendance of over 360, we consider this meeting in the Royal Tropical Institute (Koninklijk Instituut voor de Tropen), Amsterdam, to have been a great success.

A total of seventeen lectures was presented by renowned scientists mostly from abroad, among which a keynote lecture by the 1989 Nobel Prize recipient and former NIH director Harold E. Varmus (see program). In addition, 26 young CGC investigators presented their research during the poster sessions.
Laurent Knoops of the Netherlands Cancer Institute was awarded the first CGC poster prize for his contribution 'Low dose radiation induces a highly effective p53 and immune response in follicular lymphoma' (see abstract).

Low dose radiation induces a highly effective p53 and immune response in follicular lymphoma*

Award winning abstract of Laurent Knoops

Involved field radiation therapy with 30 to 40 Gy is a valuable local treatment for follicular lymphoma (FL) that is routinely used in clinical practice. We previously showed that, in contrast to other malignancies, very low dose radiation (2x2 Gy, days 1 and 3) is also effective, with rapid and often long lasting remissions in up to 90% of FL patients (Haas et al, JCO, 2003). However, the biological mechanism of this extreme sensitivity is not known. To study the molecular response to low dose radiation therapy in FL, gene-expression profiling using 35K spotted 60-mer oligo-arrays was performed from lymph node biopsy samples taken before treatment and 24 hours after the second dose of 2 Gy irradiation, in fifteen patients. The clinical response was excellent (10 CR, 5 PR).

Presentations

Genomic Approaches and Molecular Mechanisms in Cancer

Reuven Agami	'Genetic screens uncover cancer related functions of microRNAs'
Nick Hastie	'The Wilms' tumour suppressor WT1 - and the Wnt connection - in cancer and development'
Rene Medema	'Restarting the cell cycle when the checkpoint is silenced'
Guillio Draetta	'The role of CDC25 in cell cycle control'
Nick La Thangue	'Effector pathways in DNA damage response'
Richard Marais	'The role of B-raf in tumor formation'
Titia de Lange	'Safeguards of human telomeres'

Keynote Lecture

Harold Varmus 'Oncogenes and the future of cancer therapy'

Jan Klijn	'Microarray profiling of breast tumors'
Oli Kallioniemi	'Translational genome-scale cancer research'
Matthias Mann	'Proteomic approaches to study stem cell differentiation'
Roel Nusse	'Wnt signaling and growth control'
Boudewijn Burgering	'Forkhead transcription factors and the control of cellular oxidative stress'
Alan Ashworth	'Therapeutic exploitation of the DNA repair defect in BRCA mutant tumours'
Arnie Levine	'Positive and negative feedback loops in p53 signaling'
Frank McCormick	'Drug targets in the Ras pathway'

Keynote Lecture

Charles Sawyers 'Kinase inhibitors and the treatment of cancer'

In all patients, a major and consistent induction of p53 target genes was seen, reflecting both proliferation arrest (e.g., P21, repression of cell-cycle regulated genes) and apoptosis induction (e.g., NOXA, PUMA, BAX, TRAIL-R2/DR5 and FAS). The increase in apoptotic-related genes was confirmed by MLPA. P53 upregulation, p53-mediated proliferation arrest and apoptosis were substantiated using immunohistochemistry with dramatic increase of p53 protein levels in B-cells, less in T-cells and accessory cells, and no increase in macrophages. There was also a significant increase in the numbers of cleaved-caspase eight positive cells (death receptor/extrinsic apoptosis pathway) with a minor increase of cleaved-caspase nine positive cells (mitochondrial/intrinsic apoptosis pathway), suggesting a major role of the extrinsic apoptotic pathway in the hypersensitivity. The other induced genes revealed an 'immune signature', with a whole set of biologically meaningful genes related to macrophages (e.g., CD68, TLR4), TH1 immune response (e.g., IL18, CXCL9, 10, 11), clearance of apoptotic cells (e.g., C1Q, lysosomal enzymes), tolerance (ILT-3, IL-4, IDO) and death receptor ligand (TRAIL). Immunohistochemical analysis did not show an increase in T-cell subsets and macrophages density. CD68/p53 double staining showed no increase in p53 in macrophages. These data rather suggest an activation or differentiation of resident macrophages by apoptotic cells than recruitment of novel cell populations.

