Analyses of Rat Tumor Models for DMBA-induced Fibrosarcoma and Spontaneous Endometrial Carcinoma

Emma Samuelson

Department of Cell and Molecular Biology - Genetics
Lundberg Institute, Faculty of Science
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Emma.Samuelson@cmb.gu.se

Department of Cell and Molecular Biology - Genetics
Lundberg Institute, Faculty of Science,
University of Gothenburg

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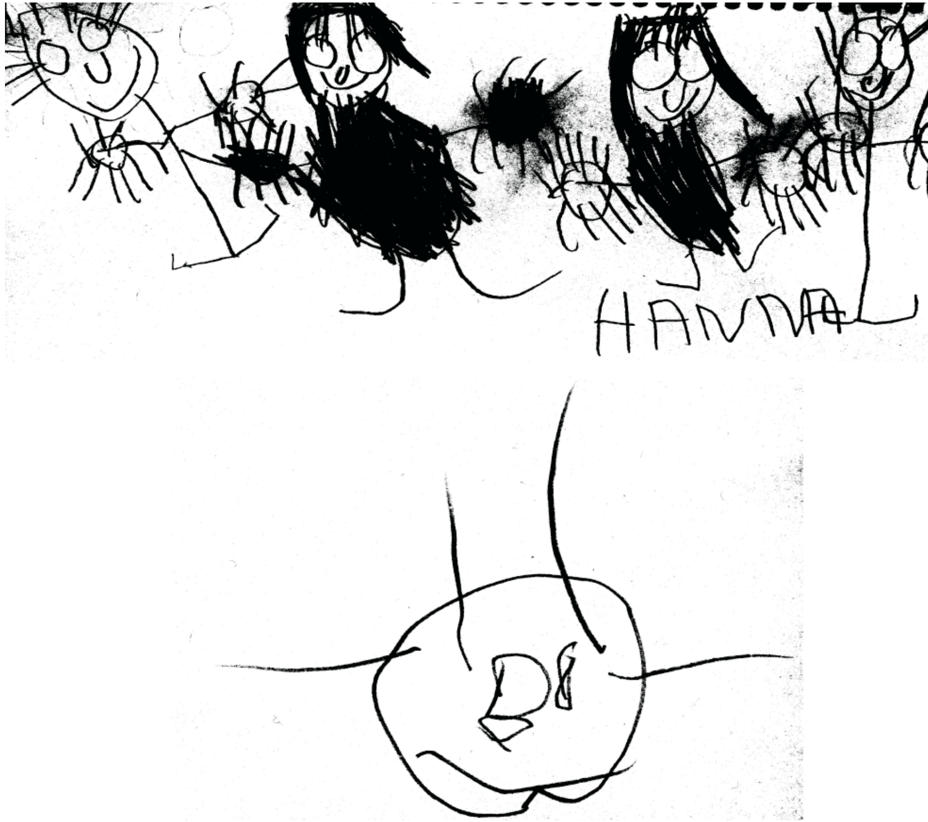
To Niklas, Hanna and Emil



"Tandem musca cacavit"

ABSTRACT

Analyses of Rat Tumor Models for DMBA-induced Fibrosarcoma and Spontaneous Endometrial Carcinoma

Emma Samuelson
Department of Cell and Molecular Biology - Genetics
Lundberg Institute, Faculty of Science,
University of Gothenburg
Göteborg, 2008.

Cancer is a disease of genes. Uncontrolled cell growth is the outcome from genetic as well as epigenetic alterations, resulting in a tumor cell mass that harbors a cancer genome. During progression, the tumor acquires self-dependence and the ability to invade other tissues and metastasize. Genetic predisposition and environmental factors such as life style, diet and exposure to carcinogenetic compounds promote initiation of tumors. The laboratory rat (*Rattus norvegicus*) has been used as an animal model in medical research for over 150 years. By using a genetically well-defined rat model in a controlled environment, we have studied two cancer models for DMBA-induced fibrosarcoma and spontaneous endometrial cancer.

In the fibrosarcoma model an F1 progeny from two inbred rat strains, BN and LE, was used and tumors were induced by a single injection of the carcinogenic agent DMBA. The tumors were used for Allelic Imbalance analysis as well as identifying putative candidate genes on chromosome1 displaying a region with gene amplification. We could successfully identify *Jak2* as a candidate gene for the amplification at the distal part of chromosome1 in one of the fibrosarcoma cell lines. Adjacent to this region on chromosome1, the Allelic Imbalance analysis displayed signs for loss of heterozygosity in the *Pten* locus. No mutation was found in the remaining allele, suggesting that *Pten* is contributing to the fibrosarcoma development in these DMBA-induced tumors by a haploinsufficient mechanism.

The endometrial tumor model is composed of the BDII rat strain, predisposed to spontaneously develop endometrial cancer. Tumors obtained from progeny from intercrosses and backcrosses between the BDII strain and two strains not prone to develop endometrial cancer, were used to classify and characterize the BDII tumors according to the human classification system. We could conclude that the BDII tumors resemble the human hormone dependent type I tumors, best. This conclusion was confirmed when we tested some of our result from the BDII model on human type I tumors in a FISH study for amplification of specific genes located on HSA2p and HSA7q. In summary, we found similar patterns of amplification in the human type I tumors as was previously found in the BDII rat tumors.

In addition, we were able to improve the rat ideogram and anchor DNA sequences (*i.e.* genes) to the physical rat gene map.

The molecular profiling of tumors at different levels, *i.e.* DNA, RNA and epigenetic, has provided an efficient tool for identifying and characterizing cancer related genes. Furthermore, the use of animal tumor models provides an important route to identify molecular biomarkers for prognosis as well as new targets for drug discovery in cancer treatment.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Hamta A*, Adamovic T*, **Samuelson E**, Helou K, Behboudi A and Levan G.
Chromosome ideograms of the laboratory rat (*Rattus norvegicus*) based on high-resolution banding, and anchoring of the cytogenetic map to the DNA sequence by FISH in sample chromosomes. *Cytogenetics and Genome Research*. 2006;115(2):158-68.

II. Sjöling Å, Lindholm H, **Samuelson E**, Yamasaki Y, Watanabe TK, Tanigami A, Levan G. Analysis of chromosomal aberrations involving chromosome 1q31-->q53 in a DMBA-induced rat fibrosarcoma cell line: amplification and overexpression of *Jak2*. *Cytogenetics and Cell Genetics*. 2001;95(3-4):202-9.

III. Sjöling Å, **Samuelson E**, Adamovic T, Behboudi A, Röhme D, Levan G. Recurrent allelic imbalance at the rat *Pten* locus in DMBA-induced fibrosarcomas. *Genes Chromosomes, and Cancer*. 2003 Jan;36(1):70-9.

IV. **Samuelson E**, Hedberg C, Nilsson S and Behboudi A. Molecular classification of spontaneous endometrial adenocarcinomas in BDII rats. 2008. *Provisionally accepted for publication in Endocrine-Related Cancer*.

V. **Samuelson E***, Levan K*, Adamovic T, Levan G and Horvath G.
Recurrent gene amplifications in human type I endometrial adenocarcinoma detected by *in situ* hybridization. *Cancer Genetics and cytogenetics*. 2008;181:25-30.

* These authors contributed equally to this work.

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Other publications not included in this thesis book:

Samuelson E, Nilsson J, Walentinsson A, Levan G, Szpirer C and Behboudi A. Absence of *Ras* mutations in DMBA-induced mammary tumors from SPRD-derived crosses. *Molecular Carcinogenesis*. 2008. In Press. (Early view available on line).

Sjöling Å, Walentinsson A, Nordlander C, Karlsson Å, Behboudi A, **Samuelson E**, Levan G and Röhme D. Assessment of allele dosage at polymorphic microsatellite loci displaying allelic imbalance in tumors by means of quantitative competitive-polymerase chain reaction. *Cancer Genetics and cytogenetics*. 2005 Mar;157(2):97-103.

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ABBREVIATIONS

aa	amino acid
AI	allelic imbalance
air	allelic imbalance ratio
BAC	bacterial artificial chromosome
BN	inbred rat strain (Brown Norway)
bp	base pair
Cdk	cyclin dependent kinase
CGH	comparative genome hybridization
CIN	chromosomal instability
cDNA	complementary deoxyribonucleic acid
DAPI	diamidino-2-phenylindole
DM	double minutes (extra chromosomal material)
DMBA	7,12-dimethylbenz[a]anthrazene
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EAC	endometrial adenocarcinoma
EC	endometrial cancer
F1	first generation of a cross, first filial
F2	second generation intercross (F1xF1)
FISH	fluorescence *in situ* hybridization
HSA	human chromosome (*Homo sapiens*)
HSR	homogeneously staining region
kb	kilo base pairs
Mb	mega base pairs
Met	hepatocyte growth factor receptor
MIN	microsatellite instability
MMU	mouse chromosome (*Mus musculus*)
NME	non-malignant endometrium
NUT	backcross generation of rat uterine tumor
N1	first backcross generation (F1xP)
PAC	P1-derived artificial chromosome
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RUT	rat uterine tumor, first and second filial
RNO	rat chromosome (*Rattus norvegicus*)
Real-time PCR	reverse transcriptase polymerase chain reaction
SKY	spectral karyotyping
SNP	single nucleotide polymorphism
SPRD-Cu3	inbred rat strain (Sprague Dawley-Curly 3)
ST	solid tumor
TC	tumor cell culture
TSG	tumor suppressor gene
WKy	inbred rat strain (Wistar Kyoto)

Genes and loci are in *italics* in the text. Genes, loci and gene products from rat are represented in, first letter UPPER CASE and the rest in lower case letters. Human genes, loci and gene products are written in UPPER CASE LETTERS in the text.

INTRODUCTION

Genetics

Genetics is the science of variations in living organisms that are transmitted through generations by the genes. The genes, organized in the chromosomes situated within the cell nucleus, are blueprints for an organism, giving production directions to the factory – the cell – mediated by the messengers (*i.e* RNA) during the transcription process. These directions instruct the cell what, how and when to produce the products, the RNA and protein, during the transcription and translation processes (Fig. 1).

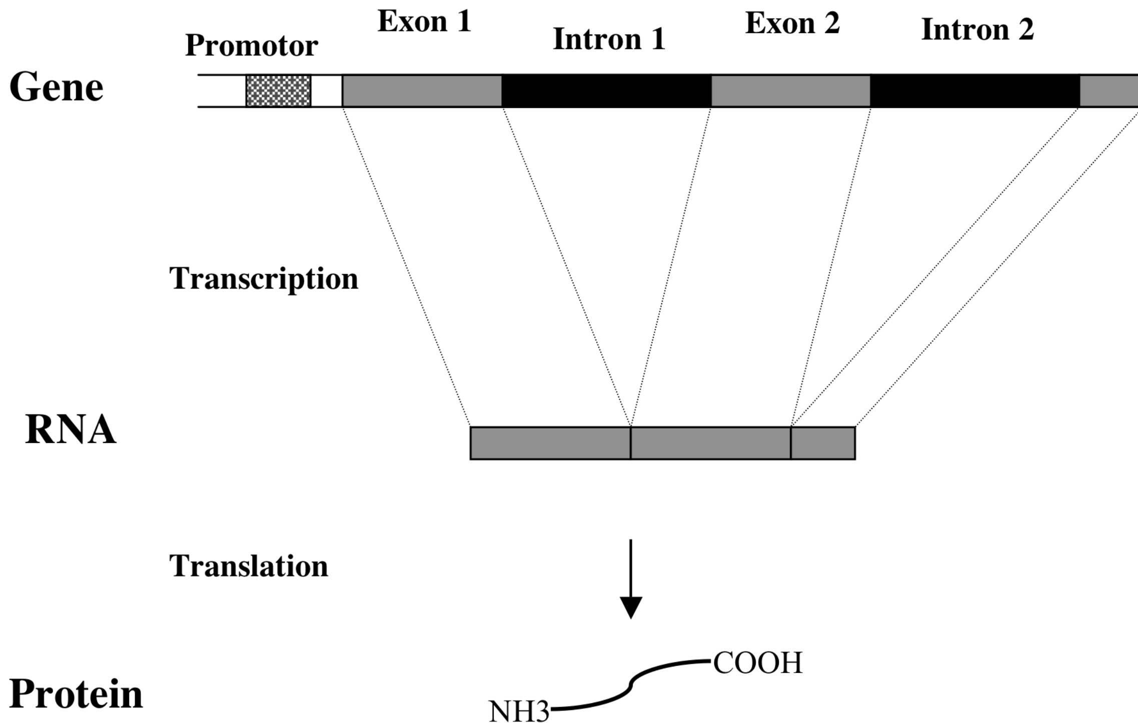

Figure 1. Schematic presentation of how information is transmitted from gene (DNA), to RNA and protein.

Each gene exists in duplicate in every diploid cell: one variant – allele – is inherited from each parent. The two alleles give a specific dosage of the product – RNA and protein – in a normal cell. An error in the gene may produce a defective product, excess or reduced amount of the product or no product at all. The consequences from these errors may result in diseases, such as cancer.

Tumor and cancer

Tumor is defined as uncontrolled growth of cells that may happen in virtually all cells and tissues of the body. Tumor classification is based on two criteria, the behavior and the cell type of origin. The behavior of the tumors is divided into two categories, benign (mostly harmless) and malignant (aggressive).Benign tumors do not spread to other tissues and organs in the body however, they may still produce negative health effects. The aggressive malignant tumors (or cancer) may invade other tissues and metastasize in distant sites.

Tumors are further classified depending on the cell of origin. A few main tumor types are:

- Carcinomas, accounting for the majority of cancers, originate from the epithelial tissues.
- Leukemia and lymphoma, develop in blood cells and lymphatic system, respectively.
- Sarcomas, originating from the connective tissues of the body (*i.e* bone, fat, muscle etc).

Cancer genes

Cancer is, in essence, a disease of genes [1]. Uncontrolled cell growth is the outcome from genetic as well as epigenetic alterations, resulting in a tumor cell mass that harbors a cancer genome. Evolution of the cancer genome is a stepwise process: more genetic damages and behavior that is more aggressive develops on the path to full malignancy [2]. The cancer genome usually contains genes with changes in copy number. Reduced number of copies is referred to as loss while increased copy number is called gain. A cell can lose maximum two gene copies, while it can gain several copies of a gene. In general, alterations in three major groups of genes are suggested to be responsible for tumorigenesis: oncogenes, tumor-suppressor genes and stability genes [1].

Oncogenes

A proto-oncogene is a gene that under normal conditions controls normal cell growth and differentiation. The proto-oncogene may become activated via a gain-of–function mutation and thus turn the proto-oncogene into an active oncogene. This activation may occur through mutational event, such as point mutation, chromosomal rearrangement or gene amplification. The activation usually confers alterations in structure and/or expression level of the oncogene [3]. Well-known examples of oncogenes are *MET,* and *MYCN*.

Tumor suppressor genes

Tumor suppressor genes (TSG) are involved in inhibition of cell proliferation and control of cell differentiation in normal cells. Loss or mutation of both alleles is usually required to inactivate a TSG through loss-of-function mutations [3]. In rare cases, when still one intact allele is retained, the single allele dosage is not enough to suppress tumorigenesis. This situation is referred to as haplo-insufficiency [4]. Well characterize examples of TSGs are *PTEN* and *TP53*.

Stability genes

Stability genes normally keep genetic alteration in a cell to a minimum and when they inactivated, mutations in other genes become more frequent. This class of genes includes the mismatch-repair (MMR), nucleotide-excision repair (NER) and basepair-excision repair (BER) genes with responsibility to repair subtle mistakes produced during normal DNA replication or from induced response to mutagens. This group of genes may also include genes that are responsible for mitotic recombination and chromosomal segregation, for example *BRCA1* and *MSH2* [5].

Genome instability

The cancer genome can contain a variety of aberrations in form of microsatellite instability (MIN) and/or chromosomal instability (CIN). CIN cancers are likely to have a poorer prognosis compared to MIN cancers, as the extent of genetic aberrations correlates with the severity of the disease [6].

Microsatellite instability

MIN gives rise to genetic instability at the nucleotide sequence level such as point mutations as well as small scale deletions or inversions [7]. The mutation rate in MIN cancer cells is two to three orders of magnitude greater than in normal cells. In these cells point mutations are

common events that may occur in both oncogenes and TSG. Often, MIN cancers retain a diploid or near-diploid chromosome content/karyotype [6].

Chromosomal instability

CIN gives rise to genetic instability at the chromosomal level, including structural alterations such as gene amplifications, translocations, large-scale deletions and inversions as well as numerical abnormalities resulting in aneuploidy [7]. CIN can accelerate the rate of loss of tumor suppressor genes through chromosomal deletion and translocation as well as cause amplification of oncogenes by duplicating the chromosomes harbouring them and thereby contribute effectively to tumorigenesis.

Gene amplification

Gene amplification is an extreme form of gain of DNA material of a specific locus or of a region containing several loci. The size of the amplified region (amplicon) can range from few kilo bases (kb) to several mega bases (Mb) of DNA [8]. Cytogenetically, these amplicons may be visible as elongated chromosomes with homogenously staining regions (HSRs), extra small chromosomal segments known as double minutes (DM), ring chromosomes (R), or as giant marker chromosomes, containing the amplified genes. HSR regions may be located at the native gene site and/or on other chromosomes. DMs are small circular stretches of DNA in the nucleus. HSRs and DMs rarely occur in the same cell. A ring chromosome (R) is a chromosomal segment whose ends have fused together to form a ring, usually larger in size compared to a DM and might contain several copies of an amplified gene. Amplification of a gene may result in an increase in the gene dosage, often leading to up-regulation of gene expression [9]. As the result, the cell can synthesize an amount of gene product exceeding normal dosage [10]. In clinical aspects, ploidy and amplification may be used as a molecular marker for prognosis as well as selection of the most appropriate therapeutic strategy. For example amplification of *MYCN* in neuroblastoma [9, 11] as well as *MYCN* and *ERBB2* in breast cancer [12], serve as prognosis factors leading to adopting suitable treatments for the diseases.

