Novel Tumor Suppressor Gene Candidates in Experimental Endometrial Carcinoma

– From Cytogenetic to Molecular Analysis

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Ph.D. thesis
Department of Cell and Molecular Biology
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UNIVERSITY OF GOTHENBURG

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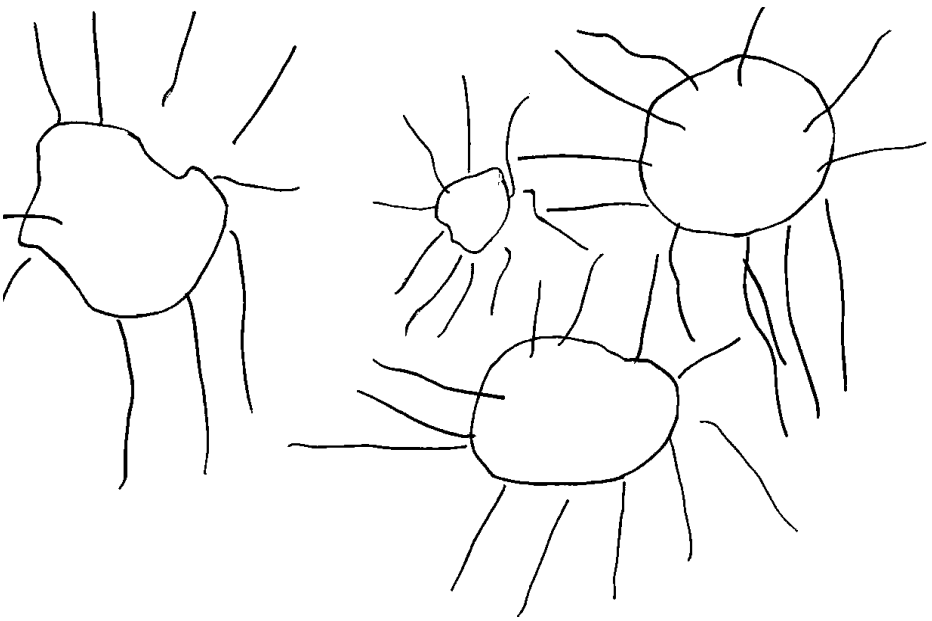
To Per, Linus and Simon



av Linus Hedberg 2009

You are the sunshine of my life……..

ABSTRACT

Novel Tumor Suppressor Gene Candidates in Experimental Endometrial Carcinoma – From Cytogenetic to Molecular Analysis

Carola Hedberg

Endometrial carcinoma (EC) is the most common form of gynecological malignancy, ranking fourth in incidence among tumors diagnosed in women. As is the case with other complex diseases, detailed analyses of the underlying mechanisms of cancer are difficult, due mainly to the genetic heterogeneity of the human population and differences in the environment and lifestyle of individuals. In this sense, analysis in animal models may serve as a valuable complement. The inbred BDII rat strain is genetically prone to spontaneous hormone-related EC and it has been used as a powerful model to investigate molecular alterations in this tumor type. BDII female rats were crossed with males from two non-susceptible rat strains and tumors were developed in a significant fraction of the progeny. We subjected a subset of BDII rat tumors to detailed analysis based on the molecular data used for the classification of human ECs. Our analysis revealed that this tumor model can be related to higher grade human type I ECs, i.e. a subgroup of ECs that constitutes more than 80% of this tumor type in humans.

Earlier work using comparative genome hybridization (CGH) revealed that rat chromosome 10 (RNO10) was frequently involved in cytogenetic aberrations in BDII rat tumors. To identify the potential target region(s)/gene(s) for these changes, we subjected a panel of rat ECs to allelic imbalance (AI) analysis. Four distinct regions of recurrent AI were identified. By deriving evolutionary tree models based on AI data, we demonstrated that one of these AI regions (located adjacent to *Tp53*) was close to the root in the derived onco-tree models, indicating that this segment might harbor early important events. In combined FISH, chromosome paint, gene expression and gene sequencing analyses, we found that, instead of *Tp53*, the main selection target was a region close and distal to *Tp53*. We developed a detailed deletion map of this area and substantially narrowed down the size of the candidate region. We then subjected all 19 genes located within this segment to qPCR analysis, followed by statistical analysis of the results, and thus identified the *Hic1*, *Skip* and *Myo1c* genes as potential target(s). By subjecting these genes to DNA sequencing, analysis of protein expression and of epigenetic silencing, we ruled out *Hic1* and confirmed *Skip* and *Myo1c* as the candidates. Interestingly, it appears that *Skip* and *Myo1c* perform overlapping roles in PI 3-kinase/Akt signaling, which is known to have implications for the survival and growth of cancer cells. In conclusion, starting from cytogenetic findings and applying a candidate gene approach, we introduced two attractive candidate genes within the independent region of tumor suppressor activity distal to *Tp53*.