This is the first global analysis of the direct molecular effect of radiotherapy and p53 related apoptosis in vivo in human lymphoma. Moreover, the 'immune signature' suggests that radiation-induced apoptosis in FL is not an immunologically silent process, but rather an early event that could contribute to the death and clearance of tumor cells. These insights may have important implications for modulation of the cancer-related immune response and for immunotherapeutical approaches in FL.

Laurent Knoops (NKI) (at right) receives the CGC poster prize from CGC scientific director Hans Bos. At left, NKI director Anton Berns

***Knoops L., R.L. Haas, S. de Kemp, A. Broeks, E. Eldering, D. Majoor, J.P. de Boer, L.J. van 't Veer, D. de Jong**

Netherlands Cancer Institute, Amsterdam and the Academic Medical Center, Amsterdam

Patent applications

Year	Subject	CGC group
2002-2003	TCF target genes to identify drugs for the treatment of cancer	Clevers
	Tumor development genes as targets for anti-cancer drugs	Clevers
	Novel markers for isolation of intestinal stem cells	Clevers
	Epac-specific compounds	Bos
	Mismatch repair	Plasterk
2004	Tamoxifen expression profile	Klijn
	New use for cancer antigen	Bernards
	Treatment of an intestinal adenoma/adenocarcinoma by inhibition of Notch pathway activation	Clevers
	Functional genomics using transposition	Grosveld
	Nucleic Acid Analysis	Grosveld
	Novel TFIIH subunit	Hoeijmakers
	Mouse models for the role of DNA damage in aging	Hoeijmakers
	Predicting response and outcome of metastatic breast cancer anti-estrogen therapy	Klijn
2005	Methods for detecting and/or staging follicular lymphoma	Bernards
	Prognostic marker for predicting therapeutic response / survival period of breast cell proliferative disorder patient	Klijn (Foekens)
	Methods and nucleic acids for the improved treatment of breast cancers	Klijn (Foekens)
	Prognostic markers for prediction of treatment response / survival of breast cell proliferative disorder patients	Klijn (Foekens)
	Prognosis diagnosing marker for predicting treatment response / survival time of patient suffering from mammary cell growth disorder	Klijn (Foekens)
	Improvements in cancer treatment and cancer treatment efficacy prediction by blocking protease inhibitors	Klijn (Foekens)
	Epigenetic markers for the treatment of breast cancer	Klijn (Foekens)
	Novel urokinase inhibitors	Klijn (Foekens)
	Predicting bone relapse of breast cancer	Klijn
	Gonadotropin Releasing Hormone and Luteinizing Hormone Receptor variants: implications for the diagnosis and treatment of breast cancer	Klijn (Berns)
	Biomarkers for prognosis in breast cancer	Klijn (Foekens)
	Diagnostic screens of genome rearrangements	Grosveld

Every year, 69,000 people in the Netherlands are diagnosed with cancer and 37,000 die from the disease. It is expected that cancer will soon be the primary cause of death in all age groups, young and old. Therefore the primary aim of the CGC is to obtain complete insight of the genetic changes that turn a healthy cell into a tumor cell, in order to contribute to improving the diagnosis and treatment of cancer. Due to the great resemblance of cancer cells with normal cells in our body, developing medicines that kill only cancer cells is not an easy task. Completely curing or surpressing the disease has therefore proven to be difficult if not almost impossible. However, genomics research now enables us to detect the very small differences that exist between healthy cells and cancer cells and to understand why a normal cell turns into a tumor cell.
The research activities of the CGC have resulted in various applications in the clinic and contributed to new economic activities, such as a long list of patent applications, new collaborations with industry and several new start-up companies. This demonstrates not only the clinical but also the societal and economic importance of cancer genomics research.