Chromosome translocation

Chromosome translocation is an abnormality caused by rearrangement and exchange of parts between non-homologous chromosomes. The translocation may be balanced (*i.e.* an even exchange of material with no genetic information extra or missing) or unbalanced (*i.e.* the exchange of chromosome material is unequal resulting in extra or missing DNA). A balanced translocation does not confer gene copy number change in the cell, however it may result in interruption of genes and/or fusion of genes, making a new one – a fusion gene. The unbalanced translocation on the other hand, confer copy number changes of the affected chromosome segment and may result in gain or loss of genes and/or whole chromosome segments contributing to tumorigenesis.

Allelic imbalance (AI)

Normally, one genome contains two alleles (*i.e.* variants of a gene) for every locus, inherited from each parent. Allelic imbalance (AI) is the altered intensity ratio between the two alleles at a (heterozygous) locus and may represent either allelic loss or gains. By using a polymorphic marker (*i.e.* a marker with different product size for each allele at the locus), in a PCR reaction, it is possible to distinguish between the two parental alleles. However, if the locus contains two identical alleles, it is not possible to discern the individual alleles. Allelic imbalance is often observed in cancer and can be an indication to why the expression level of a specific gene in a sample is changed, compared to the normal condition. An extreme situation of AI is Loss of heterozygosity (LOH) where one allele in a locus is completely lost. As mentioned earlier, deletions or other loss-of-function mutations inactivate TSGs in the tumor material. According to the Knudson's "two-hit" theory of TSG inactivation, loss or

inactivation of both alleles of a gene in a tumor suppressor locus is required for tumorigenesis to proceed [13]. In a number of typical familial cancers, inherited mutations in TSGs (the first hit) lead to highly increased probability of developing certain types of cancer. The second hit, whether in inherited or sporadic cancers, takes place at the somatic cell level and usually leads to loss/elimination of the intact allele of the TSG. The phrase "loss of heterozygosity" (LOH) is applied to genetic loci in which two distinguished alleles are present in normal tissue (locus heterozygosity), whereas only one allele is present in the tumor tissue (*i.e.* LOH) [14]. Potentially, elimination of the second TSG allele could be the result of a wide range of cytogenetic alterations, such as interstitial deletion (leading to loss of a small part of the chromosome), mitotic recombination (leading to unequal exchange of DNA between chromosomes), or non-disjunction (leading to loss of the whole chromosome). In each case, depending on the size of the affected chromosomal segment, one allele of a number of genetic markers in the vicinity of the TSG is lost/eliminated as well. Thus, detection of non-random LOH (using microsatellite allelotyping) in a certain chromosome position and tumor type may provide a practical tool to pinpoint the chromosomal localization of a TSG.

Ideogram

Successful animal studies require reliable platforms, *i.e.* a chromosomal ideogram, for information exchange between species to become possible. An ideogram is the schematic construction of the karyotype showing chromosomes with banding of different patterns and sizes for each chromosome, just as a barcode. In the laboratory, these bands are visualized from mitosis cells by using staining techniques, giving the chromosome a specific pattern. The banding patterns are unique for each chromosome of that species. The number of visible cytogenetic bands depends on the degree of chromosome condensation, where more bands give better resolution. To be able to study and identify chromosomal aberrations in a sample, an accurate and correct ideogram is needed. The ideogram is useful for interpreting biological data in a species, such as exact descriptions of the location of chromosomal breaks in CIN or specifications of the physical position of FISH signals on the chromosomes. Through FISH and with aid of an accurate ideogram, position of a gene with an estimated genetic value (centimorgan, cM) in the genetic map will have a physical base pair (bp) position as well as a cytogenetic location on the chromosome. The ideograms are also very useful tools for exchange of molecular data between species, *i.e.* to translate the findings in model systems to human disease. These aspects are discussed further in *Paper I*.

Genetic modeling of cancer

The human genome consists of approximately 21 000 different genes. In addition, each gene has allelic variants, making the human genome extremely complex [15]. This heterogeneous genetic composition of the human population that additionally is under influence of diverse external environmental factors, make identification of genes underlying human polygenic traits very difficult. By using a genetically well-defined animal model, these obstacles may be well circumvented. The laboratory rat (*Rattus norvegicus*) has been used as an animal model for physiology, pharmacology, toxicology, nutrition, behavior, immunology and neoplasia for over 150 years [16, 17]. Today, some 500 inbred rat strains have been developed for a wide range of biochemical and physiological phenotypes and different disease models [16]. In an inbred strain all animals have the same genetic composition. In essence each member of the strain is an “identical twin” of every other member of the strain. This fact, combined with the similarity in physiology and hormone responsiveness patterns between humans and rats at the organ level makes the rat an excellent model for human cancer genetic investigations [18]. By using evolutionary conservation of gene segments as a guide, animal models are powerful tools to discover pathways and genes involved in tumorigenesis in cancer. Since these types

of genes are generally strongly conserved between species, the findings in model systems can easily be to translated to human disease through comparative mapping and analysis of homologous genomic segments [19].

The DMBA-induced fibrosarcoma model

Various chemical compounds can be used to accelerate tumor development in rodent models for cancer. Some rat strains show inherited susceptibility for spontaneously developing tumors, while others may be well suited for induction of tumors by a specific carcinogenic agent. In rat models, the carcinogenic compound 7,12-dimethylbenz[a]anthrazene (DMBA) is frequently used to induce tumors. DMBA belongs to the group of polycyclic aromatic hydrocarbons, which are commonly found in cigarette smoke. DMBA interacts directly with the DNA and provokes A to T and, to a lesser extent, A to G base substitutions [20, 21]. Depending on the mode of administration, DMBA may induce different types of tumors. For instance, papillomas and squamous cell carcinomas arise when DMBA is applied to the skin of mouse [20]. In rats, leukemia develops when DMBA is administrated by intravenous pulse injections [22], oral administration will give rise to mammary tumors [23] and fibrosarcomas with various degree of differentiation, develops when DMBA is injected subcutaneously in the limbs [24]. Previous studies of DMBA-induced rat fibrosarcomas revealed pattern of recurrent chromosomal changes, including trisomi RNO2, gain of the proximal part of RNO4 and frequent Allelic Imbalance in RNO1 [25, 26]. In the present thesis, RNO1 genetic aberrations were selected for a more detailed analysis in a subset of tumors and the results are presented in *Papers II* and *III*.

Endometrial carcinoma

The endometrium consists of a single layer of columnar epithelium, resting on a layer of connective tissue. The endometrium functions as a lining for the uterus, preventing adhesions between the opposed walls of the myometrium and maintaining the patency of the uterine cavity. During the menstrual cycle or estrous cycle, the endometrium grows to a thick, blood vessel-rich, and glandular tissue layer. This represents an optimal environment for the implantation of blastocysts upon its arrival in the uterus at time for implantation, resulting in pregnancy.

Human endometrial carcinoma (EC) also called corpus cancer of the uterus, is ranked fourth among invasive tumors in women. In Sweden, some 1300 women are diagnosed annually with this disease [27]. EC is often divided in two groups, type I and type II. Type I tumors are of endometrioid type and represent 75-80% of all EC, including endometrial adenocarcinoma (EAC). These tumors are well differentiated at low-stage and follow the estrogen-dependent pathway. Type II tumors account for approximately 10-20% of the cases, are of non-endometroid type, usually poorly differentiated and follow the estrogen-unrelated pathway [28, 29]. However, in many cases it is not always easy to discern these subtypes. In fact, there is evidence for yet another, large group of endometrial carcinomas with overlapping clinical and molecular features of types I and II. In addition, it has been clearly demonstrated that genetic predisposition is of critical importance in at least 5% of the endometrial cancers [30-32]. Familial clustering of endometrial carcinoma is reported to be the most common extra colonic malignancy in hereditary non-polyposis colorectal cancer (HNPCC), a multi organ cancer syndrome with mismatch repair (MMR) deficiency.

When a woman is diagnosed with EC, the standard procedure is to remove the tumor surgically. .Her case is referred to one of the risk groups for further treatment based on prognostic factors such as the tumors stage, grade, DNA-ploidy status and tumor type (I or II) [33]. To find the most suitable treatment for a patient, it is urgent to classify the tumor correctly. Today, the available molecular tools for this purpose have proven to be inadequate. Growing knowledge of biological processes behind the development of EC would certainly

improve molecular classification of the tumors and thus aid finding specific targets for developing new treatment procedures. Identification of molecular biomarkers for the disease will additionally allow early detection, provide a more specific diagnosis and prognosis as well as predict clinical outcome (response to therapies) [19, 34].

The BDII/Han rat model for human endometrial cancer

The BDII/Han inbred rat strain (hereafter BDII) is highly prone to spontaneously develop endometrial carcinomas, often of the endometrial adenocarcinoma (EAC) subtype. More than 90% of virgin females develop this tumor within two years of age (average life time for a laboratory rat is three to four years). EAC is the only early-onset tumor, indicating a genetic hereditary component behind the development of this cancer type in BDII rats. Since no tumors will develop after ovariectomization, development of EAC in BDII rats is considered to be hormone (estrogen) dependent [35]. In this respect, the inbred BDII rat represents a unique model for human hormone-related (type I) endometrial carcinogenesis [28]. Roshani and co-workers found three chromosomal regions implying susceptibility for developing endometrial adenocarcinoma [36, 37], in crossbreeding experiments of female BDII rats with males of two strains (BN and SPRD-Cu3) with a low incidence of endometrial adenocarcinoma. Interestingly, the genes affecting susceptibility to endometrial adenocarcinoma were different in the two crosses, suggesting that genes behind the susceptibility in BDII animals may interact with various genes in different genetic backgrounds. Data in the literature suggest that this feature of heritability of endometrial carcinogenesis in BDII rats is shared by at least two forms of human endometrial cancer [30, 32, 38]. To determine if this heritability of endometrial carcinogenesis resembles the heritable phenotype in human or other subtypes of human endometrial carcinomas, the work presented in *Paper IV* was designed. In this paper, the molecular template used for classification of human tumors, was used for molecular analysis of tumors developed in the BDII rats. We were able to provide systematic molecular data for endometrial adenocarcinogenesis in this tumor model.

Finally, previous CGH analysis suggested that amplifications of two independent amplified regions in the proximal part of RNO4 were common among tumors. Analysis of the amplified regions revealed highest levels of amplification of *Cdk6* (cyclin dependent kinase 6) and *Met* (hepatocyte growth factor receptor) among the genes tested [39, 40]. These data suggested that up-regulation of *Cdk6* and/or *Met* may contribute to endometrial cancinogenesis in BDII rats. Furthermore, RNO6 amplification was recurrently found among EAC tumors. Eight cancer-related genes located in this region, including the *MycN* proto-oncogene, apolipoprotein B, the DEAD box gene, ornithine decarboxylase, proopiomelanocortin, ribonucleotide reductase, M2 polypeptide and syndecan were selected for further study These investigations suggested *MycN* amplifications to be a common event among BDII rat EAC tumors [41, 42]. CGH analysis of human type I endometrial carcinomas suggested chromosomal segments homologous to RNO4 and 6 were similarly amplified in a number of human tumors [43]. To investigate whether the observed amplified genes in the rat model were also targets for chromosomal amplifications in human endometrial carcinomas, the work presented in *Paper V* was designed.

Cancer genetics, a tool in diagnosis, prognosis and therapy

Today, several hundred subtypes of cancer are known, developing from virtually every tissue of the body [44], often with several different subtypes within each tissue and/or organ. Depending on clinical and molecular features of the tumor, different treatment strategies may be chosen. Mode of tumor development and its aggressiveness as well as tumor response to the treatment (clinical outcome) vary significantly among different tumor types and individuals. Traditionally, classification of tumors relies on histopathological features.

However, this system does not discriminate between tumor types within the same histopathological group, which may potentially display different clinical outcomes. Thus, pointing to an urgent need for more accurate and stringent methods for tumor classification. Molecular profiling of tumors at different levels, *i.e*. DNA, RNA and epigenetic, has provided an efficient tool for subcategorizing tumors within the same tumor type. Furthermore, this approach provides an important route to identify molecular biomarkers for prognosis as well as to identify new targets for drug discovery in cancer treatment (discussed and reviewed in [45-47]). Thus, animal tumor models provide useful tools for these purposes.

AIMS

The overall objective of this thesis that involves cytogenetic and molecular cancer genetic analyses was to characterize the molecular mechanisms involved in human fibrosarcomas and endometrial carcinoma using unique rat models as tools.

Specific aims are:

- To produce an improved high-density ideogram for rat to aid anchoring the genetic map to the physical map. *Paper I*.
- To investigate the involvement of the cancer-related genes *Jak2* and *Omp* in the development of DMBA-induced fibrosarcomas. *Paper II*.
- To map the tumor suppressor gene, *Pten* in the rat genome and to investigate the involvement of this tumor suppressor gene in DMBA-induced fibrosarcomas using allelic imbalance analysis. *Paper III*.
- To increase the density of the physical and genetic map on RNO1q22-q53, using gene specific PAC clones for FISH mapping. *Papers II* and *III*.
- To determine features of molecular alterations in EAC developed in the BDII rat model, using the molecular classification system for human endometrial cancer. *Paper IV*.
- To translate some of the findings in the BDII rat model to human endometrial cancer. *Paper V*.

EXPERIMENTAL BACKGROUND

The material and methods are described in detail in the papers and are only briefly explained here.

Material

DMBA-induced fibrosarcoma (Papers II and III)

Tumors were induced by a single subcutaneous injection of DMBA into the thigh of F1 animals from the two reciprocal crosses, BNxLE and LExBN (each animal designated BL or LB and a number, respectively). Tumors were surgically removed when they reached the size of 0.5 cm in diameter (Table 1). DNA was extracted from the solid tumors and the livers using standard phenol/chloroform procedures. Cell cultures were established from solid tumor and used for chromosome analysis, as well as for DNA and RNA extraction and cDNA preparation.

Table 1. Tumor material used *in Paper II* and *III*.

Tumor name	Cross and progeny	Average Chr. No	Paper
BL150	(BNxLE)F1	52	II, III
BL151	(BNxLE)F1	86	II, III
BL153	(BNxLE)F1	ND	II, III
BL170	(BNxLE)F1	49	II, III
BL172	(BNxLE)F1	62	II, III
LB10	(LExBN)F1	71	II, III
LB20	(LExBN)F1	87	II, III
LB30	(LExBN)F1	ND	II, III
LB31	(LExBN)F1	93	II, III
LB32	(LExBN)F1	47	II, III
LB130	(LExBN)F1	42	II, III
LB131	(LExBN)F1	54	II, III
LB132	(LExBN)F1	ND	II, III
LB133	(LExBN)F1	121	II, III
LB135	(LExBN)F1	64	II, III
LB136	(LExBN)F1	103	II, III
LB137	(LExBN)F1	43	II, III
LB140	(LExBN)F1	75	II, III
LB142	(LExBN)F1	91	II, III

ND, not determined

BDII rat EAC tumors (papers IV and V)

BDII females, prone to endometrial carcinoma (EC), were mated to males from two different strains not prone to developing EC (SPRD-Cu3/Han and BN/Han). Brother-sister mating of F1 animals was performed in order to generate F2 offspring. In addition, F1 males were backcrossed to BDII females to generate a backcross (N1) population (Table 2). The animals were maintained in a specific pathogen-free environment and were examined weekly for signs of tumor formation. After two years, or in case of suspected tumor, the animals were killed and subjected to necropsy. Tumors and tissues were removed surgically.

Table 2. Tumor cell cultures used *in paper IV*.

Sample name	Tumor or tissue type	Cross (progeny)	Chromosome No.	Paper
NUT6	EAC	BN (N1)	60	IV
NUT31	EAC	BN (N1)	67	IV
NUT43	EAC	BN(N1)	46	IV
NUT46	EAC	BN (N1)	58	IV
NUT50	EAC	BN (N1)	66	IV
NUT51	EAC	BN (N1)	71	IV
NUT52	EAC	BN (N1)	42	IV
NUT76	EAC	BN (N1)	42	IV
NUT81	EAC	BN (N1)	42	IV
NUT82	EAC	BN (N1)	84	IV
NUT97	EAC	BN (N1)	52	IV
NUT98	EAC	BN (N1)	52	IV
NUT99	EAC	BN (N1)	67	IV
NUT100	EAC	BN (N1)	67	IV
NUT127	EAC	BN (F1)	70	IV
NUT128	EAC	BN (N1)	64	IV
RUT12	EAC	BN (F1)	51	IV
RUT30	EAC	BN (F2)	61	IV
NUT4	EAC	SPRD (N1)	62	IV
NUT7	EAC	SPRD (N1)	46	IV
NUT12	EAC	SPRD (N1)	64	IV
NUT14	EAC	SPRD (N1)	42	IV
NUT39	EAC	SPRD (N1)	39	IV
NUT42	EAC	SPRD (N1)	44	IV
NUT47	EAC	SPRD (N1)	48	IV
NUT84	EAC	SPRD (N1)	42	IV
RUT2	EAC	SPRD (F1)	40	IV
RUT6	EAC	SPRD (F2)	58	IV
RUT13	EAC	SPRD (F2)	62	IV
NUT122	NME	BN (N1)	ND	IV
NUT123	NME	BN (N1)	ND	IV
NUT129	NME	BN (N1)	ND	IV
NUT18	NME	SPRD (N1)	ND	IV
NUT118	NME	SPRD (N1)	ND	IV
NUT58	NME	SPRD (N1)	ND	IV

EAC, endometrial adenocarcinoma; NME, non-malignant endometrium; F1, first filial; F2 second filial; N1, backcross; BN, SPRD are inbred rat strains; ND, not determined.

The histo-pathlogical investigation of the rat EC tumors revealed mainly endometrial adenocarcinomas and a few samples of other malignant type. In some cases however, no malignant cells were detected in the removed tissues when pathologically characterized. They are referred to as non-malignant endometrium (NME) in this thesis. Spectral karyotyping analysis revealed only minor numerical chromosomal changes in these samples (unpublished data). Normal tissue samples from liver were collected from all animals for DNA extraction.

Primary tumor cell cultures were developed from a subset of fresh NME and EC tumors. DNA was extracted from NME, tumors, tumor cell cultures and livers. Good quality total RNA was extracted from cultured rat embryonic cells, and tumor cell cultures; subsequently cDNA was synthesized according to standard procedures after DNaseI treatment of the RNA.