Key words: cancer, endometrial cancer, rat models, rat chromosome 10, deletion, allelic imbalance, FISH, gene expression, tumor suppressor gene, *Hic1*, *Skip*, *Myo1c*

LIST OF PUBLICATIONS

The thesis is based on the following papers that are referred to in the text by their Roman numbers:

I. Samuelson E, **Hedberg C**, Nilsson S and Behboudi A. Molecular classification of spontaneous endometrial adenocarcinomas in BDII rats. *Endocrine-Related Cancer*: *2009*; *16: 99-111*.

II. **Nordlander C**, Behboudi A, Levan G and Klinga-Levan K. Allelic imbalance on chromosome 10 in rat endometrial adenocarcinomas. *Cancer Genetics and Cytogenetics*: *2005*; *156: 158-166*.

III. Chen L, **Nordlander C**, Behboudi A, Olsson B and Klinga-Levan K. Deriving evolutionary tree models of the oncogenesis of endometrial adenocarcinomas. *International Journal of Cancer*: *2006*; *120: 292-296*.

IV. **Nordlander C**, Karlsson S, Karlsson Å, Sjöling Å, Winnes M, Klinga-Levan K and Behboudi A. Analysis of chromosome 10 aberrations in rat endometrial cancer – evidence for a tumor suppressor locus distal to *Tp53*. *International Journal of Cancer*: *2007*; *120: 1472-1481*.

V. **Hedberg C**, Garcia D, Linder A, Samuelson E, Ejeskär K, Abel F, Karlsson S, Nilsson S and Behboudi A. Analysis of the independent tumor suppressor loci telomeric to *Tp53* suggests *Skip* and *Myo1c* as novel tumor suppressor gene candidates in experimental endometrial carcinoma. *Manuscript*.

Other publications not included in this thesis:

- 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*; *157: 97-103*.

- Cronkhite J, **Norlander* C**, Furth J, Levan G, Garbers D and Hammer R. Male and female germaline specific expression of an EGFP reporter gene in a unique strain of transgenic rats. *Developmental Biology: 2005*; *284: 171-183*.
  * Nordlander

- Falk E, **Hedberg C**, Klinga-Levan K and Behboudi A. Specific numerical and structural chromosome changes contributed to endometrial carcinogenesis revealed by SKY analysis in a rat model for the disease. *Manuscript*.

TABLE OF CONTENTS

ABSTRACT **4**

LIST OF PUBLICATIONS **5**

TABLE OF CONTENTS **6**

ABBREVIATIONS **8**

INTRODUCTION **9**

GENETICS 9
GENETIC BASIS OF CANCER 11
GENES INVOLVED IN CANCER 12
Proto-oncogenes *12*
Tumor suppressor genes *13*
DNA repair genes *13*
GENETIC INSTABILITY IN CANCER 14
Chromosomal instability *14*
Microsatellite instability *14*
EPIGENETIC CHANGES IN CANCER 15
COMPLEX DISEASES AND ANIMAL MODELS 15
THE LABORATORY RAT AS A POTENT MODEL ORGANISM 16
ENDOMETRIAL CARCINOMA AND A POWERFUL RAT MODEL FOR THIS MALIGNANCY 16
Molecular basis of distinction between types I and II tumors *17*
Treatment and prognosis of endometrial carcinoma *19*
The inbred BDII rat tumor model *19*
Rat chromosomes 10 ..... *20*
FROM WHOLE GENOME TO IDENTIFICATION OF CANDIDATE GENE(S) 21

AIMS OF THE STUDY **23**

EXPERIMENTAL BACKGROUND **24**

MATERIAL 24
Animal crosses *24*
Tumor material *25*
METHODS 28
Polymerase chain reaction (Papers I, II, IV and V) *28*
Allelic imbalance/loss of heterozygosity (Papers I-III) *29*
Mutation screening by DNA sequencing (Papers I, IV-V) *31*