From scientific discovery to economic value

Microarray tests provide insight into the risk of metastasis for breast cancer patients

An example of a new clinical application are the microarray-tests developed by CGC researchers at the NKI, René Bernards en Laura van 't Veer, and at the Erasmus MC, Jan Klijn, John Foekens and Els Berns. With these tests, the activity of many genes in breast cancer cells are registrered, resulting in expression profiles with which patients can be subdivided into different risk classes. In the NKI study that focusses on patients younger than 55, it was found that 30-35% of patients has a minimal risk to develop metastasis after an operation to remove the tumor. Thus it may not be neccesary to treat these patients with adjuvant chemotherapy, which at present is still given to almost all breast cancer patients, just to be safe. Apart from sparing these patients the burden of chemotherapy, such a test will lead to a significant decrease in treatment costs. These findings were the basis for the establishment of Agendia, a spin-off company that commercializes these diagnostic expression profiles.
The Eramus MC group developed expression profiles applicable to all breast cancer patients. In addition, they found profiles that predict the response to the cancer drug tamoxifen. In collaboration with Veridex (Johnson & Johnson), these profiles are also exploited commercially. Both teams are now collaborating to optimize the current tests and to develop the next generation of tests. This includes both national studies as well as large European clinical trials. The outcome is obviously of great importance for breast cancer patients.

Second start-up, DNage, focuses on DNA repair and aging

In addition to Agendia, a second start-up company DNage was established based on technology and knowledge developed by CGC researcher Jan Hoeijmakers in the field of DNA repair, aging and cancer. DNage has identified several safe, small molecules with an inhibitory effect on premature aging and aging-related diseases. It is expected that these molecules will soon be tested in the clinic. Very recently, DNage has been acquired by the Dutch biotech company Pharming.

CGC collaborations with academia and industry

CGC member(s)	Organization	Purpose of collaboration
All CGC members	Netherlands Bioinformatics Centre (NBIC); BioRange and BioAssist Programs	Bioinformatics
Bernards	Cancer Research UK	RNAi library
	Rosetta Inpharmatics, Seattle, USA	RNAi library
	Merck Research laboratories, USA	Mouse shRNA library construction
	Sanger Centre, Cambridge, UK	High throughput sequencing of shRNA libraries
	UCSF & Lawrence Livermore Berkeley Laboratories, USA; Supported by NIH	Functional analysis of EGF signaling pathway
	Prolifix/Topotarget, Ltd	Collaboration on the development of anti-cancer drugs
	EU FP6 Network of Excellence	Role of ubiquitin and ubiquitin-like modifiers in cellular regulation (proteomics, genetics)
	University of California, San Francisco, USA Supported by NIH	Functional genomics
Bernards, Van 't Veer	EU INTACT (FP6 Integrated Project)	Functional genomics for the identification of novel targets for cancer therapy
Bernards, Van 't Veer, Bos	EU TRANSFOG (FP6 Integrated Project)	Functional genomics
Bernards, Klijn, Van 't Veer	Veridex LLC/ Johnson & Johnson, San Diego/New Jersey, USA; Rosetta Inpharmatics, Seattle, USA; Agendia, Amsterdam	Expression profiling in breast cancer
Berns	Sanger Centre, Cambridge, UK	High throughput sequencing of insertion sites
Bos, Clevers	Semaia Pharmaceuticals/ Hybrigenics SA	Collaboration on the development of anti-cancer drugs
	Hybrigenics SA, Parijs	Two-hybrid screens
Bos, Clevers, Grosveld	Netherlands Proteomics Centre (NPC)	Proteomics
Clevers	ICCB, Harvard, USA	High-throughput screening of chemical compounds
Grosveld	OncoMethylome Sciences, Luik België	Methylation of genes
	Minos Biosystems, UK	Transposition in mice
Hoeijmakers	DNage; Start-up company	Aging
Klijn	7 European clinical partners Sponsored by Johnson & Johnson	Validation of prognostic gene signature in breast cancer
	EORTC - RBG; Sponsored by Johnson & Johnson	Multicenter validation 76-gene prognostic profile in untreated LNN and tamoxifen treated patients
	EU TRANSBIG; Sponsored by Johnson & Johnson	Independent multicenter validation 76-gene prognostic profile in untreated LNN patients
	Epigenomics, Berlin Germany/Seattle, USA; 9 European clinical partners; Supported by EU-FP6	Methylation profiling in breast cancer
	Pamgene, Den Bosch	PamChip kinase profiling in tumor cell lines and tissue samples
	Pamgene/Bionavigator; Supported by Senter	Kinase profiling
	Dr. A.J. Minn & Dr. J. Massagué, Memorial Sloan-Kettering Cancer Center, NY	Lung/bone metastasis profile
	Dr. G. Narla & Dr. J. Martignetti, Mount Sinai School of Medicine, NY, USA	Clinical relevance of KLF6 and isoforms
	Dr. L. Pasa-Tolic, Pacific Northwest National Laboratory, Richland, WA, USA; Supported by Dutch Cancer Society	MS in tamoxifen-treated patients by nLC-FT-ICR
	Dr. E. Petricoin & Dr. L. Liotta, George Mason University, Washington, USA	Phophoprotein detection for prognosis and therapy response
	Veridex LLC/Johnson & Johnson, San Diego/New Jersey, USA Sponsored by Johnson & Johnson	SNP analysis & RT-PCR in paraffin-embedded tissues
	R. Zeillinger, Ludwig Botzmann Institute, Vienna Supported by EU 6FP	Ovarian Cancer Diagnosis of a Silent Killer (OVCAD)
Plasterk, Cuppen	Dr. Claire Allen University of Sheffield, UK	Functional characterization of mismatch repair-deficient zebrafish
	Dr. Derek Stemple, Sanger Institute, Hinxton, UK	High-throughput zebrafish mutant library screen
Van 't Veer	EU TRANSBIG (FP6 Network of Excellence)	Molecular profiling