Human type I EC tumors (Paper VI)
Human type I endometroid carcinomas (EC), were surgically removed at primary surgery and stored at -80°C. All tumors were classified as EC by the same pathologist and according to the regional treatment guidelines. Any cases of endometrial hyperplasia were excluded and only samples with normal/tumor cell ratio content below 50% were accepted for further analysis. Tumors were from various stages and grades (described in *paper VI*). A panel of 13 tumors (Table 3) was selected for analysis. The median age of the patients was 69 years (ranging from 42 to 89 years) and the median follow-up time was 7.5 years (range 5 to 11 years).

Table 3. Human type I endometrial solid tumors used in *Paper VI*.

Patient/ tumor designation	**Stage**	**Differentiation**	**DNA Index**
301	Ia	poor	2,52
396	IIb	poor	1,16
1265	IIb	moderate	1,09
683	IIa	moderate	1,00
1138	Ib	well	1,00
292	Ia	poor	1,93
649	IVb	poor	1,68
1123	Ib	well	1,09
146	Ia	moderate	1,00
326	IIb	poor	0,92
54	Ib	poor	2,42
816	Ic	poor	1,83
183	Ib	moderate	0,68

Methods

Chromosome preparations and G-banding (Papers I and VI)
For normal rat chromosome slides, cell cultures from rat embryo fibroblasts (RE) were set up according to standard procedures. Banded metaphase and prometaphase spreads were prepared from early passages. The slides were stained with Giemsa and karyotyped.
For normal human chromosome metaphase slides, blood from healthy donors was cultured and metaphase spreads were produced according to standard air-dry technique.

Cytogenetic analysis (karyotyping) (Papers I and VI)
A cytogenetic analysis gives an overview of the chromosomes in a sample; it can detect large structural rearrangements as well as numerical aberrations. The karyotype is the observed characteristics (number, type, shape, banding pattern etc) of the chromosomes of an individual. All normal somatic cells from the same species exhibit identical diploid

karyotypes. Aneuploidy is a change in the normal number of chromosomes. Syndromes, such as Downs Syndrome (trisomi 21) in humans, displaying an abnormal (aneuploid) karyotype can be identified by a cytogenetic analysis. Further, gross chromosomal abnormality and translocations may be detected in this analysis.

Tumor imprints (Paper V)

Tumor imprints is a technique were imprints containing interphase nuclei from the solid tumor obtained. Fresh frozen tumor tissues were pressed to a clean glass slide. The cell material was then fixed in Carnoy´s fixative, dried in ethanol series and stored in absolute ethanol, at -20°C. The slides were treated according to standard procedures before FISH. The imprint method is rapid and simple and gives very clear and specific signals in analysis. However, it is only possible to detect numerical information from this platform and detection of structural aberrations or organization and order of the genes would be difficult with this technique.

Fluorescence in situ Hybridization (Papers I, II, and V)

Fluorescence *in situ* Hybridization (FISH) is a technique in which fluorescence labeled probes hybridized with specific DNA structures, such as chromosomes or cell nuclei fixed on slides (Fig. 2). The probes consist of DNA sequences, genes, segments of chromosomes, or the whole specific chromosomes (*i.e.* chromosome paint). The resolution of FISH depends on the hybridization target. The less condensed target the higher the resolution: resolution of 1-3 Mb for metaphase chromosomes, 0.5-1 Mb for prometaphase chromosomes, 50kb-1Mb for resting nuclei (interphase) and 1-500kb for free chromatin fibers. By using dual-color FISH, it is possible to determine the position or organization of several probes simultaneously. FISH is often used for physical mapping, to determine gene order and copy number, characterize gene amplification as well as organization of genes in amplified regions. Furthermore, in chromosome paint, one chromosome is labeled with a fluorochrome and used as probe in FISH to detect single chromosome aberrations. Using multi-fluorochrome technique to label all chromosomes in a sample, it is possible to detect aberrations in all chromosomes simultaneously and in one round of hybridization. This is referred to as combinatorial labeling and the technique is called spectral karyotyping (SKY). SKY has the advantage to discover hidden structural translocations undetectable with conventional cytogenetics in regions with similar banding patterns.

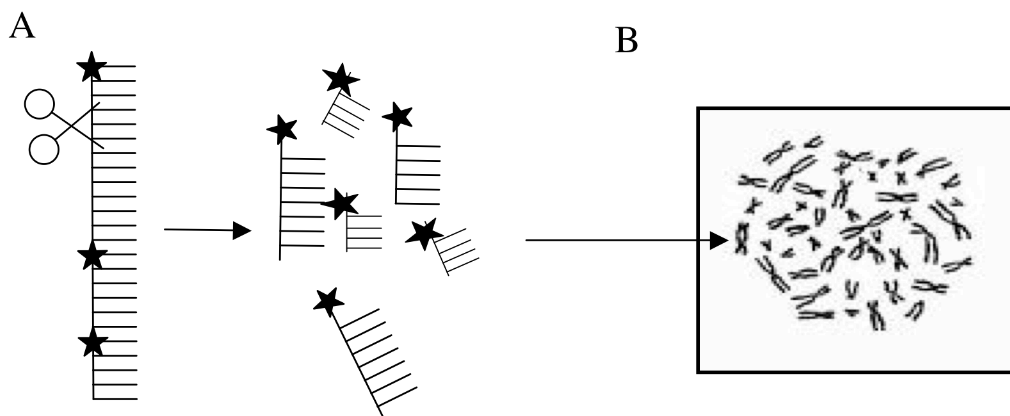

Figure 2. Schematic presentation of FISH. **A.** The probe DNA is labeled by polymerase with a fluorescent dye, and cleaved by DNase enzyme into smaller pieces of labeled DNA. **B**. The labeled probe is hybridized to the target DNA. Here the target is metaphase chromosomes.

Southern Blot (Paper II)
Amplification of genomic DNA may be detected by southern Blot, where DNA is digested with restriction enzyme (*i.e*. EcoRI) and the DNA fragments are separated on an agaorose gel with a DNA size standard. The DNA is transferred onto a nylon filter and radioactively labeled probes, consisting of specific DNA from the gene of interest, are allowed to hybridize to the filter. The filters are then exposed to X-ray films and the films developed using standard photographic procedures and analyzed. This method reveals a copy number approximation if a normal sample (*i.e* with two gene copies) is included.

Rat PAC filter screening (Paper II)
Rat PAC filters from the RPCI-31 rat PAC library were screened using radioactively labeled gene-specific PCR products as probes. PAC clones giving positive signals according to the PAC filter key were cultured and purified according to the manufacturers instructions (BAC-PAC Resources, Roswell Park Memorial Institute, Buffalo, NY USA). Each PAC clone was subjected to PCR and the PCR product from each PAC clone was sequenced in both forward and reverse direction to verify that the PAC clone contained the specific gene.

Polymerase chain reaction (Papers II, III, IV and V)
Polymerase chain reaction (PCR) is one of the most frequently used techniques in molecular research today. This method allows exclusive amplification of a defined target DNA sequence using flanking specific primers complementary to each DNA strand. To achieve this selective amplification, knowledge of the target sequence is necessary. The primers come in pairs and are 20-25bp long oligonucleotide sequences. Depending on how the primers are designed (i.e. in exon or intron sequences) it is possible to amplify targets in genomic DNA and/or cDNA. The method works by an initially denaturation step of the DNA, annealing of primers to the single stranded DNA complementary sequence and elongation of the primers to build a new DNA strand by copying the complementary strand. This procedure is repeated for several cycles. Since the newly produced DNA strands will function as a new target for the primers in subsequent cycles, the reaction is exponential. The enzyme working in the reaction is the thermostable DNA Taq polymerase, which originates from microorganisms originally found in hot springs. Eventually, enough PCR productsare generated to be visual on an agarose gel stained with ethidium bromide. Several applications are available to this technique by modifying specificity of the primers to the target sequence and optimization of reaction temperatures.

Expand PCR (Paper III)
A standard PCR reaction supplemented with 1 x Expand Long template PCR buffer was used to amplify the intron 8 sequence of the rat *Pten* gene together with the flanking sequences, exon 8 and exon 9. This method is used to amplify extra long segments.

Reverse transcriptase PCR (RT-PCR) (Papers II and III)
Total RNA was extracted from the fibrosarcoma tumor cell cultures or from cultured rat embryo cells. The enzyme Reverse transcriptase makes it possible to produce complementary DNA copies (cDNA) of the RNA, which is more stable than RNA. cDNA was made using random priming in RT-PCR according to the manufacturers instructions (Invitrogen). The relative levels of expression of candidate genes (i.e. *Jak2*, *Pten* and *Omp*) were measured in co-amplification PCR with *β-actin* (comparative quantitative PCR), visualized on an agarose gel together with a DNA size marker. This method gives an approximation of the expression level of the target gene compared to the reference gene within the same PCR reaction (multiplex PCR). The reference gene postulated to be expressed at a normal level.

Real-Time quantitative PCR (Paper IV)
Quantitative Real-Time PCR is a technique used for simultaneous amplification and quantification of targeted (often) cDNA molecules in a sample. When normalized to cDNA input or additional normalizing genes (often housekeeping genes) in a cDNA sample, it is possible to determine the absolute copy number or relative amount of a specific sequence (gene) in a test sample. This method is frequently used to determine and compare the expression levels of specific genes in two or more diverse groups of samples.
The TaqMan system was the first quantitative Real-time PCR assay developed. This system based on dual labeled fluorogenic oligonucleotide probes that emit a fluorescent signal upon cleavage between the reporter (F) and the quencher (Q) dyes, based on the principle of fluorescence resonance energy transfer [48]. Taq polymerse has, apart from its polymerase activity, also a 5´-3´exonuclease activity. Consequently, since the Taq polymerase is cleaving a probe in every PCR cycle, it is possible to measure the PCR product in every cycle and thereby quantify the amount of PCR product after finalized amplification, which in turn is a measurement of the expression of the specific gene (Fig. 3). In every PCR cycle, an increase in fluorescence intensity proportional to the amount of PCR product is obtained.

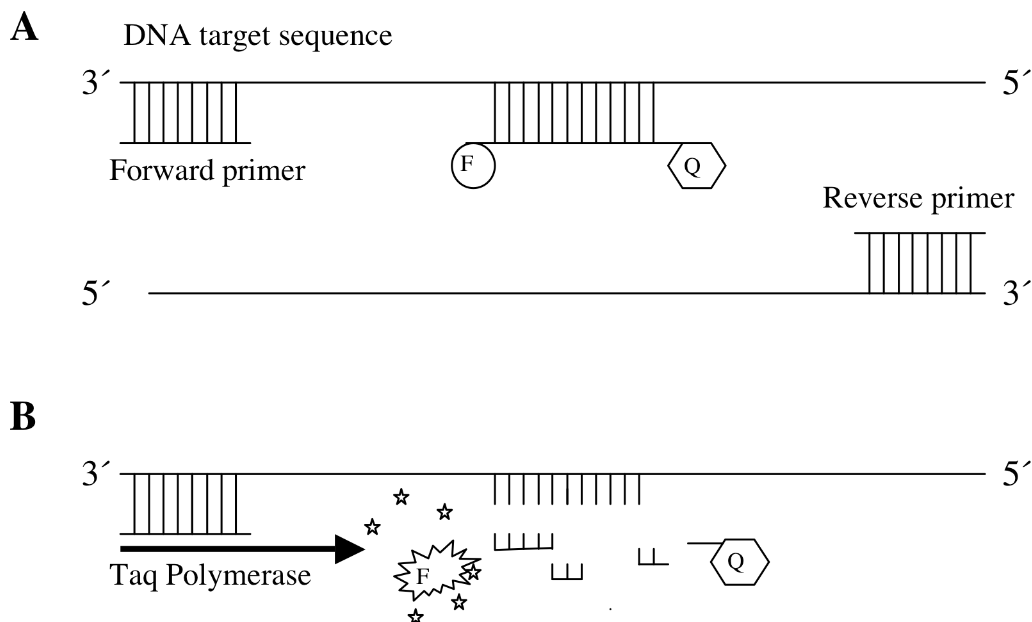

Figure 3. Schematic presentation of the TaqMan principle. **A**. Primers and probe anneal to the target sequence of the gene. **B**. When the Taq polymerase cleaves the probe by 5´-3´-exonuclease activity, fluorescence emission occurs (only one strand is shown). F, fluorophore; Q, quencher.

In paper *IV* we used a cDNA plate consisting of cDNA from EC and NME samples as well as a serial dilutions of commercial available control rat RNA for production of the standard curve to be amplified with every TaqMan gene assay. Furthermore, we used an internal reference gene (*Rps9*) for normalization in each quantitative Real-time PCR analysis.

Analysis of Real-Time PCR data (Paper IV)
Each sample was PCR amplified and analyzed in triplicate to account for any possible differences between wells in the plate, due to, for instance, laboratory handling (*i.e.* pipetting, etc). The fluorescence emission measured continuously during the PCR amplification (ΔRn = fluorescence increase after the subtraction of the fluorescence background) and was plotted against the PCR cycle number. In this diagram, the threshold line is set at the exponential

phase of the amplification curve. From the plot it is possible to read out the C_T value (threshold cycle), which is the cycle value obtained for each sample from the point where the threshold line crosses the amplification curve (Fig. 4). A high C_T value is indicative of low level of transcription from the targeted gene.

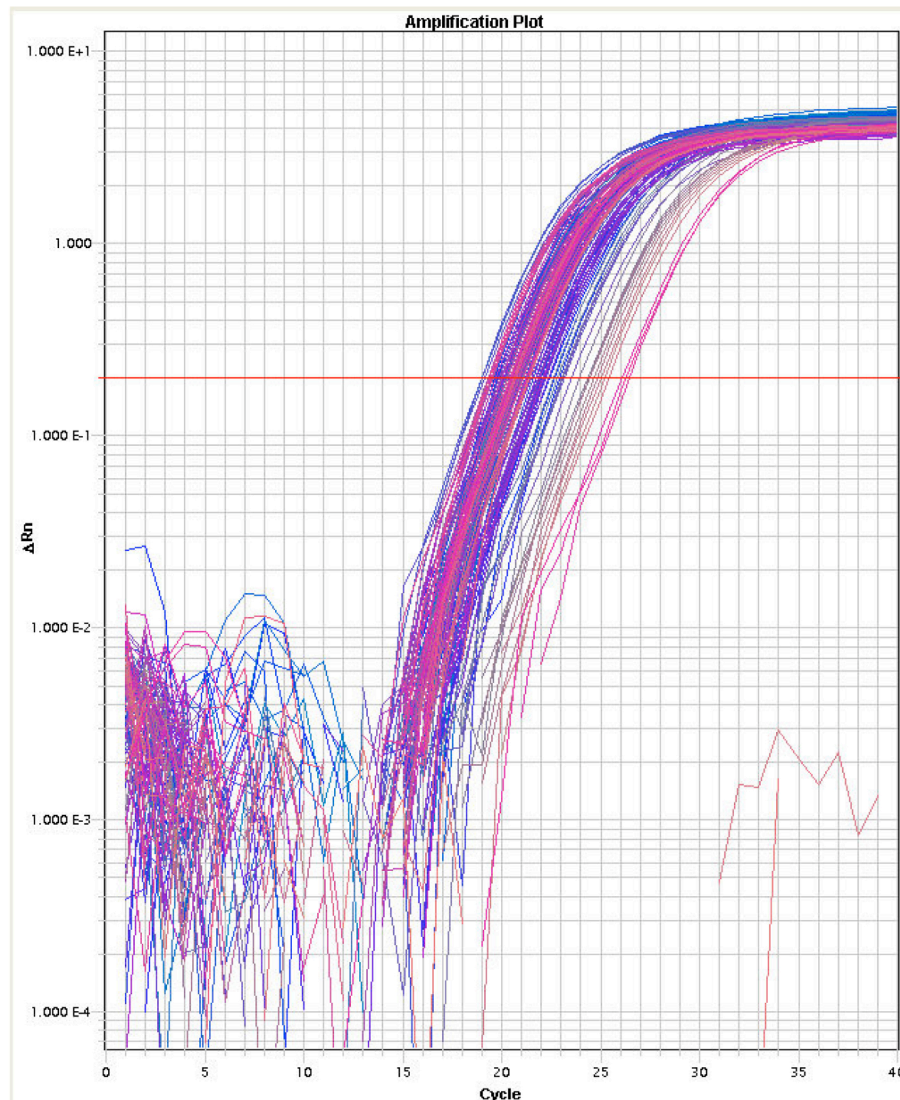

Figure 4. Real-time PCR amplification plot. Increased fluorescence emission is plotted against the cycle number. The threshold line (red vertical line) is set in the exponential phase of the fluorescence emission curves.

Quantification and normalization (Paper IV)

To calculate the relative quantification of gene expression, we used the Relative Standard Curve Method. The standard curve is used to determine the efficiency of the PCR amplification in both the target gene and the internal control sample. The standard curve is set up by a serial dilution of a reference RNA/cDNA. The serial dilution must be accurate while the absolute amount of the reference RNA/cDNA is not required. The amounts of targets and gene products are determined from the standard curve in each experiment.

In this project, a standard curve was prepared in each PCR assay for all genes using serial dilutions (1:1, 1:3, 1:9, 1:18, 1:36, and 1:72) of a calibrator cDNA (obtained from rat reference RNA, Stratagene, La Jolla, CA). For each gene, the mean C_T-value for triplicates or

duplicates was calculated, and the gene concentration (or gene copy number) of test samples was determined, based on the standard curves.
Correction for experimental variations, such as different amount of starting RNA was done by normalization to a housekeeping gene, *Rps9*. The target amount is divided by the reference gene (*Rps9*) amount to generate a normalized target value.

Statistical analysis (Paper IV)
Welch's t-test
This test is similar to an ordinary two-sample t-test, but allows for unequal variances in the two samples. Here, it is used to compare the gene expression levels in two groups, NME and EC. Since expression levels have a positively skewed distribution the levels are first log transformed and the Welch's statistic is applied on $\log_{10}$ transformed levels. This implicitly means that expression levels are assumed to have a lognormal distribution. Individual expression levels are presented as fold changes, calculated as the ratio between the expression level and the geometric mean of expression levels in the NME group.