Tech transfer: how to use and protect your scientific discovery?

As part of our educational task, the CGC organizes valorization workshops on a regular basis. The first one was held on 11 May 2005 for PhD students, postdocs and research staff of the 'Utrecht' branch of the CGC. The workshop was organized in collaboration with the Utrecht Graduate School of Developmental Biology and the Netherlands Industrial Property Office (Octrooicentrum Nederland). It included speakers from the CGC (Frank Grosveld), the Netherlands Industrial Property Office (Nikki Rethmeier) and Cancer Research Technology Ltd (Simon Youlton). Topics discussed during the workshop covered Dutch and international patent law and procedures, international patent database search and experiences from the floor. A second and similar workshop for the 'Rotterdam' branch of the CGC was held on 18 May 2006 in collaboration with the Rotterdam/Leiden Medical Genetics and Molecular Medicine Graduate Schools.

Alzheimer drug against intestinal cancer?

Recently, CGC investigator Hans Clevers discovered that blocking the Notch-pathway can inhibit the growth of intestinal cancer cells in mice. This finding may have a major impact on the treatment of intestinal cancer, since the drugs needed to block this pathway, the so-called γ-secretase inhibitors, already exist. They were originally developed for the treatment of Alzheimer's disease, but turned out to have severe side-effects in the intestine and therefore unsuitable for this application. Within a few years, these medicines (with an adapted treatment strategy) could be tested in the clinic for their effectivity in the treatment of intestinal cancer. Clevers is currently in the process of starting a spin-off company to commercialize this finding.

New targets voor specific medicines

The genomics research in the CGC has lead to novel knowledge on the genetic changes that occur when healthy cells turn into tumor cells and on the molecules that act as switches during these changes. Apart from the aforementioned new diagnostic methods, this knowledge also provides new targets for specific, targeted medicines. A successful example of such a targeted approach is trastuzumab, a medicine that is used for the treatment of breast cancer tumors that overexpress the HER2/neu protein. Originally used only for the treatment of metastasized breast cancer, this inhibitor also proved very effective as adjuvant therapy after operative removal of the tumor, radiation and chemotherapy, resulting in a spectacular 50% reduction in the risk of reoccurrence of the tumor.

It now appears that in order to fight cancer successfully, a range of medicines adapted to the specific properties of the tumor will be needed. CGC researchers have identified specific molecules in different tumor types that appear to act as switches in the change from healthy cells to tumor cells. These molecules are now being validated as targets for the development of new, specific medicines. An example are the TCF en β-catenin molecules that play a crucial role in the development of intestinal cancer and which are an important subject of investigation at Semaia Pharmaceuticals, a biotech company started by CGC researchers Hans Clevers and Hans Bos. This company is currently a full daughter of the French biotech company Hybrigenics (Paris).

Although this new knowledge provides the necessary basis for the development of novel therapeutics, it must be stressed that this process normally takes ten to fifteen years and that the identification of targets only is a first step requiring many years of research before new medicines actually become available for the patient. Very recently, several CGC researchers have entered into new collaborations with Dutch companies (Organon, Kiadis) in the framework of the TopInstitute Pharma (TI Pharma), a new Dutch initiative to perform high-profile pharmacologic research.

CGC scientific director Hans Bos presents the first copy of ‘Genes, faulty DNA and cancer’ to Els Borst-Eilers, chairperson of the Netherlands Federation for Cancer Patient Organizations (NFK) and former Minister of Health

Societal communication

During the existence of the CGC, the activities of its researchers have attracted high media interest. As an example, press exposure in 2005 included publications on microarray profiles related to tamoxifen resistance, a possible new treatment for intestinal adenoma/ adenocarcinoma, as well various awards from national and international organizations, such as the Spinoza award, the Prix Louis D. of the Institut de France and the Louis-Jeantet-Prize for Medicine.

CGC scientists also contributed to societal communication through various presentations and publications for the public and (ex)patients through participation in national TV programs (such as ‘Nieuwslicht’) and columns in TV programs and papers (e.g. ‘Buitenhof’ and ‘Volkskrant’). Interactive communication with the public regarding (the impact of) life sciences research is further stimulated by monthly presentations and discussions by CGC and other researchers in Science café ‘De Doelen’ in Rotterdam, an initiative of Reflexie that is financially supported by the CGC (http://www.reflexie.org/wetenschapscafe.htm).

Discussions after the presentations of Laura van 't Veer & Els Berns

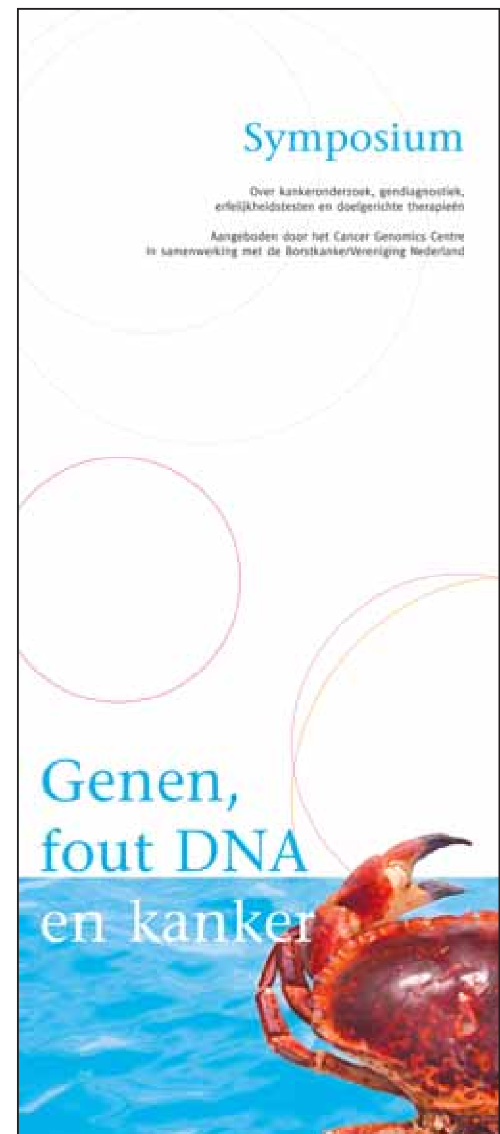
First step: reaching out to breast cancer patients

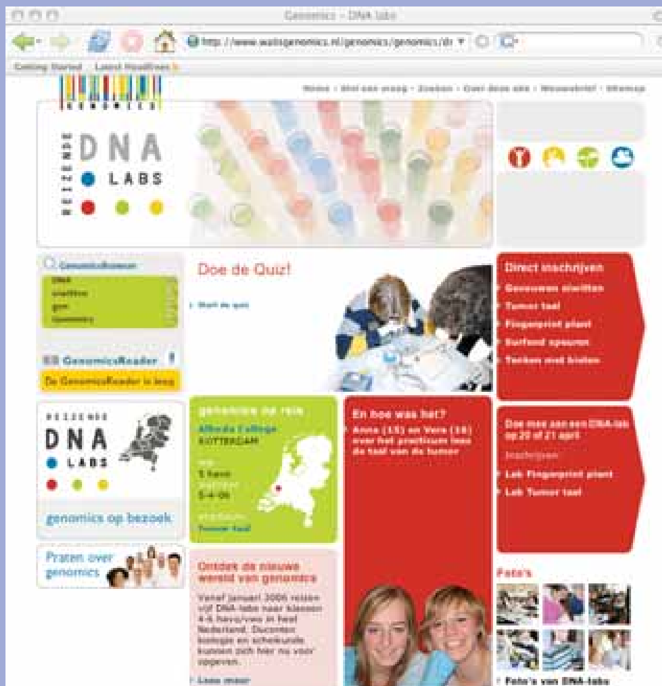
One of the highlights of the CGC communication activities over the period 2002-2005 was the symposium entitled 'Genes, faulty DNA and cancer' ('Genen, fout DNA en kanker'). This meeting was organized for (ex) breast cancer patients on 15 October 2005 in collaboration with the Netherlands Breast Cancer Organization (BVN). It focussed on cancer research, new diagnostic tests, screening for hereditary forms of breast cancer and new, targeted therapies. A similar symposium is presently being organized for all cancer patients.