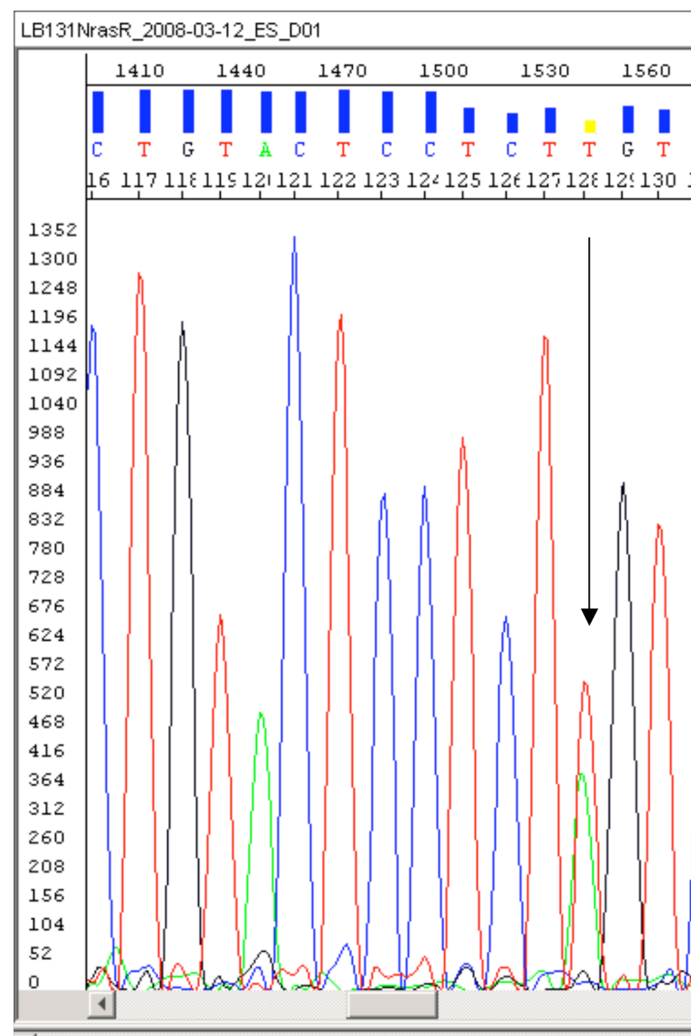

Figure 5. Partial DNA sequence as a result from Sanger´s direct sequencing method. This sequence was obtained in a tumor sample and a heterozygous mutation, A/T was detected (arrow).

DNA sequencing (Papers III and IV)

DNA sequencing is a method where the base composition of a specific DNA segment is determined. Often, the DNA sequencing is preceded by PCR amplification of the sequencing target. A frequently used method for DNA sequencing is the Sanger's direct sequencing method [49], which also referred to as dideoxy sequencing or chain termination. It is based on the use of dideoxynucleotides (ddNTP's) in addition to the normal nucleotides (NTP's) found in DNA. Dideoxynucleotides are essentially the same as nucleotides except they contain a hydrogen group on the 3´ carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides, because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide and thus the DNA chain is terminated [49]. The pool of terminated DNA fragments are separated by electrophoresis and ordered by size, the fluorescent-labeled dideoxynucleotides are scored by a laser beam and analyzed by the computer software to complete the DNA sequence. Reactions are performed in each direction (*i.e.* forward and reverse) and are analyzed separately to conform the final sequence. This method can be used to search for SNP or single base pair substitutions (Fig. 5), large deletions, or frame shift mutations.

In a simple experimental design, we could show that this method is capable of detecting mutations/polymorphisms even when present in only 25% of the test sample (Fig. 6) [50].

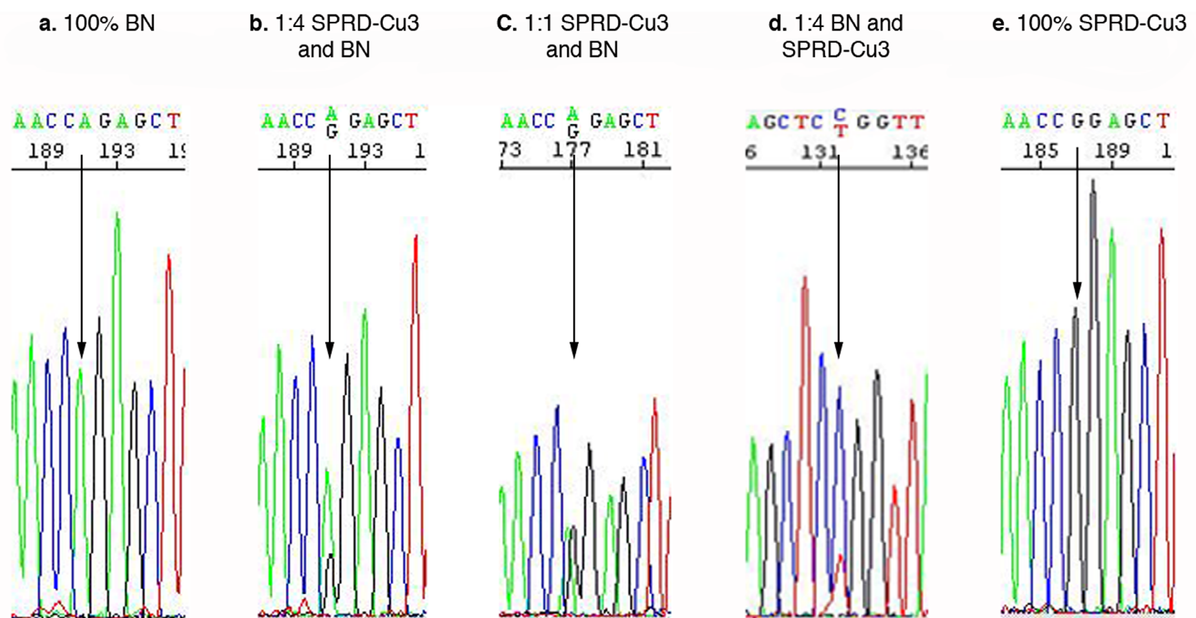

Figure 6 . Sensitivity test of the direct sequencing method [50]. BN and SPRD-Cu3 rat strains show polymorphism for a single nucleotide (SNP). We used different mixtures of normal DNA from BN and SPRD-Cu3 rats as template for PCR amplification of a segment of genomic DNA containing the SNP and sequenced the fragments in both forward and reverse directions. The result showed that SNP was detectable/visible even in a mixture of 1:4 of the two DNA samples equally good with both forward and reverse primers (see the peaks in b and d). (a) pure BN DNA; (b) 1:4 mixture of SPRD-Cu3 and BN DNA; (c) 1:1 mixture of SPRD-Cu3 and BN DNA; (d) 1:4 mixture of BN and SPRD-Cu3 DNA; (e) pure SPRD-Cu3 DNA. Note that in (d) the sequencing result from the reverse primer is shown .

Constructing a linkage map (Paper II)

Normal DNA from a set of 32 (BNxLE)F2 animals was genotyped for microsatellite markers located on RNO1, including the D1Lev1 *Pten* intragenic marker. The alleles present in each

animal from each marker were determined after the PCR products had been separated on PAGE. The programs Genescan and Genotyper (PE, Applied Biosystems) were used to calculate peak size, area and height of each allele product. The linkage map was constructed using JoinMap version 2.2 [51]. This program constructs a linkage map based on the input data and group the markers accordingly. Care was taken to ensure that each marker was placed in the most appropiate position according to previous linkage maps.

Analysis of allelic imbalance (Papers III and IV)

Allelic Imbalance (AI) analysis using DNA markers in PCR amplification is based on polymorphism between the parental strains. An F1 cross is ideal since the animals are isogenic and heterozygous for all polymorphic loci, making them well suited for AI analysis. Backcrosses and F2 crosses may be used for AI as well, however, in these cases not all marker loci will be heterozygous.

To search for allelic imbalance of the *Pten* gene we used informative polymorphic markers inside and in the vicinity of the *Pten* locus. DNA from paired tumors and normal liver were used as template in fluorescent PCR reactions with the polymorphic markers. The PCR products were separated on polyacrylamide gel electrophoresis (PAGE) and the allele peaks were analyzed using Genescan and Genotyper softwares (PE, Applied Biosystems, Foster City, CS, USA) as well as by visual inspection. The area of the peaks was used to calculate the Allelic Imbalance Ratio (AIR), using the equation (T1/T2)/(N1/N2), where T1 is one tumor allele and T2 is the other tumor allele. N1 and N2 corresponds to the two normal alleles from the liver DNA. If the AIR value was above 1, the inverted value was used. A ratio below 0.65 was considered indicative of loss or gain of allelic material (Fig. 7).

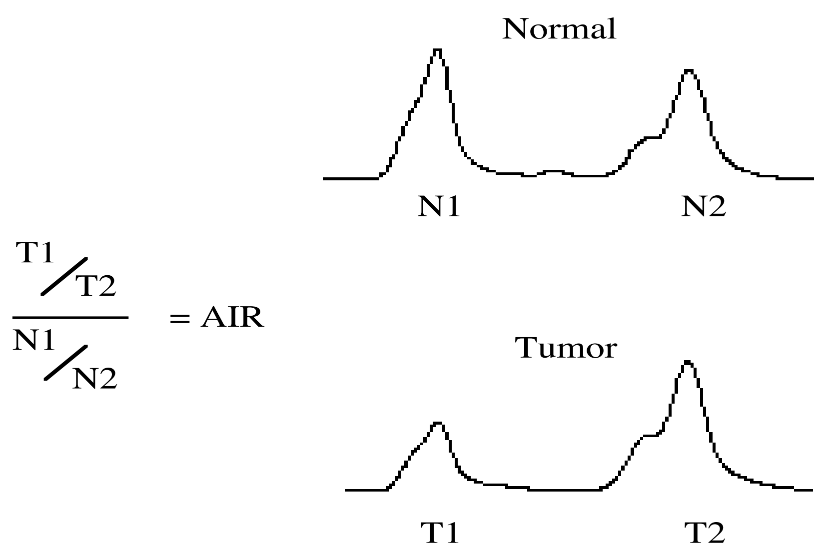

Figure 7. Two allele peaks from a polymorphic microsatellite marker. The AIR value is calculated by dividing the ratio from the two tumor allele peaks (T1/T2) to the ratio from the normal allele peaks (N1/N2).

RESULTS AND DISCUSSION

Paper I: **Chromosome ideograms of the laboratory rat (*Rattus norvegicus*) based on high-resolution banding and anchoring by FISH of the cytogenetic map to the DNA sequence in sample chromosomes.**

Accurate ideograms provide a valuable tool for transferring biological data between species. High-density ideograms facilitate the search for and identification of physical map locations, as anchorage of DNA segments and genes to the physical maps are essential for proper comparative mapping and cytogenetic analyses. Furthermore, detailed ideogram is very useful in determining exact location of chromosomal break points or gene specific FISH signals. In this paper, we could substantially improve the existing rat ideogram as well as successfully connect the genetic map to the physical map in three sample chromosomes: RNO6, RNO15 and the proximal part of RNO4.

Construction of the rat ideogram

The rat ideogram from 1974 was based on chromosomes in early-to-mid metaphase and displayed a total number of 236 cytogenetic bands [52]. The present improved ideogram is based on prometaphase and/or elongated G-banded metaphase chromosomes that can be found in rare spreads and thus exhibits 535 defined G-bands (Fig. 8). Photographs were taken of selected prophase and prometaphase chromosomes and the images were stored electronically. New ideograms were prepared, by drawing the new bands in the correct positions in the ideogram using the computer software. An average of all images for each individual chromosome was used as the master ideogram for the corresponding chromosome. All master ideograms were then adjusted to the chromosome size standards. After finalizing the ideograms, the correlation between the relative sizes of the chromosomes and the corresponding DNA molecules was satisfactory. In the new ideogram, several small dark and light bands (dark-light-dark) emerged from single larger dark bands. A few light bands were also split compared to the older ideogram.

Anchoring the DNA sequence to the physical map

We used the newly produced high-density rat ideogram to test features of anchorage of the DNA sequences to the physical map in three sample chromosomes using dual-color FISH. Seventeen genes mapped in the 22.6 Mb proximal region of RNO4 (Fig. 9b), 26 genes along the entire telocentric RNO6 (Fig. 9a), and 16 genes distributed along the metacentric RNO15 (Fig. 9c) were used in this study. The segment on RNO4 showed consistent co-linearity between the FISH data and the sequence data. Similarly, gene positions derived from FISH correlated very nicely with the ideogram alignment on RNO6, with only minor irregularities in the middle part of the chromosome. In RNO15, however, the DNA sequence data was deceptive compared to the physical location of the gene sequences. The locus positions on the p-arm correlated quite well, whereas there was less accurate correlation in the centromere region and the q-arm. A few genes misplaced in the sequence database compared to the FISH mapping data (Fig. 9c). This divergence may be due to the fact that RNO15 is a metacentric chromosome and the correlation between the DNA sequence and the ideogram would be further improved if the alignment between the chromosome and DNA molecule starts from the centre proceeding outwards rather than from the end of the short arm. The discrepancies probably arise because the centromere contains large regions of repetitive DNA sequences with only few genes. In general, it is difficult to sequence the repetitive DNA since the primers will constantly “slip” from the template strand during PCR amplification. As a

consequence, it will generally be difficult to accurately align the DNA sequences as well as to determine the length of each segment in the centromeric regions.

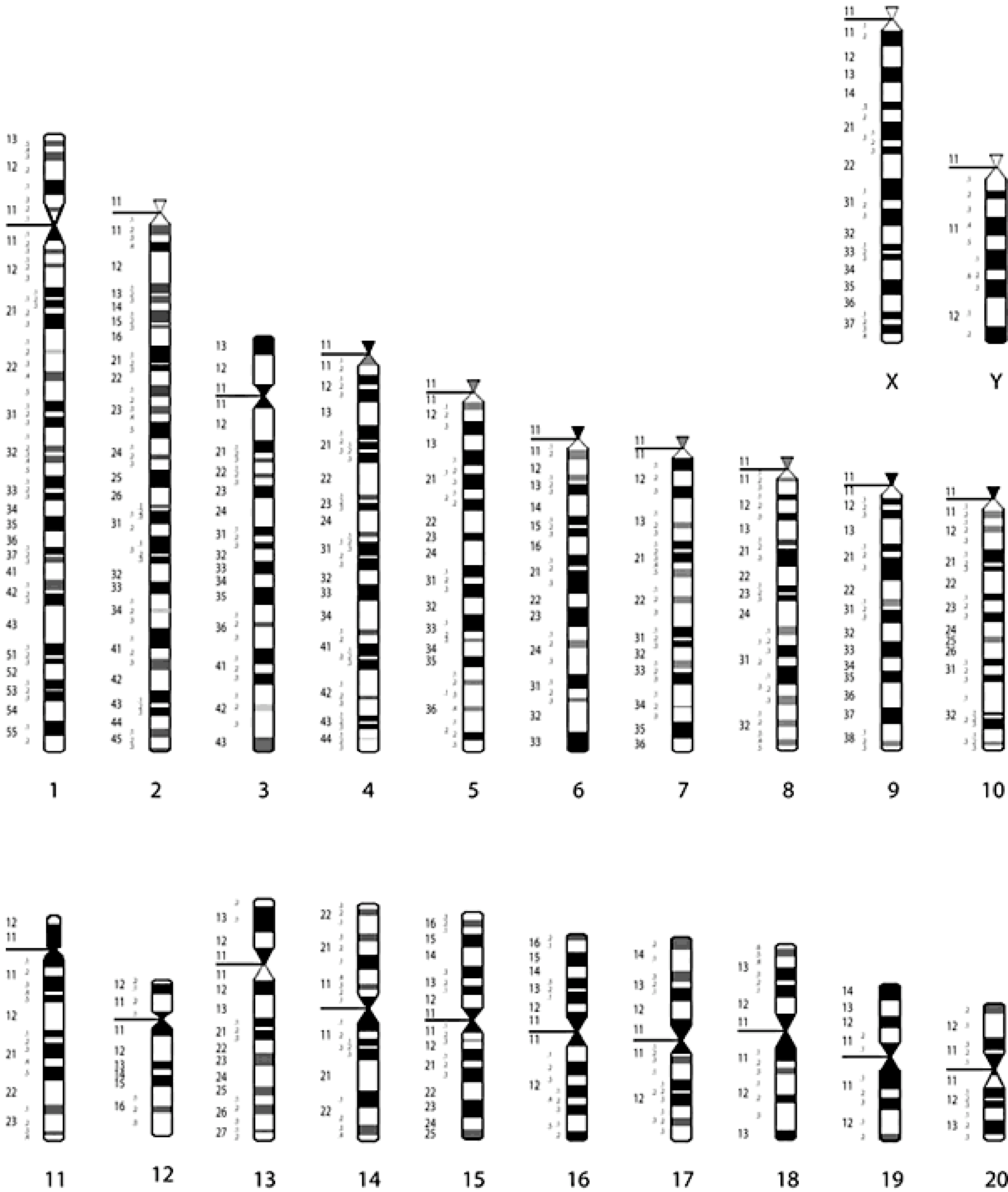

Figure 8. The improved rat ideogram displays 535 defined G-bands on 20 autosomal and two sex chromosomes (XY).