Overview in book

The CGC published a book on the occasion of the symposium, which provides an overview of the lectures and workshops presented by CGC researchers at the symposium. It was printed in an edition of 10,000 copies and sent free of charge to all 7,000 members of the BVN as well as the patient information departments of all 120 hospitals in the Netherlands.
It can be downloaded from http://www.cancergenomics.nl/files/Genen_foutDNA_kanker.pdf or requested by sending a message to a.speksnijder@med.uu.nl .

BorstkankerVereniging Nederland
vereniging van borstkankerpatiënten en erfelijk belasten





Mobile labs on www.watisgenomics.nl

Cancer and genomics theme on www.watisgenomics.nl

Genomics education

In the current high school curriculum, DNA is an important yet rather isolated topic that pupils often perceive as complex and highly abstract. In order to introduce modern DNA-research to schools and to make pupils at least acquainted with and hopefully enthousiastic about current genomics topics and their implications for society, five genomics Centres of Excellence have each developed a mobile DNA-lab that is offered free of charge to all secondary schools in the Netherlands. The four-hour educational modules contain a two-hour practical lab taught at the schools by university students with up-to-date genomics equipment and techniques.

Read the language of the tumor

For upper-secondary school pupils (4-6 havo/vwo), the CGC developed the mobile DNA-lab 'Read the language of the tumor' (Lees de taal van de tumor). The pupils are given the role of DNA-researchers and are asked to compare DNA from cancer cells of a (fictive) patient with DNA of healthy cells. Based on their results, they have to advise the physician on the optimal treatment of this particular patient. The module was developed in collaboration with the Centre for Science Education of Utrecht University in line with the recommendations of the Committee Innovation Biology Education ('Commissie Vernieuwing Biologie Onderwijs') regarding the context-concept approach. The module was tested at schools in the Utrecht area in the fall of 2005.

Large demand of the lab

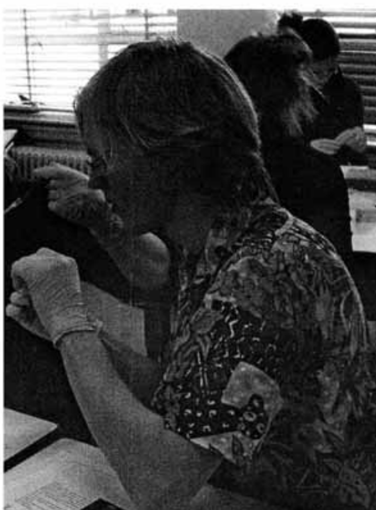
From 1 January 2006 onward, the lab and its educational materials are offered to all schools in the Netherlands. Teachers can obtain additional information and register at the dedicated web-

Junior Science Molecular Medicine initiative

site www.dnalabs.nl. The lab 'Read the language of the tumor' is fully booked until 1 July 2006 and will then have reached 100 schools and 3800 pupils. Due to its large demand, the lab will continue to be offered to schools in the school year 2006-2007. Parts of the lab were presented to the public during the BIOPOP 2006 event on April 1-2 in Delft, which attracted close to 3,000 visitors. The lab was also present at the public event DNAmazing on April 20-23 2006 in Nemo Science Centre, Amsterdam.

'iiieuw, slierten!'

Reizende DNA-Labs introduceren bovenbouwleerlingen in de wereld van genomics. Op een dinsdagmiddag lezen leerlingen van 6 VWO de taal van tumoren.