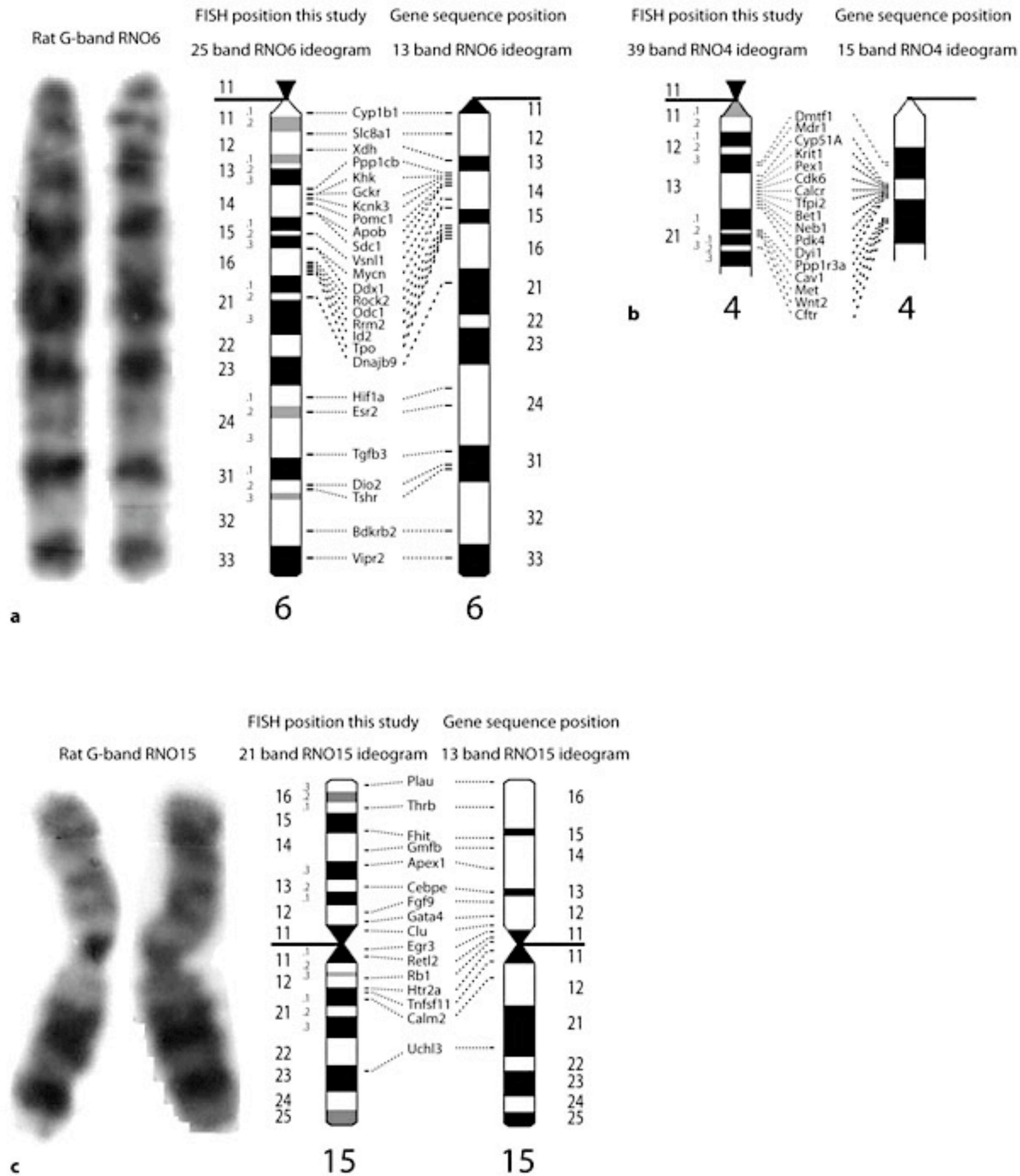

Figure 9 Comparison of dual-color FISH mapping results and ideogram positions derived from the Ensembl database. (**a**) 26 gene loci on RNO6; (**b**) 17 gene loci in a 22.6-Mb segment of RNO4; (**c**) 16 gene loci on RNO15.

Our results clearly suggest that the alignment of the ideograms as presented in the databases provides a good first approximation of the cytogenetic position of the corresponding sequence. However, in many cases it will be valuable to confirm the sequence position with high-resolution FISH. In particular, errors in the alignment of the BAC/PAC clones to each chromosome sequence in the Rat Genome Sequencing project may be circumvented and/or corrected if high-quality dual-color FISH hybridization are performed using the same sequenced BAC clones as FISH probes. It also appears that the centromeric region may distort the linear relationship between sequence and cytogenetic position, particularly of the metacentric chromosomes.

Paper II: Analysis of chromosomal aberrations involving chromosome 1q31-q53 in a DMBA-induced rat fibrosarcoma cell line: amplification and overexpression of *Jak2*.

Comparative mapping

Genome wide screening methods (AI and CGH) revealed gain of RNO1q31-q52 in one fibrosarcoma cell line, LB31TC [26]. In order to identify the potential target gene(s) for the amplification, the rat gene map needed to be improved. Information from available rat, mouse and human gene maps (*i.e.* microsatellite marker, radiation hybrid and physical maps) was integrated and used as template to construct a physical gene map covering RNO1q22-q52 (Fig. 10).

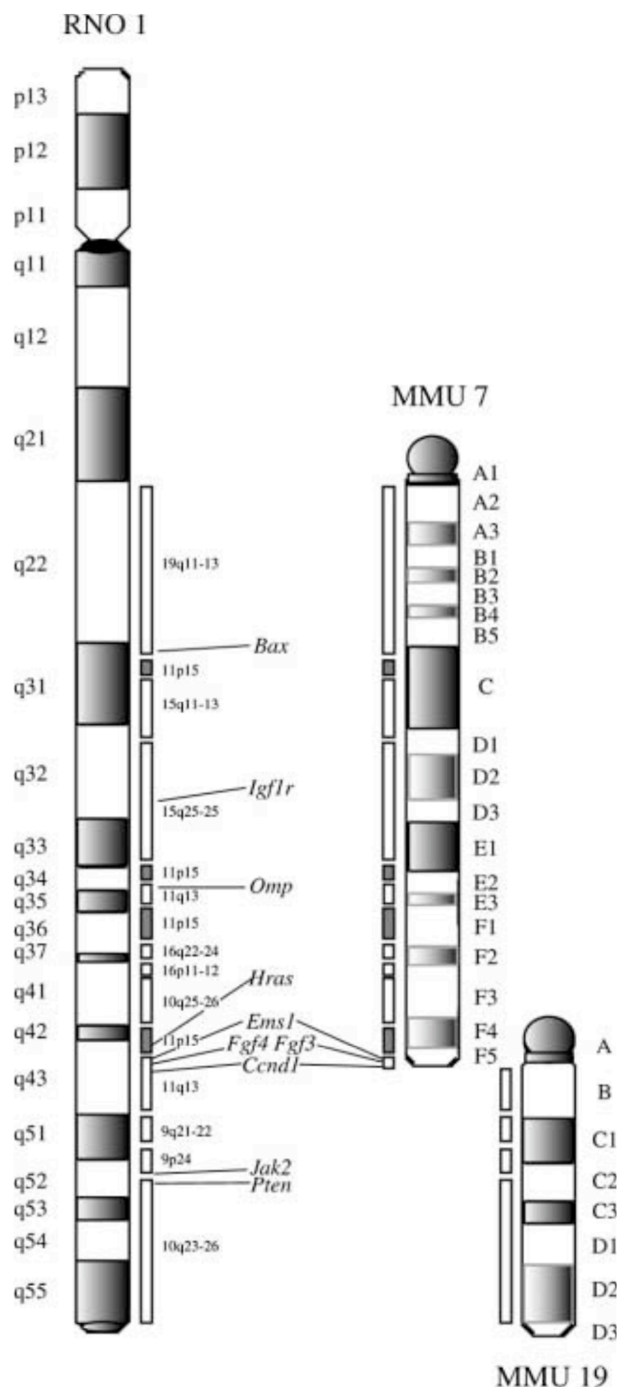

Figure 10. Comparative analysis of the distal part of RNO1. The homologous mouse chromosomes are shown on the right and the corresponding human segments are shown as white or grey boxes next to the chromosomes. The genes mapped by FISH (in rat) and RH mapping (in rat and/or mouse) are shown in the center and their physical positions are indicated. *Fgf3* and *Fgf4* are positioned together, since the exact order of these genes was not resolved in the rat. The evolutionarily conserved segments have been anchored to specific chromosome positions in the rat based on single and dual-color FISH and on comparative data available from RATMAP, OMIM and MGD.

Amplification of RNO1 segment in LB31TC

The CGH profile suggested gain of material in the region RNO1q31-q52 in one tumor, and possibly in the form of two independent amplified regions at RNO1q31-q35 and RNO1q43-q53. Probes from nine genes (*Ccnd1*, *Fgf4*, *Fgf3*, *Ems1*, *Hras, Omp*, *Igf1r, Jak2* and *Bax*) were generated using PCR amplification and used in Southern blot experiments, to look for copy number increases in 19 fibrosarcoma solid tumors and 17 fibrosarcoma cell lines. Amplification of two genes, *Omp* and *Jak2,* was detected in tumor LB31TC. We used the same gene specific PCR products from the Southern blot for screening PAC filters and gene specific PAC clones suitable for FISH were identified. The PAC clones for all genes were labeled pair-wise with two different fluorochromes and hybridized together on normal rat metaphase chromosomes. The positional data obtained were used to generate the physical gene map. The PAC clones were then used as probes in dual-color FISH on tumor metaphase spreads. The cytogenetic analysis revealed that there were sub-populations of cells in the tumor cell culture of LB31. The major population (accounting for approximately 85% of the cells) was near-tetraploid, and displayed three normal-appearing RNO1 and one long RNO1-derived marker chromosome. Both *Omp* and *Jak2* showed normal signals, on their native positions (*Omp* maps to RNO1q34-q35 and *Jak2* to RNO1q52), on two of the normal-looking RNO1. However, on the third copy of RNO1, an inverted pattern of *Omp* and *Jak2* was present, showing that this chromosome derived a paracentric inversion. In addition, the FISH analysis showed that both *Omp* and *Jak2* were amplified in a ladder-like pattern on the long marker chromosome (Fig. 11).

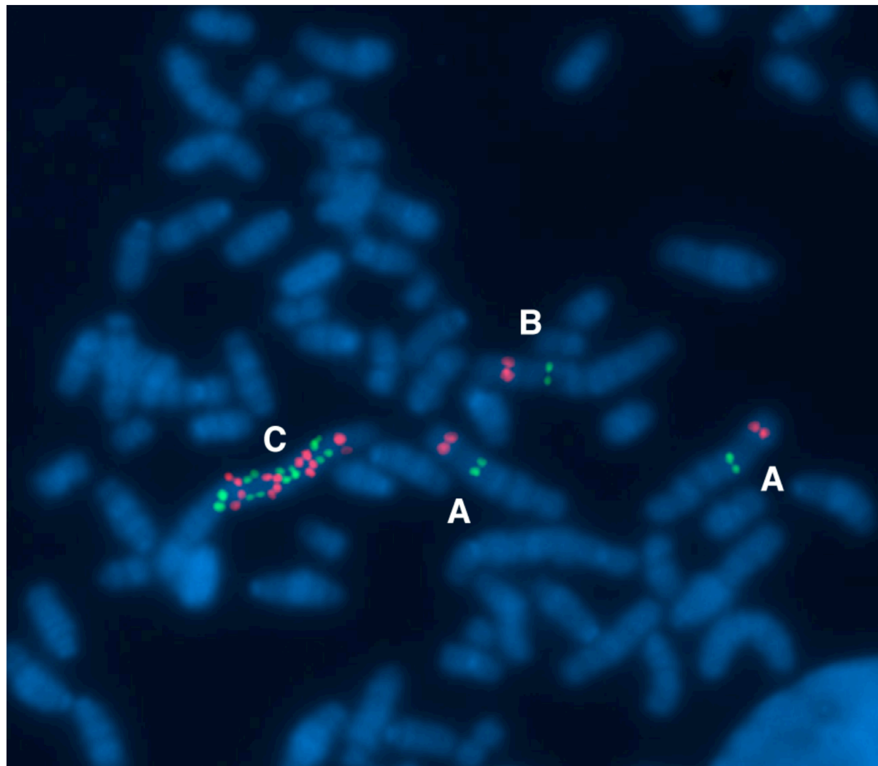

Figure 11. Four RNO1 positive chromosomes with *Omp* labeled in green and *Jak2* in red, respectively. A) Two normal RNO1 showing *Omp* and *Jak2* in the native sites. B) One RNO1 chromosome with an inversion of the region RNO1q22–q52 containing *Omp* and *Jak2* . C) A long banded marker chromosome shows an inverted repeat pattern of *Omp* and *Jak2* signals that are indicative of amplification through chromatid break-fusion bridges during mitosis.

Genes normally located between *Jak2* and *Omp* were absent on the marker chromosome placing *Omp* and *Jak2* next to each other. The ladder-like amplification pattern was interpreted as a sign of repeated cycles of break-fusion-bridge (BFB) events during mitosis. Amplification through BFB mechanism will lead to simultaneous loss of chromosome regions situated distally of the break point. Thus, if a tumor suppressor gene is positioned distally of the amplified genes, this might favor amplification through BFB cycles and give selective advantage to cells harboring the amplification. Interesting to notice, RNO1 is homologous to human chromosome 11q13 for which amplifications that were assumed to emerge from BFB events were discovered in human oral squamous cell carcinoma cell lines [53].

Further, it should be noted that the *Pten* tumor suppressor gene was mapped distal to *Jak2* and our AI analysis suggested that *Pten* might been involved in LOH in this fibrosarcoma tumor; this finding is discussed in *Paper III*.

Expression studies of *Omp* and *Jak2* showed that there was no expression of *Omp*, which is in concordance with previous reports [54] stating that *Omp* is mainly expressed in olfactory neurons. These findings suggest that another gene close to *Omp* might be the actual target for the observed amplification at RNO1q31-q35. *Jak2* on the other hand, was strongly over-expressed. *Jak2* is a member of the JAK's (Janus Kinases) gene family, which induces the JAK-STAT pathway that mediates cell growth and proliferation. In human, the *JAK2* homologue has been mapped to a region reported to be recurrently amplified in human leiomyosarcomas [55]. Consequently, *Jak2* appears to be a good candidate target gene for the observed amplification in RNO1q43-q53.

Paper III: **Recurrent allelic imbalance at the rat *Pten* locus in DMBA-induced fibrosarcomas.**

Indications for LOH in the distal part of RNO1 was previously found [26]. The fact that *Pten* had been physically mapped distal to *Jak2* at RNO1q52 [56] and has previously been reported to function as a tumor suppressor gene in other types of tumors [57, 58] prompted us to further investigate the role of *Pten* in this set of fibrosarcomas.

Mapping of the Pten gene

Since there is a microsatellite including CA-repeats in the human intron 8 sequence of *PTEN*, we looked for a microsatellite marker sequence in the homologous position of the rat *Pten* gene. A marker within the gene itself would be very useful to prove involvement of *Pten* in the observed AI. Using an expand PCR amplification system, it was possible to amplify the entire rat *Pten* intron 8 (2040 bp) and determine its DNA sequence content. Rat intron 8 did not contain any CA-repeat, however, an ID-repeat was detected in the intron sequence. ID-repeats in rats consist of a core region of 75 bp followed by a poly-A tail and they belong to the group of rodent-specific Short INterspersed Element (SINE) repetitive sequences, of which about 150 000 copies are present in the rat genome. Primer pairs were designed to match regions flanking the ID-repeat sequence, making it possible to PCR-amplify the poly-A tail. The marker, designated as D1Lev1, was found to be highly polymorphic in nine different rat strains due to variation in the length of the poly-A tail. The D1Lev1 marker was genetically mapped in conjunction with 20 RNO1 commercial markers using DNA from a set of 32 rats from a (BNxLE) F2 cross and subsequently, a linkage map of the segment was constructed (Fig. 12.).

Allelic Imbalance at the Pten locus

The same set of RNO1 markers was then used to perform a detailed AI analysis on the distal part of RNO1 on DNA from 19 fibrosarcoma tumors. Liver DNA from the corresponding animals was used as normal control. In total, 12 out of 19 tumors (63%), showed AI in the *Pten* region. Allelic imbalance affecting only the *Pten* marker (D1Lev1), but not the adjacent markers, was detected in four individual tumors (21%).

The peak size and base composition of the PCR products generated with the D1Lev1 marker was found to show a uniform pattern. On closer inspection it became obvious that there was often loss of the LE allele whenever AI was detected at the *Pten* locus. In fact in two tumors, LB20TC and LB31TC, a complete loss of the LE allele at the D1Lev1 marker was detected. LB31TC represented a special case showing absence of the LE allele at the *Pten* locus whereas the nearest proximal marker (D1Rat300) showed AI with reduction of the BN allele. This allele shift was suggested to be due to amplification in the region proximal to the *Pten* locus. FISH analysis showed that the relative reduction of the BN allele was in fact due to amplification of the LE allele (discussed in *Paper II*). The entire coding sequence of *Pten* was examined in the tumors showing AI at the *Pten* locus using direct DNA sequencing.

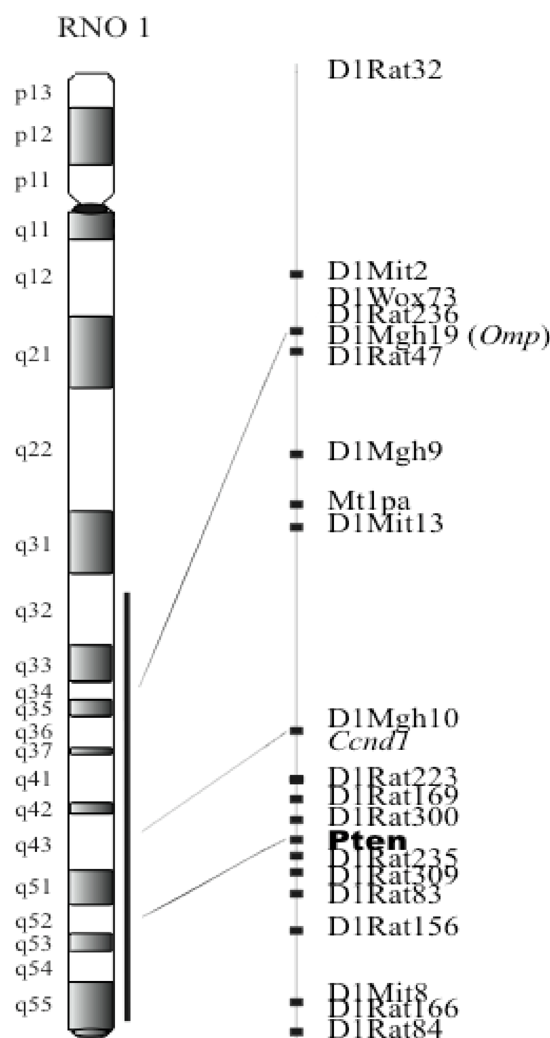

Figure 12. The linkage map constructed on the distal part of RNO1, RNO1q32-q55. The intragenic *Pten* marker (D1Lev1) was mapped between the two markers D1Rat300 and D1Rat235.

The result showed normal *Pten* sequence in all samples tested. We then examined the gene expression of *Pten* in four selected tumors, three with AI at the *Pten* locus (LB153TC, LB20TC, LB31TC) and one (LB133TC) without AI in this gene. Interestingly, it was found that the expression level of *Pten* in these four tumors was similar, suggesting that the retained allele must be inactivated by a mechanism other than mutation of the coding sequence. Since *Pten* was expressed in the tumors tested, it appears that promoter methylation and other epigenetic silencing can be excluded. Taken all data into consideration, the most likely explanation would be that *Pten* is a tumor suppressor gene that acts in a dose-dependent manner (haploinsufficiency). In fact, *Pten* along with several other tumor suppressor genes, including *Tp53, $p27^{Kip1}$*, *Tgfβ* and *Dmp1* have previously been suggested to act in a haploinsufficient mode [59-63]. Dose-dependent activity of *Pten* is in agreement with our results and thus would be a reasonable explanation for the contribution of this gene to the sarcomagenesis in this set of DMBA-induced fibrosarcomas [64].

In conclusion, our molecular analysis of RNO1 aberrations in the DMBA-induced rat fibrosarcoma provided evidence for:
- *Jak2* as a good candidate target gene for the observed amplification in RNO1q43-q53.
- *Omp* is not the main target for the observed amplification in RNO1q31-q35 and thus yet another unidentified proto-oncogene located close to *Omp* might be the main target.
- The *Pten* tumor suppressor gene is recurrently inactivated in DMBA-induced rat fibrosarcomas and most likely through the haploinsufficiency mechanism.

<u>*Paper IV*</u>: **Molecular classification of spontaneous endometrial adenocarcinomas in BDII rats.**

Cancers of the reproductive system are significant causes of morbidity and mortality among women worldwide. Although the incidence of the disease has remained stable, the death rate has doubled over the last two decades [65]. Hence, there is definitely a need for developing new strategies for the treatment of this cancer type. Most of the endometrial carcinomas (EC) are sporadic, although it has clearly been demonstrated that a genetic predisposition is of critical importance in about 5% of the cases [30-32]. Based on the biology, clinical and molecular features, two different pathways have been distinguished for the tumorigenesis of sporadic tumors: the majorities are designated as type I, with endometrioid histology and follow the estrogen-related pathway. Type II tumors show non-endometrioid histology and follow the estrogen unrelated pathway [29, 66-68]. At molecular level type I show frequent microsatellite instability accompanied by *PTEN*, *K-RAS* and *CTNNB1* mutations. Type II tumors are featured by recurrent *TP53* mutation, *P16 (CDKN2A*) inactivation, over-expression of *ERBB2* (*Her2/neu*) and reduced *CDH1* expression (summarized in Table 4). However, many tumors do not fully fit in either of these categories and there is a large group of endometrial carcinomas with overlapping clinical, morphologic, immunohistochemical, and molecular features of types I and II.

The BDII rat strain represents a unique experimental tumor model for molecular analysis of EC and provides a valuable tool for design, development and testing of novel substances to combat this disease [28]. Tumors developed in this model are from endometrioid type and hormone–dependent [35, 69], thus resemble human type I tumors. In this study, we aimed to classify the BDII EC tumors further according to the molecular guidelines used for classification of human EC tumors. The guidelines are based on the results from several

research groups around the world, including information on status of microsatellite instability, gene expression, mutation, and allelic imbalance of specific genes and pathways thought to be involved in endometrial tumorigenesis (summarized in Table 4).

We took this data on human EC into consideration and selected seven genes (*Pten, Ctnnb1, Tp53, P16, Cdh1, Erbb2* and *Irf1*) for detailed molecular analysis in a panel of 29 EAC tumor and seven non-malignant endometrium (NME) samples. The *Pten* locus was screened for allelic imbalance (AI) in the rat tumors; three out of seven informative tumors showed AI in this gene. We additionally subjected all seven genes to gene expression analysis. Statistical analysis revealed that *Pten, Ctnnb1, Cdh1, P16,* and *Erbb2* were expressed at significantly lower levels (nominal p-value < 0.05) in the EC compared to the NME samples. *Tp53* showed a significant lower expression level if one outlier was removed (Fig. 13). Furthermore, we subjected the entire coding sequences of the *Pten, Ctnnb1* and *Irf1* genes to gene mutation analysis. No mutation was found in *Pten* and *Ctnnb1* genes. Thus, it appeared that the observed lowered expression of *Pten* and *Ctnnb1* was not due to inactivating mutations in the coding region of these genes. Inactivation/lower expression of *PTEN* and *CTNNB1* is characteristic for human type I EC tumor, and thus, endometrial carcinomas in the BDII rat model can be related to human type I tumors.

Table 4. Summary of molecular analysis of EC developed in the BDII rat tumor model compared to the different subgroups of human EC.

Gene alteration	**Pathway**	**Human endometrial carcinoma** [64, 67, 70, 71]				**Endometrial carcinoma in BDII rats** [72, 73]
		Type I	Type II	Arisen from HNPCC	Familial site-specific	
Microsatellite instability	MMR	20–45%	0–5%	90–95%	8–9%	0-5%
PTEN inactivation	Akt	50–80%	10%	60–85%	N	2.3 fold down-regulation, no mutation [a]
K-RAS mutations	Akt	10–30%	0–5%	10%	N	No
ERBB2 over-expression	Akt	10–30%	45–80%	N	N	No [a]
TP53 mutations	P53	10–20%	90%	Low	N	67% and 2.1 fold down-regulation [a, b]
P16 inactivation	P53	10%	40%	N	N	57% deletion and 28 fold down-regulation [a]
CDH1 down-regulation	Wnt	10-20%	80-90%	N	N	Yes, 23 fold down-regulation [a]
CTNNB1 mutations/ over-expression	Wnt	20%	0–5%	N	N	No [a]

N = not known or no information available; [a] Paper II; [b] after removing one outlier from the NME samples.

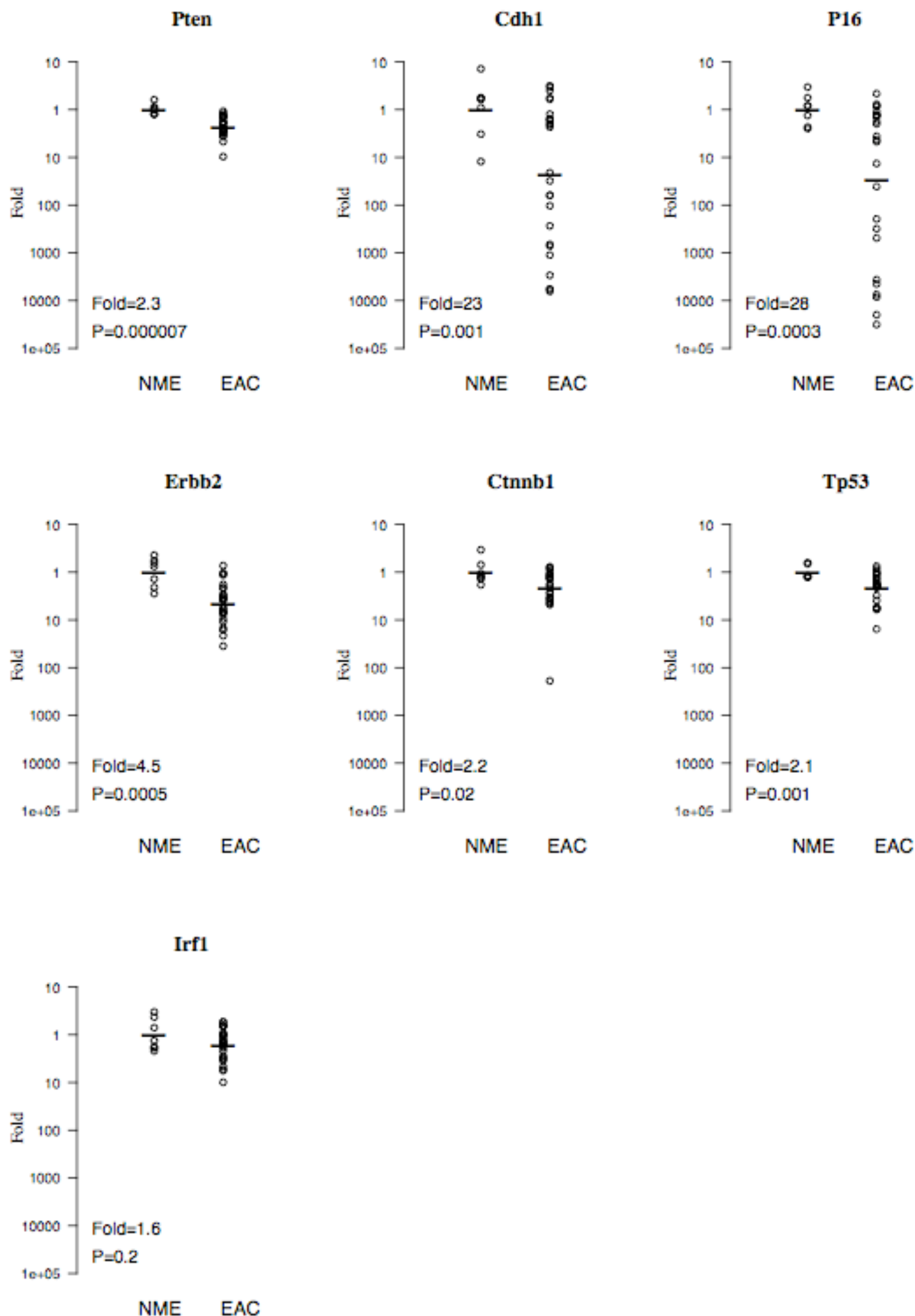

Figure 13. Scatter plot showing the seven transcripts from the Real-time PCR analysis of the 29 EAC tumors and seven NME samples. Expression presented as fold change of C_T (log2).

Irf1 showed sequence alterations in only two EC samples, and the corresponding expression level did not differ markedly between EC and NME sample groups, suggesting that *Irf1* to be of less importance in development of EC tumors in this rat model. *P16* displayed significant lower expression in the EC compared to the NME samples; this data is in agreement with the results from a recent study indicating frequent hemi- or homozygous deletions of this gene in BDII rat EC tumors [74]. *CDH1* is a member of Cadherins that are adhesion molecules essential for tight connection between cells. Significant reduced expression of this gene was found among rat EC tumors. Lower expression of both *P16* and *CDH1* is characteristic for human type II tumors, but is also frequently reported in high-grade type I tumors. Significant lower expression of *P16* and *Cdh1* along with previously reported high frequency of *Tp53* mutations [75] and absence of *K-ras* mutations [76], as well as lack of microsatellite instability [72, 77] in BDII rat tumors suggest that this model can be related to the high-grade type I tumors in human.

In conclusion, BDII rat EAC tumors can be related to human type I tumors in histology, hormone-dependency as well as in frequent inactivation of *Pten* and *Ctnnb1*. In frequency of *Tp53* mutation, down-regulation of *P16*, lack of microsatellite instability as well *K-ras* mutation, based on the literature, this tumor can well be related to human high-grade type I tumors. Hence, our data suggest that the BDII rat EAC represents a suitable tumor model for modeling human type I endometrial cancer, in particular for those with an aggressive clinical course and poor prognosis that are life threatening.

Paper V: **Recurrent gene amplifications in human type I endometrial adenocarcinoma detected by fluorescence *in situ* hybridization.**

Identification of genes that actively involved in tumor development is very important, since they may provide new targets for prognosis and therapy. Previously, we reported frequent RNO4 and RNO6 amplifications in a subset of rat endometrial carcinomas [39-42]. In molecular genetics analysis of these regions, a number of candidate genes as the main targets for the observed chromosomal amplifications were found and defined.

CGH analysis of human EC

In CGH analysis of 98 human type I endometrial carcinomas, Levan and co-workers could provide evidence for recurrent gains on HSA2p in five tumors, in three tumors gain of HSA2p and HSA7q was seen and additionally five tumors showed gain on HSA7q [43]. These chromosomal segments are homologous to parts of RNO4 and RNO6 for which amplifications were reported in BDII rat tumors. To determine whether the reported target genes for chromosomal amplifications in the rat EAC were targets for the observed amplifications in human EC, 13 tumor samples were selected for FISH analysis on tumor imprints. Twelve genes from HSA2p21-25 and three genes from HSA7q21-31 were selected for the analysis (Table 5).

RNO6 amplifications in the BDII rat EAC

We showed that proximal part of RNO6 was frequently amplified in BDII rat EAC tumors, in the form of two independent amplified regions situated between RNO6q11-q16 in several tumors [41, 42]. Molecular analysis suggested that *MycN* and *Slc8a1*, located at RNO6q14-16 and RNO6q12, respectively, were main targets for the observed amplifications. Proximal part of RNO6 is homologues to short arm of human chromosome 2 (HSA2p21-p25, Fig. 14).

Table 5. Cytogenetic position in human and rat for genes used for FISH analysis.

Gene	Cytogenetic position (HSA)	Cytogenetic position (RNO)
RRM2	2p25-p24	6q16
OCD1	2p25	6q16
DDX1	2p24	6q15
MYCN	2p24.1	6q15
SDC1	2p24.1	6q14
POMC	2p23.3	6q14
GCKR	2p23	6q14
PPP1CB	2p23	6q14
XDH	2p23-p22	6q12
SLC8A1	2p23-p22	6q12
CYP1B1	2p21	6q11
PRKCE	2p21	6q12
CDK6	7q21	4q13
TAC1	7q21-q22	4q13
MET	7q31	4q21.2

HSA, human chromosome; RNO, rat chromosome.

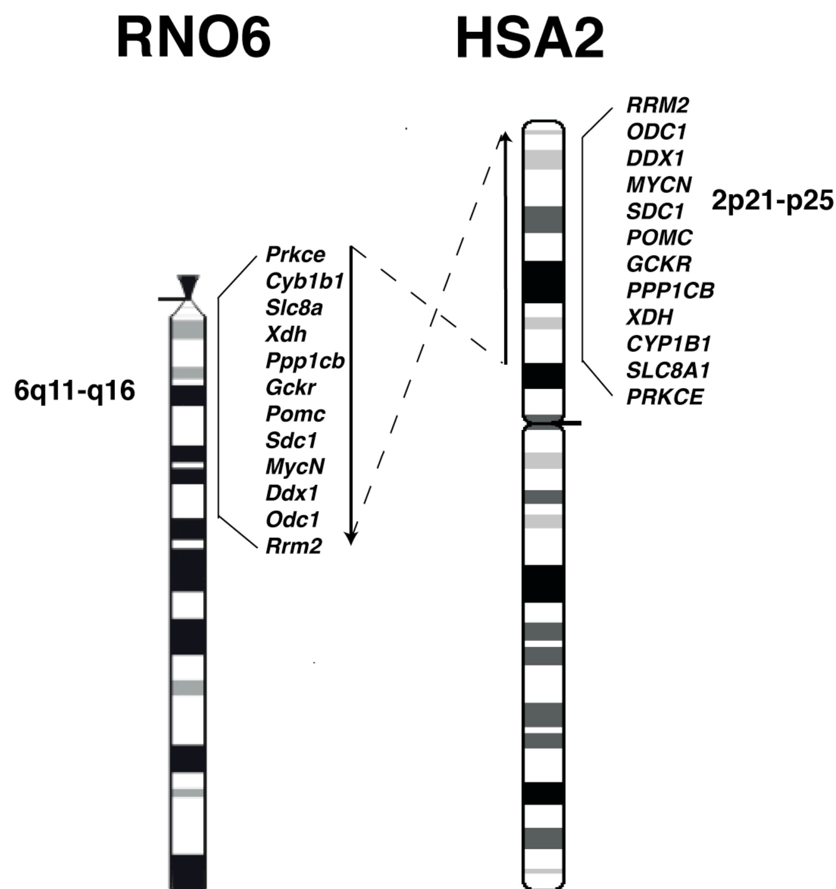

Figure 14. Homologous regions on rat chromosome 6 and human chromosome 2 and order of the genes in each species, respectively.

FISH analysis of HSA2p

Among the 12 genes located on HSA2p, we found *SDC1* to be the most frequently amplified gene in this region (Table 6). In general, *SDC1* exhibited a low level of amplification, with 5 to 10 copies per cell. In three tumors, *SDC1* was found to be co-amplified with two other genes, *MYCN* and *POMC*. The three genes *SDC1, MYCN*, and *POMC* are located very close together at HSA2p23-p24, within a region spanning about 9 Mb. Amplification of *MYCN* has been reported in human endometrial carcinomas and is quite common in neuroblastoma, in which there exist a significant correlation between the degree of *MYCN* amplification and poor prognosis [78]. Accordingly, *MYCN* amplification is used as a molecular biomarker and as a guideline to decide for the level of aggressiveness of the treatment of neuroblastoma [9, 11]. In our study, however, it appeared that *MYCN* was not the main target, as amplification of *SDC1* alone was detected in two tumors. The *SDC1* gene product (Syndecan 1) is a cell surface proteoglycan, an integral membrane protein acting as a receptor for the extra cellular matrix [79]. Tsanou *et al*. found high expression levels of SDC1 in breast cancer cells and speculated *SDC1* might under certain circumstances promote tumorigenesis [80]. The *POMC* gene is occasionally expressed in nonpituitary tumors leading to Cushing syndrome [81], but has not previously been reported to be involved in endometrial carcinogenesis. In summary, our finding clearly suggests that the main target for the observed amplification at HSA2p23-24, is the SDC1 gene, or a gene at very close vicinity of *SDC1*.

In a second and more distal part of HSA2p, the *CYP1B1* gene showed highest level of amplification among a cluster of genes located at HSA2p21, including three other genes: *XDH, SLC8A1* and *PRKCE*. In several cases, it was found that *CYP1B1* was amplified together with adjacent genes within a region spanning ~17 Mb. Involvement of *CYP1B1* in human endometrial cancers has earlier been reported, mainly in form of DNA sequence polymorphism [82, 83] and/or promoter hypomethylation [84]. Although the *Prkce* gene was not included in the rat study, the authors suggested that this gene might have been the main target for the observed amplifications. In our study, we could exclude this possibility, since we found higher level of *CYP1B1* amplification compared to that detected for *PRKCE*.