Marc en Femke sjouwen gelbakjes, pipetten en een PCR-apparaat over de gang van het Revius Lyceum in Doorn. Een vitrinekast met opgezette egels, een vleermuis op sterk water en de schedel van een rund verklappen dat we bij het biologielokaal aankomen. In het kabinet staat een plastic penis prominent op tafel naast een kooi met hamsters. 'Als jullie ergens een zwarte hamster voorbij zien schieten, geef dan even een gil want die zijn we kwijt,' vertelt een leraar die vlug in en uit banjert.

Marc van Mil van het Cancer Genomics

Media attention for DNA-labs

Latest insights

The DNA-lab 'Read the language of the tumor' is supported by a series of 23 articles within the theme 'Cancer & Genomics' on the public website www.watisgenomics.nl (in Dutch). Here the visitor can find the latest insights in the cellular mechanisms that drive cancer, new diagnostic tools, interviews with renowned cancer researchers and the use of microarrays in cancer research and their potential for application in the clinic. The site was launched on October 1st, 2005 and is targeted primarily at upper-secondary school pupils but is also visited frequently by (relatives and friends of) cancer patients. For the first group, suggestions for research projects ('profielwerkstuk') have been included as well as an interactive quiz and a map of the Netherlands showing this week's DNA-labs.

The CGC furthermore supports the Junior Science Molecular Medicine initiative of CGC partners at the Erasmus MC that aims to interest upper-secondary school pupils (5-6 vwo) in scientific research by offering two-week practicals in their university labs (www.erasmusmc.nl/juniorscience). In 2005, 34 pupils have thus been introduced to biomedical research. Due to its enormous success, the number of available practicals is greatly extended in the year 2006 and as of June another 26 pupils have participated.

Apart from conducting research to understand the molecular and cellular basis of cancer, the CGC contributes to two research projects aiming to determine the societal impact of cancer genomics research and to develop new genomics educational programs for secondary high school students. These projects are carried out in collaboration with the Centre for Society and Genomics and involve CGC researchers as well as social and educational scientists at other research institutes, such as the VU Medical Centre in Amsterdam, the Centre for Science Education of Utrecht University and the University of Humanistics in Utrecht.

Societal research

Cancer genomics: benefits and risks for society

The rapid developments in the field of genomics are expected to lead to a further increase in the potential for early diagnosis, the fine-tuning of prognostic features of specific tumors and the detection of cancer predisposition. Each new development, each new technique raises the question of how to assess its consequences for putative users. Little is known yet about how these genomics-related developments will affect the ways in which doctors, patients or the lay-public perceive, define and cope with cancer risks, diagnosis and therapy. In order to determine how these new diagnostic tools might shape their likely future actions, studies are needed of how aforementioned groups have used genetic information in the past and at present in conceptualising cancer risks and defining medical coping strategies.
To this end, a collaborative research project has been initiated to study the following question: The benefits and risks of cancer genomics for society: How new genomics-related technologies affect the perception and handling of cancer risks and prognosis by patients, doctors, nurses and the public. The objectives of the project are made operational in two related studies, which run parallel. A historical study focuses on mechanisms and patterns in the ways in which scientists, physicians, nurses, patients and the media have dealt with cancer risks and prognosis related to genetic factors and heredity. The second study, which concentrates on contemporary data, aims to explore and describe the dynamics of the understanding and coping by users of new genomics-related clinical technologies. The results of these interrelated studies will be jointly analyzed and translated into a matrix model. This model envisages the information and communication on cancer related to genetic and genomical aspects in medical practice, the media and public health policy.
The coordinator of the project is prof. T. (Toine) Pieters (VU Medical Centre) and the participants include prof. F.J. (Frans) Meijman (VUMC), prof. N.K. (Neil) Aaronson (NKI, VUMC), prof. F. E. (Floor) van Leeuwen (NKI, VUMC) and dr. L.J. (Laura) van 't Veer (NKI). Funding for the project is provided by the Centre for Genomics and Society and the CGC.