Table 6. Gene symbols represents the genes located at chromosome 2p. The following classification was used: - 1-4 copies, A - 5-10 copies, AA - 11-15 copies, AAA - 16-20 copies, AAAA - >20 copies.

Gene / **Tumor**	*RRM2*	*ODC1*	*DDX1*	*MYCN*	*SDC1*	*POMC*	*GCKR*	*PPP1CB*	*XDH*	*CYP1B1*	*SLC8A1*	*PRKCE*
146	**A**	**A**	**A**	**A**	**A**	**-**	**A**	**-**	**A**	**A**	**A**	**A**
292	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
326	**A**	**-**	**-**	**-**	**A**	**-**	**A**	**A**	**A**	**A**	**-**	**-**
649	**-**	**A**	**A**	**A**	**A**	**AA**	**-**	**A**	**A**	**AA**	**AA**	**A**
1123	**A**	**A**	**-**	**AA**	**AA**	**A**	**A**	**A**	**A**	**A**	**A**	**A**
54	**-**	**-**	**-**	**A**	**A**	**-**	**-**	**AAAA**	**A**	**AA**	**A**	**-**
183	**-**	**-**	**-**	**-**	**A**	**A**	**-**	**-**	**-**	**-**	**-**	**A**
816	**AA**	**AAA**	**-**	**AAAA**	**AAAA**	**AAAA**	**A**	**AAAA**	**A**	**AAA**	**AAA**	**AAAA**

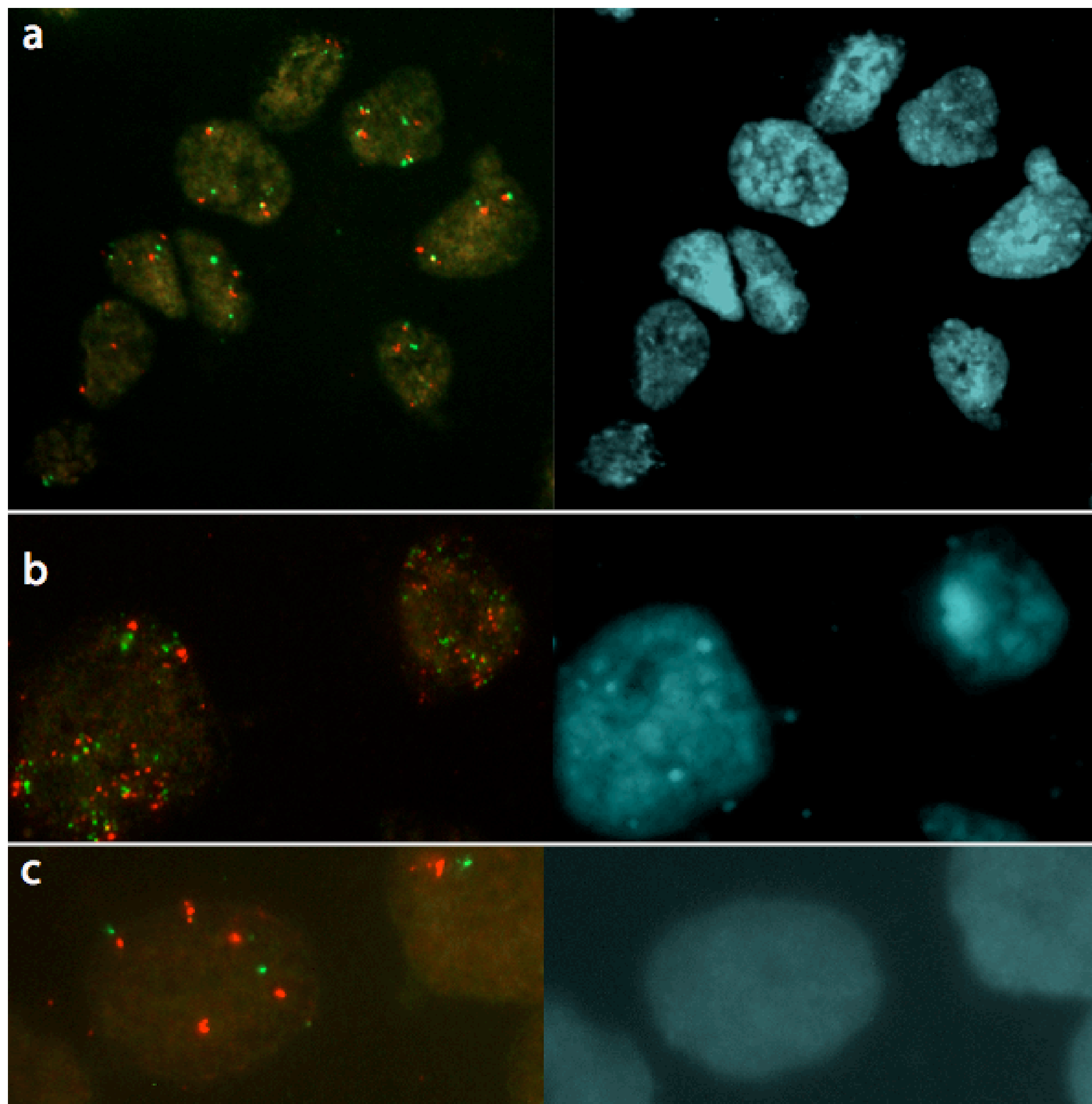

Figure 16. Dual-color FISH image representative for the tumor tissue imprints of human EC tumors. Left panel showing FITC and Texas Red images and right panel shows corresponding DAPI stained cell nuclei. a) tumor 816, PRCKE is labeled in green and GCKR is labeled in red, no amplification. b) tumor 816, POMC1 and SDC1 is labeled in green and red, respectively, showing amplification of both genes. c) tumor 292, XDH showing normal copy number, is labeled in green and SDC1 showing increased copy number, is labeled in red.

RNO4 amplifications in the BDII rat EAC

Similarly, two independent amplified regions were identified on RNO4, for which *Cdk6*, located at RNO4q13, and *Met*, located at RNO4q21 were suggested as the main targets [39, 40] It was also found that the *Tac1* gene, situated in between the two amplification regions, had a normal copy number in the majority of samples tested, but in few cases signs of loss/deletions at the *Tac1* locus was detected. Proximal part of RNO4 (RNO4q13-21) is homologues to the middle part of long arm of human chromosome 7 (HAS7q21-q31) as presented in Figure 15.

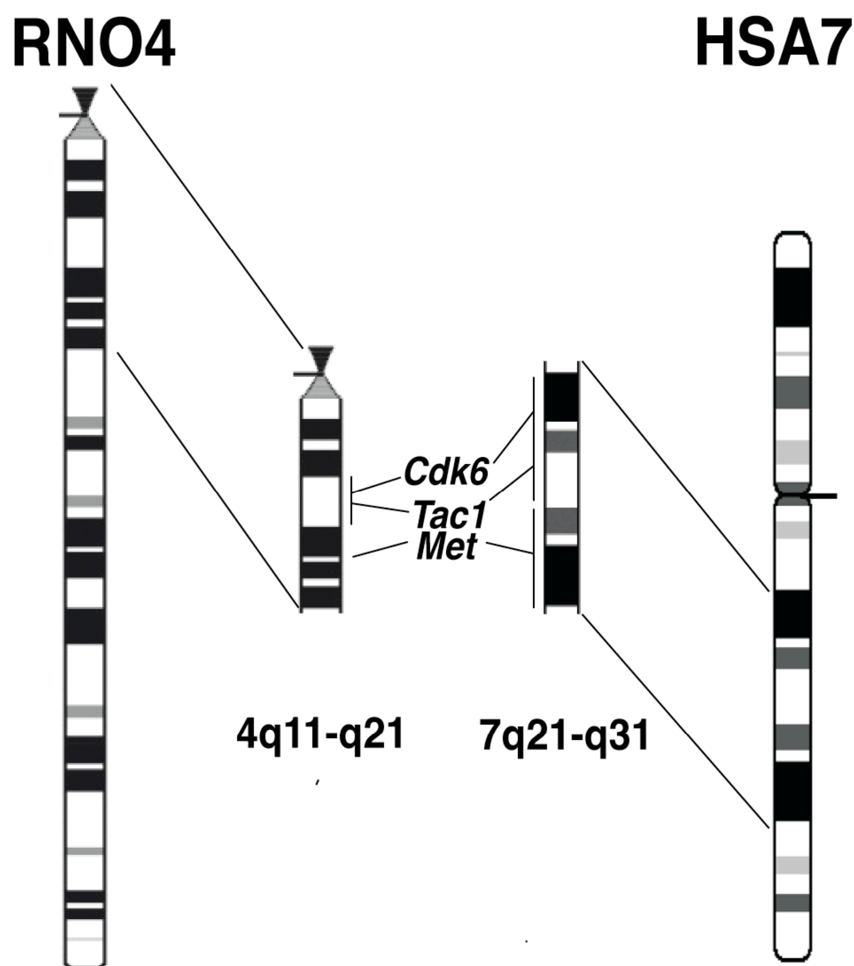

Figure 15. Homologous regions on rat chromosome 4 and human chromosome 7 and the gene order.

FISH analysis of HSA7q
We found both *CDK6* and *TAC1* to be frequently amplified in human EC tumors (Table 7). *CDK6* has been suggested to have oncogenic features, play an important role in cell cycle control mainly through providing a link between growth factor stimulation and onset of cell cycle progression [85]. Amplification of *CDK6* was found in nodal metastasis from endocervical adenocarcinomas of the uterus [86] and in human gliomas [87]. In addition, *CDK6* was found to play an important role in the development and/or progression of human prostate cancer [88]. *TAC1*, which encodes for four products of the tachykinin peptide hormone family, has not been previously studied for gene amplifications in human tumors, most likely because *CDK6* has always been the most attractive candidate gene in this region. Interestingly, we found higher level of *TAC1* amplification in three tumors compared to that observed for *CDK6*. Thus, whether the *TAC1* and/or *CDK6* are/is the main targets for the observed chromosomal amplification at HSA7q21, or yet the main target remains to be identified, is not clear.

We additionally found that the proto-oncogene *MET*, located at HSA7q31, was frequently amplified in human EC tumors (Table 7). *MET* encodes a hepatocyte growth factor receptor exhibiting tyrosine kinase activity and is involved in scattering, angiogenesis, proliferation, and invasion. It has been suggested that aberrant *MET* signaling plays a significant role in the pathogenesis of many types of solid tumors as well as in several hematological malignancies

[89]. Against this background and taking our FISH results into consideration, we suggested that *MET* was the main target gene for the observed amplifications at distal part of HSA7q.

Gene / Tumor	*CDK6*	*TAC1*	*MET*
54	**A**	**AAA**	**-**
183	**A**	**-**	**A**
816	**A**	**AAA**	**A**
301	**AAA**	**AAAA**	**AAAA**
396	**A**	**A**	**A**
683	**AA**	**A**	**AA**
1138	**AAA**	**A**	**AAA**
1265	**-**	**-**	**A**

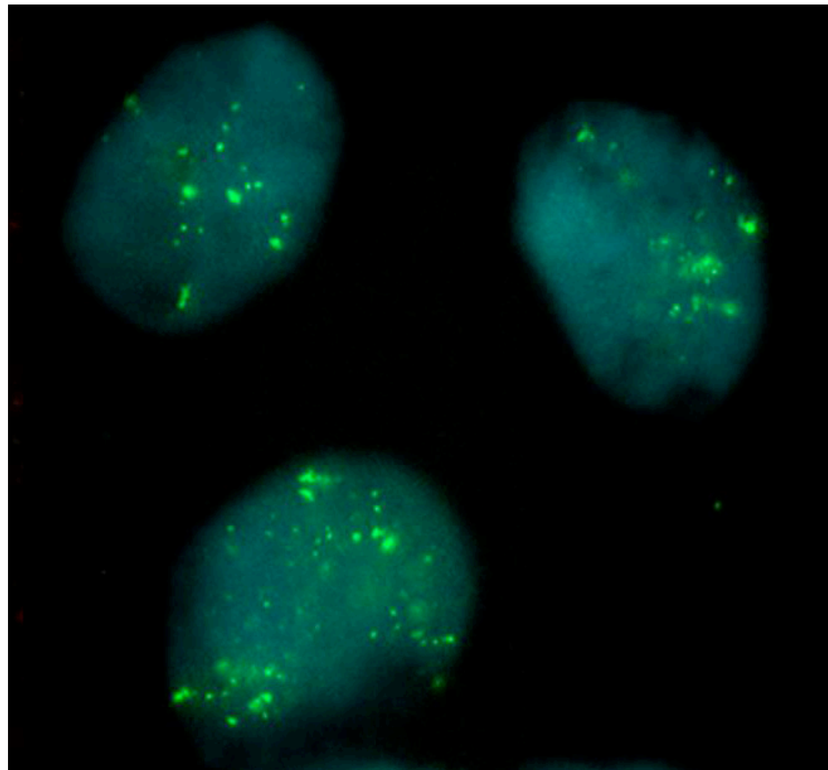

Figure 17. Amplification of the *MET* oncogene in tumor 301. The BAC probes is labeled in green and the nucleus is counterstained with DAPI (blue).

In conclusion, using the data available for gene amplifications in the rat model for endometrial carcinogenesis, we studied in a subset of human EC tumors and could find clear evidence of gene amplifications in the two chromosome regions tested, HSA2p21-p25 and HSA7q21-31. We additionally suggested a number of candidate target oncogenes in these regions, all with documented association with tumorigenesis in several other tumor types. Our analysis clearly indicate that specific findings in genetically well-defined rat cancer models may well provide guidance for targeted molecular analysis of the corresponding human tumors, for which there usually exist the problem of shortage of tumor material and/or difficulties to obtain tumor cell cultures.

CONCLUDING REMARKS

It is known that the cancer genome comprises diverse genetic changes that occur based on individual genetic background and environmental/life style factors and are selected for during tumor development. In the search for key genes and biomarkers for drug discovery and testing purposes, animal models serve as valuable complement to human studies. Through use of animal model systems, it is expected that influences of genetic background heterogeneity and environmental factors on analysis of disease to be reasonably reduced. Today, rat models are widely used as a tool to improve, enhance and facilitate human medical research in a broad spectrum of appliance. The work presented in this thesis looks into the use of rat tumor models for analysis of human fibrosarcomas and endometrial cancer.

The effects of a polycyclic aromatic hydrocarbon (PAH) called DMBA (7,12-dimethyl-benz[a]anthrazene) were investigated in a rat fibrosarcoma model. PAH:s are often found in air pollution and cigarette smoke and are known to interact directly with the DNA bases. Although, we found no point mutations due to the PAH induction, we could show that the tumor genomes are unstable, we found LOH at the distal region of RNO1, and that several different genes are involved in different chromosomal lesions in the tumors. One fibrosarcoma cell line was found to harbor a marker chromosome with a ladder like amplification pattern consisting of only two genes, *Jak2* and *Omp*.

The BDII rat serve as a unique model for genetic investigations of the biological processes underlying development of human endometrial carcinoma. During the last two decades, the research group at the University of Gothenburg has subjected this model to detailed cytogenetic and molecular analysis. Through these works, the BDII rat tumor model had become genetically well characterized, but some important pieces of information was missing. By using cytogenetic methods and FISH, combined with molecular techniques, including Allelic Imbalance, quantitative Real-time PCR and gene sequencing as well as statistical methods, we aimed to address some of these questions. We could substantially produce systematic data on molecular features of BDII rat endometrial carcinogenesis based on the classification system used for human endometrial carcinomas. Furthermore, we were able to successfully translate part of the genetic data found in the BDII rat model into the human system and finally, add some valuable data to the knowledge of molecular pathways involved in BDII rat endometrial tumorigenesis.

In addition, we manage to improve the rat ideogram and anchor DNA sequences (*i.e.* genes) to the physical gene map.

In summary, with the aid of molecular studies, knowledge of the pathogenesis of cancer in general has broadened extensively over the last decades. Nevertheless, a considerable amount of work remains to be done to get a better understanding of the biological processes behind the development of this disease. Genome-wide association studies in human populations have recently provided a direct approach for finding robust genetic associations in common diseases, but identifying the precise genes and their mechanisms of action remains problematic. In the context of significant progress in rat genomic resources combined with recent advances in molecular techniques could certainly help provide an increasing rate of discovery of new disease genes, pathways and mechanisms, ultimately leading to the development of new drugs and cancer treatments.

SWEDISH SUMMARY
POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

En individ består av celler. Cellerna innehåller DNA som utgör ritningar/instruktioner för cellens aktiviteter. DNA är organiserat i olika sammanhängande delar, generna. Generna är i sin tur ordnade i kromosomer och utgör individens genom. En normal cell hos människan innehåller 46 kromosomer, medan råttans celler innehåller 42 kromosomer.
Cancer är en sjukdom som beror på förändringar i gener och DNA i kroppen. Cellens normalt fungerande tillväxtkontroll sätts ur spel och den kan börja dela sig ohämmat (en tumör bildas) och kan spridas till andra delar av kroppen (bilda metastaser). För att underlätta forskningen om cancer har olika modellsystem utvecklats. Ett ofta använt modellsystem utgörs av olika råttstammar där varje individ i en stam har identiska gener och DNA.
Målsättningen med mitt arbete har varit att identifiera och karaktärisera gener och genmekanismer som bidragit till tumörutvecklingen i två former av cancer, fibrosarkom och livmoderkroppscancer.

Jag har deltagit i ett projekt för att förbättrat råttans ideogram (*delarbete I*). Ett ideogram är en schematisk förteckning och beskrivning över alla kromosomer hos en art. Detta används för att identifiera och räkna antalet kromosomer i en cell från den arten. Det är vanligt att kromosomerna i en cancercell skiljer sig åt mot dem i en normal cell från samma art, med avseende på antal kromosomer samt deras utseende. Det är därför viktigt att kunna identifiera vilka delar av cellens kromosomer som blivit förändrade och hur.

På 1970-talet utvecklades ett modellsystem för fibrosarkom (cancer i mjukdelarna). I den råttmodellen används DMBA för att inducera cancer. DMBA är att ämne som påminner om de ämnen som bildas i cigarettrök och som finns i luftföroreningar, därför är det viktigt att studera dess påverkan på kroppen. Den här avhandlingen fokuserar på ett avgränsat område på råttans kromosom 1 i modellen för fibrosarkom. Vi undersökte först en kopieökning av två gener på sista delen av kromosom 1 (*delarbete II*), därefter koncentrerades arbetet på en förlust av en gen i samma område (*delarbete III*). Vi lyckades identifiera gener som var inblandade i kopietalsökningen (*Omp* och *Jak2*), samt förlusten av en annan gen (*Pten*) på råttans kromosom 1.

Vidare har vår grupp etablerat ett modellsystem för livmoderkroppscancer i råtta. Honor från råttstammen BDII utvecklar spontant cancer i livmodern. Eftersom livmoderns uppbyggnad och hormonresponsen är likartad hos råtta och människa utgör råttan en utmärkt modell för livmoderkroppscancer hos människa. Livmoderkroppstumörerna från den etablerade råttmodellen har tidigare undersökts med flera olika genom-omfattande metoder. Denna grundläggande kunskap har jag sedan dragit nytta av i mina studier där jag har kunnat identifiera och karaktärisera ett antal målgener som bidragit till tumörutvecklingen i denna cancerform. Jag har undersökt tumörerna från råttmodellen enligt de kriterier som används för att dela in livmoderkroppsstumörer hos människa i olika typer (*delarbete IV*). Vi kunde konstatera att tumörerna från modellen stämde väl överens med de hormonberoende tumörer av typ I hos människa som representerar ca 80% av cancer i livmodern. Vidare har jag testat om ett flertal genförändringar funna i råtttumörerna också är förändrade på samma sätt i tumörer från människa (*delarbete V*). Flera gener (*SDC1*, *MYCN*, *CDK6* och *MET*) visade sig finnas i många fler kopior i tumörerna än de gör i normal vävnad. Tidigare studier på människa, i en annan tumörform (neuroblastom), har visat att *MYCN* kan användas som markör, avgörande för vilken cancerbehandling som bör sättas in.

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grund för utvecklingen av biomarkörer eller fungera som angreppspunkt i riktad behandling av cancer.

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The possession of facts is knowledge; the use of them is wisdom.
Thomas Jefferson

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Man cannot create the current of events. He can only float with it and steer.
Otto von Bismarck

But women can…..
Emma Samuelson
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REFERENCES

1. Vogelstein, B. and K.W. Kinzler, *Cancer genes and the pathways they control*. Nat Med, 2004. 10(8): p. 789-99.
2. Yokota, J., *Tumor progression and metastasis*. Carcinogenesis, 2000. 21(3): p. 497-503.
3. Haber, D.A. and E.R. Fearon, *The promise of cancer genetics*. Lancet, 1998. 351 Suppl 2: p. SII1-8.
4. Kwabi-Addo, B., et al., *Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression*. Proceedings of the National Academy of Sciences of The United States of America, 2001. 98(20): p. 11563-11568.
5. Heinen, C.D., C. Schmutte, and R. Fishel, *DNA repair and tumorigenesis: lessons from hereditary cancer syndromes*. Cancer Biol Ther, 2002. 1(5): p. 477-85.
6. Rajagopalan, H. and C. Lengauer, *Aneuploidy and cancer*. Nature, 2004. 432(7015): p. 338-41.
7. Breivik, J., *Don't stop for repairs in a war zone: Darwinian evolution unites genes and environment in cancer development*. Proc Natl Acad Sci U S A, 2001. 98(10): p. 5379-81.
8. Stark, G.R., et al., *Recent progress in understanding mechanisms of mammalian DNA amplification*. Cell, 1989. 57: p. 901-908.
9. Schwab, M., *Amplification of oncogenes in human cancer cells*. Bioessays, 1998. 20(6): p. 473-9.
10. Schwab, M., *Oncogene amplification in solid tumors*. Seminars in Cancer Biology, 1999. 9(4): p. 319-325.
11. Savelyeva, L. and M. Schwab, *Amplification of oncogenes revisited: from expression profiling to clinical application*. Cancer Lett, 2001. 167(2): p. 115-23.
12. Al-Kuraya, K., et al., *Prognostic relevance of gene amplifications and coamplifications in breast cancer*. Cancer Res, 2004. 64(23): p. 8534-40.
13. Knudson Jr, A.G., *Mutation and cancer: statistical study of retinoblastoma*. Proceedings of the National Academy of Sciences of The United States of America, 1971. 68(4): p. 820-823.
14. Cavenee, W.K., et al., *Expression of recessive alleles by chromosomal mechanisms in retinoblastoma*. Nature, 1983. 305(5937): p. 779-784.
15. Consortium, I.H.G.S., *Finishing the euchromatic sequence of the human genome*. Nature, 2004. 431(21 October): p. 931-945.
16. Aitman, T.J., et al., *Progress and prospects in rat genetics: a community view*. Nat Genet, 2008. 40(5): p. 516-22.
17. Gill III, T.J., et al., *The rat as an experimental animal*. Science, 1989. 245: p. 269-276.
18. Gould, M.N., B. Wang, and C.J. Moore, *Modulation of Mammary Carcinogenesis by Enhancer and Suppressor Genes*, in *Genes and Signal Transduction in Multistep Carcinogenesis*, e. In: Colburn NH, Editor. 1989, Marcel Dekker, Inc. 270 Madison Avenue, New York. p. 19-38.
19. Chin, L. and J.W. Gray, *Translating insights from the cancer genome into clinical practice*. Nature, 2008. 452(7187): p. 553-63.
20. Quintanilla, M., et al., *Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis*. Nature, 1986. 322: p. 78-80.
21. Sills, R.C., et al., *Mutations in ras genes in experimental tumours of rodents*. IARC Scientific Publications, 1999. 146: p. 55-86.

22. Osaka, M., et al., *Loss of heterozygosity at the N-ras locus in 7,12-dimethylbenz(a)anthracene-induced rat leukemia*. Molecular Carcinogenesis, 1997. 18: p. 206-212.
23. Russo, J. and I.H. Russo, *Experimentally induced mammary tumors in rats*. Breast Cancer Research and Treatment, 1996. 39: p. 7-20.
24. Mitelman, F. and G. Levan, *The chromosomes of primary 7,12-dimethylbenz(a)anthracene-induced rat sarcomas*. Hereditas, 1972. 71(2): p. 325-34.
25. Levan, G., U. Ahlström, and F. Mitelman, *The specificity of chromosome A2 involvement in DMBA-induced rat sarcomas*. Hereditas, 1974. 77: p. 263-280.
26. Walentinsson, A., et al., *Genome-wide assessment of genetic alterations in DMBA-induced rat sarcomas: Cytogenetic, CGH and allelotype analyses reveal recurrent DNA copy number changes in rat chromosome 1, 2, 4, and 7*. Genes, Chromosomes and Cancer, 2000. 28: p. 184-195.
27. *Statistics - Health and Diseases. Cancer Incidence in Sweden 2002, The National Board of Health and Welfare*.
28. Vollmer, G., *Endometrial cancer: experimental models useful for studies on molecular aspects of endometrial cancer and carcinogenesis*. Endocr Relat Cancer, 2003. 10(1): p. 23-42.
29. Lax, S.F., *Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification*. Virchows Archiv, 2004.
30. Gruber, S.B. and W.D. Thompson, *A population-based study of endometrial cancer and familial risk in younger women. Cancer and steroid hormone study group*. Cancer Epidemiological Biomarkers Preview, 1996. 5(6): p. 411-417.
31. Sandles, L.G., *Familial endometrial adenocarcinoma*. Clinical Obstetrics and Gynecology, 1998. 41(1): p. 167-171.
32. Sandles, L.G., et al., *Endometrial adenocarcinoma: genetic analysis suggesting heritable site-specific uterine cancer*. Gynecologic Oncology, 1992. 47(2): p. 167-171.
33. Jadoul, P. and J. Donnez, *Conservative treatment may be beneficial for young women with atypical endometrial hyperplasia or endometrial adenocarcinoma*. Fertil Steril, 2003. 80(6): p. 1315-24.
34. Wallace, C.A. and T.J. Aitman, *The rat comes clean*. Nat Genet, 2004. 36(5): p. 441-2.
35. Kaspareit-Rittinghausen, J., F. Deerberg, and K. Rapp, *Mortality and incidence of spontaneous neoplasms in BDII/Han rats*. Zeitschrift für Versuchstierkunde, 1987. 30(5-6): p. 209-216.
36. Roshani, L., et al., *Genetic identification of multiple susceptibility genes involved in the development of endometrial carcinoma in a rat model*. International Journal of Cancer, 2001. 94(6): p. 795-799.
37. Roshani, L., et al., *Genetic analysis of susceptibility to endometrial adenocarcinoma in the BDII rat model*. Cancer Genetics and Cytogenetics, 2005. 158: p. 137-141.
38. Lynch, H.T., et al., *Endometrial carcinoma: multiple malignancies, constitutional factors, and heredity*. The American Journal of the Medical Sciences, 1966. 252(4): p. 381-390.
39. Walentinsson, A., et al., *Independent amplification of two gene clusters on chromosome 4 in rat endometrial cancer: identification and molecular characterization*. Cancer Research, 2001. 61(22): p. 8263-8273.
40. Samuelson, E., et al., *Amplification studies of MET and Cdk6 in a rat endometrial tumor model and their correlation to human type I endometrial carcinoma tumors*. Hormonal Carcinogenesis V, 2008. 617: p. 511-517.

41. Karlsson, A., et al., *Amplification of Mycn, Ddx1, Rrm2, and Odc1 in rat uterine endometrial carcinomas*. Genes Chromosomes Cancer, 2001. 31(4): p. 345-56.
42. Adamovic, T., et al., *Oncogene amplification in the proximal part of chromosome 6 in rat endometrial adenocarcinomas as revealed by combined BAC/PAC FISH analysis, chromosome painting, zoo-FISH and allelotyping analysis*. Genes, Chromosomes and Cancer, 2005. 44(2): p. 139-153.
43. Levan, K., et al., *Chromosomal alterations in 98 endometrioid adenocarcinomas analyzed with comparative genomic hybridization*. Cytogenet Genome res, 2006. 115(1): p. 16-22.
44. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer*. Cell, 2000. 100(1): p. 57-70.
45. Quackenbush, J., *Microarray analysis and tumor classification*. N Engl J Med, 2006. 354(23): p. 2463-72.
46. Bild, A.H., et al., *Oncogenic pathway signatures in human cancers as a guide to targeted therapies*. Nature, 2006. 439(7074): p. 353-7.
47. Downward, J., *Cancer biology: signatures guide drug choice*. Nature, 2006. 439(7074): p. 274-5.
48. Overbergh, L., et al., *The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression*. J Biomol Tech, 2003. 14(1): p. 33-43.
49. Sanger, F., *Determination of nucleotide sequences in DNA*. Biosci Rep, 2004. 24(4-5): p. 237-53.
50. Samuelson, E., et al., *Absence of Ras mutations in rat DMBA-induced mammary tumors*. Mol Carcinog, 2008.
51. Stam, P. and J.W. Van Ooijen, *JoinMap (tm) version 2.0: Software for the calculation of genetic linkage maps*. CPRO-DLO, Wageningen, 1995.
52. Levan, G., *Nomenclature for G-bands in rat chromosomes*. Hereditas, 1974. 77: p. 37-52.
53. Shuster, M.I., et al., *A consistent pattern of RIN1 rearrangements in oral squamous cell carcinoma cell lines supports a breakage-fusion-bridge cycle model for 11q13 amplification*. Genes, Chromosomes and Cancer, 2000. 28(2): p. 153-163.
54. Rogers, K.E., et al., *Molecular cloning and sequencing of a cDNA for olfactory marker protein*. Proceedings of the National Academy of Sciences of The United States of America, 1987. 84(6): p. 1704-1708.
55. Otano-Joos, M., et al., *Detection of chromosomal imbalances in leiomyosarcoma by comparative genomic hybridization and interphase cytogenetics*. Cytogenetics and Cell Genetics, 2000. 90(1-2): p. 86-92.
56. Yamasaki, Y., et al., *Mouse chromosome 19 and distal rat chromosome 1: a chromosome segment conserved in evolution*. Hereditas, 2001. 134(1): p. 23-34.
57. Di Cristofano, A. and P.P. Pandolfi, *The multiple roles of PTEN in tumor suppression*. Cell, 2000. 100(4): p. 387-390.
58. Mutter, G.L., *Pten, a protean tumor suppressor*. American Journal of Pathology, 2001. 158(6): p. 1895-1898.
59. Kolasa, I.K., et al., *PTEN mutation, expression and LOH at its locus in ovarian carcinomas. Relation to TP53, K-RAS and BRCA1 mutations*. Gynecol Oncol, 2006. 103(2): p. 692-7.
60. Di Cristofano, A., et al., *Pten is essential for embryonic development and tumour suppression*. Nature Genetics, 1998. 19(4): p. 348-355.
61. Kwabi-Addo, B., et al., *Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression*. Proc Natl Acad Sci U S A, 2001. 98(20): p. 11563-8.

62. Kwon, C.H., et al., *Pten haploinsufficiency accelerates formation of high-grade astrocytomas*. Cancer Res, 2008. 68(9): p. 3286-94.
63. Macleod, K., *Tumor suppressor genes*. Current Opinion in Genetics and Development, 2000. 10(1): p. 81-93.
64. Ollikainen, M., et al., *Patterns of PIK3CA alterations in familial colorectal and endometrial carcinoma*. Int J Cancer, 2007. 121(4): p. 915-20.
65. Sorosky, J.I., *Endometrial cancer*. Obstet Gynecol, 2008. 111(2 Pt 1): p. 436-47.
66. Hecht, J.L. and G.L. Mutter, *Molecular and pathologic aspects of endometrial carcinogenesis*. J Clin Oncol, 2006. 24(29): p. 4783-91.
67. Liu, F.S., *Molecular carcinogenesis of endometrial cancer*. Taiwan J Obstet Gynecol, 2007. 46(1): p. 26-32.
68. Ryan, A.J., et al., *Endometrial cancer*. Cell Tissue Res, 2005. 322(1): p. 53-61.
69. Deerberg, F. and J. Kaspareit, *Endometrial carcinoma in BDII/Han rats: model of a spontaneous hormone-dependent tumor*. Journal of the National Cancer Institute, 1987. 78: p. 1245-1251.
70. Kariola, R., et al., *APC and beta-catenin protein expression patterns in HNPCC-related endometrial and colorectal cancers*. Fam Cancer, 2005. 4(2): p. 187-90.
71. Lax, S.F., *Molecular genetic changes in epithelial, stromal and mixed neoplasms of the endometrium*. Pathology, 2007. 39(1): p. 46-54.
72. Behboudi, A., et al., *High-density marker loss of heterozygosity analysis of rat chromosome 10 in endometrial adenocarcinoma*. Genes, Chromosomes and Cancer, 2001. 32: p. 330-341.
73. Nordlander, C., et al., *Recurrent chromosome 10 aberrations and Tp53 mutations in rat endometrial cancer*. Manuscript, 2005.
74. Adamovic, T., et al., *Rearrangement and allelic imbalance on chromosome 5 leads to homozygous deletions in the CDKN2A/2B tumor suppressor gene region in rat endometrial cancer*. Cancer Genet Cytogenet, 2008. 184(1): p. 9-21.
75. Nordlander, C., et al., *Analysis of chromosome 10 aberrations in rat endometrial cancer-evidence for a tumor suppressor locus distal to Tp53*. Int J Cancer, 2007. 120(7): p. 1472-81.
76. Helou, K., et al., *Analysis of genetic changes in rat endometrial carcinomas by means of comparative genome hybridization*. Cancer Genetics and Cytogenetics, 2001. 127(2): p. 118-127.
77. Nordlander, C., et al., *Allelic imbalance on chromosome 10 in rat endometrial adenocarcinomas*. Cancer Genet Cytogenet, 2005. 156(2): p. 158-66.
78. Seeger, R.C., et al., *Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas*. New England Journal of Medicine, 1985. 313(18): p. 1111-1116.
79. Maeda, T., C.M. Alexander, and A. Friedl, *Induction of syndecan-1 expression in stromal fibroblasts promotes proliferation of human breast cancer cells*. Cancer Res, 2004. 64(2): p. 612-21.
80. Tsanou, E., et al., *Clinicopathological study of the expression of syndecan-1 in invasive breast carcinomas. correlation with extracellular matrix components*. J Exp Clin Cancer Res, 2004. 23(4): p. 641-50.
81. Pascual-Le Tallec, L., et al., *Identification of genes associated with the corticotroph phenotype in bronchial carcinoid tumors*. J Clin Endocrionol Metab, 2002. 87(11): p. 5015-5022.
82. Sasaki, M., et al., *CYP1B1 gene polymorphisms have higher risk for endometrial cancer, and positive correlations with estrogen receptor alpha and estrogen receptor beta expressions*. Cancer Res, 2003. 63(14): p. 3913-8.

83. McGrath, M., et al., *Cytochrome P450 1B1 and catechol-O-methyltransferase polymorphisms and endometrial cancer susceptibility*. Carcinogenesis, 2004. 25(4): p. 559-65.
84. Tokizane, T., et al., *Cytochrome P450 1B1 is overexpressed and regulated by hypomethylation in prostate cancer*. Clin Cancer Res, 2005. 11(16): p. 5793-801.
85. Meyerson, M. and E. Harlow, *Identification of G1 kinase activity for Cdk6, a novel cyclin D partner*. Molecular and Cellular Biology, 1994. 14(3): p. 2077-2086.
86. Hirai, Y., et al., *Putative gene loci associated with carcinogenesis and metastasis of endocervial adenocarcinomas of uterus determined by conventional and array-based CGH*. Am J Obstet Gynecol., 2004. 191(4): p. 1173-1182.
87. Costello, J.F., et al., *Cyclin-dependent kinase 6 (CDK6) amplification in human gliomas identified using two-dimensional separation of genomic DNA*. Cancer Research, 1997. 57(7): p. 1250-1254.
88. Lim, J., M. Mansukhani, and I. Weinstein, *Cyclin-dependent kinase 6 associates with the androgen receptor and enhances its transcriptinal activity in prostate cancer cells*. Proc Natl Acad Sci U S A, 2005. 14(102): p. 5156-5161.
89. Ma, P.C., et al., *c-Met: Structure, functions and potential for therapeutic inhibition*. Cancer and Metastasis Review, 2003. 22: p. 309-325.

Manu Propia