DNA labs for citizenship

The societal embedment and control of genomics research and its rapid development are a major challenge in (science) education. Teachers are supposed to prepare students to function in a multicultural, democratic society with different religious traditions, i.e. to educate them for citizenship. Critical-democratic citizens take responsibility for the functioning of the community and participate in public discourses about health, moral, social and political issues like genomics research, applications and implications. Such a critical disposition should be combined with solidarity with

Learning for understanding and valuing genomics

To establish theoretically underpinned and empirically tested genomics education for citizenship, the research project 'DNA labs for citizenship: Learning for understanding and valuing genomics in upper-secondary education' will be initiated in 2006. It will also elaborate on the 'context-activity-concept' approach and test its practicability. The project is coordinated by prof. A.J. (Arend Jan) Waarlo (Centre for Science and Mathematics Education, Utrecht University) and includes the following researchers: prof. W.M.M. (Wiel) Veugelers (University of Humanistics, Utrecht), dr. J.E. (Annelies) Speksnijder and ir. M.H.W. (Marc) van Mil (Cancer Genomics Centre), prof. J.G. (Jan) Klijn (Erasmus MC) and prof. K.Th. (Kerst) Boersma (Centre for Science Education, Utrecht University and Chair of the Biology Curriculum Innovation Board, CVBO). Funding for the project is provided by the Centre for Genomics and Society and the CGC.

others. Citizenship education includes learning how to make considered judgments that are partly based on values.

Facilitating DNA labs for secondary schools is a favourite outreach activity of genomics research centres in different countries. The student laboratory in general has been given a central and distinctive role in science education, and science educators have suggested that rich benefits in learning accrue from laboratory activities. However, science education research has put these expectations into perspective and questions whether meaningful learning indeed always occurs. On the other hand, engaging in DNA lab activities may provide an opportunity to raise questions about the applications of genomics research in health care, industry, etc. and about its societal implications. These issues will need to be addressed in the classroom to accomplish contemporary science education for citizenship. Ultimately, the aim would be to involve citizens/patients in clinical practice (shared decision-making). This requires interaction of academic, professional and experiential knowledge (knowing why and knowing how) and co-construction of new meanings.

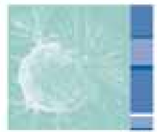
In current science education, there is a strong movement to teach science concepts in meaningful contexts, i.e. starting from its applications and implications in different practices and aiming at empowering consumers/patients and citizens of a democratic society. Yet there are still several important questions that need to be answered: If and how DNA lab initiatives can contribute to valuable lessons, i.e. engaging with the social context of genomics in schools? Which learning processes are actually taking place in terms of concept formation, value formation and acquiring opinion-forming competence, and how can these be optimized? How to adequately meet teachers' concerns and needs? How to enduringly incorporate this temporary facility in science education? And, what do genomics researchers and health care professionals learn from interaction and dialogue with teachers and students? Finally, discussions on educational innovations tend toward wishful thinking and rhetoric at the cost of theoretical underpinning and empirical testing and the DNA labs run the risk of being supply driven, technically oriented and not deeply reflected due to time pressure.

CGC investigator awards

Year	Award	Recipient
2002	1st EMBO Award for Communication in the Life Sciences	Ronald Plasterk
2004	Josephine Nefkens Prize	René Bernards
	Louis-Jeantet-Prize for Medicine	Hans Clevers
2005	Spinoza award	René Bernards
	Ernst W. Bertner Memorial Award of the University of Texas MD Anderson Cancer Centre	René Bernards
	Katharine Berkan Judd Award of the Memorial Sloan-Kettering Cancer Centre	Hans Clevers
	Prize for Science and Society	Hans Clevers
	The French Honor of 'Chevalier de la Legion d'Honneur'	Hans Clevers
	Prix Louis D. of the Institut de France	Ronald Plasterk

A recent publication in Chemisch Weekblad C2W* provides another indication of the measure of scientific esteem for CGC researchers in the Netherlands. Using a new ranking method based on the 'h-index', four CGC investigators can be found in the Top 11 of Dutch molecular scientists: Grosveld (6), Hoeijmakers (7), Bos (9) and Berns (11).

*'Wetenschappers langs een nieuwe maatlat', C2W, 11 maart 2006, p18.



Cancer | GENOMICSCENTRE
improving cure rates for cancer patients

The mission of the Cancer Genomics Centre is to improve diagnosis, therapy and cure rates for cancer patients. Genomics offers new promising opportunities for cancer research, with realistic expectations for therapy improvement in the coming decade. For instance, the genomics signature of the cancer and that of the patient may serve in the near future as a basis on which to choose the most effective therapy for the individual patient ('personalized medicine') to improve cancer patients chances of recovery and their quality of life.

Report 2002 - 2005