Real-time quantitative PCR (qPCR) and analysis of data (Papers I and V) *32*
Derivation of evolutionary tree models using AI/LOH data to select candidate chromosomal segments harboring early important events (Paper III) *34*
Chromosome painting and dual-color fluorescence in situ hybridization (FISH, Paper IV) *35*
Northern blot (Paper IV) *36*
DNA methylation analysis (Paper V) *36*
5-aza-2´-deoxycytidine (5-Aza-dC) and/or trichostatin A (TSA) treatment (Paper V) *37*
Semi-quantitative RT-PCR (Paper V) *37*
Western blot (Paper V) *37*

RESULTS AND DISCUSSION **38**

PAPER I: MOLECULAR CLASSIFICATION OF SPONTANEOUS ENDOMETRIAL ADENOCARCINOMAS IN BDII RATS 38
PAPER II: FOUR SEGMENTS SHOW ALLELIC IMBALANCE ON CHROMOSOME 10 IN RAT ENDOMETRIAL ADENOCARCINOMAS 41
PAPER III: DERIVING EVOLUTIONARY TREE MODELS OF THE ONCOGENESIS OF ENDOMETRIAL ADENOCARCINOMAS 44
PAPER IV: ANALYSIS OF CHROMOSOME 10 ABERRATIONS IN RAT ENDOMETRIAL CANCER – EVIDENCE FOR A TUMOR SUPPRESSOR LOCUS DISTAL TO TP53 45
PAPER V: DETAILED ANALYSIS OF THE INDEPENDENT TUMOR SUPPRESSOR LOCI TELOMERIC TO *TP53* SUGGESTS *SKIP* AND *MYO1C* AS NOVEL TUMOR SUPPRESSOR GENE CANDIDATES IN THIS REGION 49

CONCLUDING REMARKS **52**

SWEDISH SUMMARY – SAMMANFATTNING PÅ SVENSKA **53**

REFERENCES **56**

ABBREVIATIONS

aa	amino acid
AI	allelic imbalance
AIR	allelic imbalance ratio
5-aza-dC	5-aza-2´-deoxycytidine
BAC	bacterial artificial chromosome
BDII	rat inbred strain (*BDII/Han*)
BN	rat inbred strain (*Brown Norway*)
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CGH	comparative genome hybridization
CIN	chromosomal instability
DAPI	diamidino-2-phenylindole
ddNTP	dideoxynuclotide triphosphate
DM	double minute
DNA	deoxyribonucleic acid
dNTP	deoxynucelotide triphosphate
EAC	endometrial adenocarcinomas
EC	endometrial cancer
F1	first generation of a cross
F2	second generation intercross (F1xF1)
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
HSA	human chromosome (*homo sapiens*)
HSR	homogeneously staining region
kb	kilo base pairs
kDa	kiloDalton
LOH	loss of heterozygosity
Mb	mega base pairs
MIN	microsatellite instability
N1	backcross generation (F1xP)
NME	non-malignant endometrium
NUT	rat uterine tumor, N1 (back-cross) progeny
PAC	P1 artificial chromosome
PCR	polymerase chain reaction
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
RNO	rat chromosome (*rattus norvegicus*)
RT-PCR	reverse transcriptase polymerase chain reaction
RUT	rat uterine tumor, F1 and F2 progeny
SNP	single nucleotide polymorphism
SPRD	rat inbred strain (*Sprague-Dawley*)
SRO	smallest region of overlap
ST	solid tumor
TC	tumor cell culture
TSA	trichostatin
TSG	tumor suppressor gene

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

INTRODUCTION

Genetics

The word “genetic” comes from the ancient Greek word “genesis”, which means “origin”. Genetics is the science of heredity and variation in living organisms. Modern genetics started in the mid-nineteenth century with the work of Gregor Mendel, when he observed that organisms inherit traits in a distinct way (Weiling 1991). In 1944, Oswald Theodore Avery, Colin McLeod and Maclyn McCarty identified the molecule responsible for this transformation of traits as DNA (deoxyribonucleic acid) (Lederberg 1994). DNA contains the genetic information or the code of life and is organized in chromosomes within the cell nucleus. DNA is a large polymer composed of four different nucleotides, adenine (A), guanine (G), thymine (T) and cytosine (C). The structure of DNA is a double helix in which two DNA molecules (DNA strands) are held together by weak hydrogen bonds. According to the Watson-Crick model, hydrogen bonds occur between parallel bases of the two strands, as adenine specifically binds to thymine and cytosine to guanine (Fig. 1) (Watson and Crick 1953). Each strand of the DNA molecule can act as a template for creating a new partner strand by a process called DNA replication.

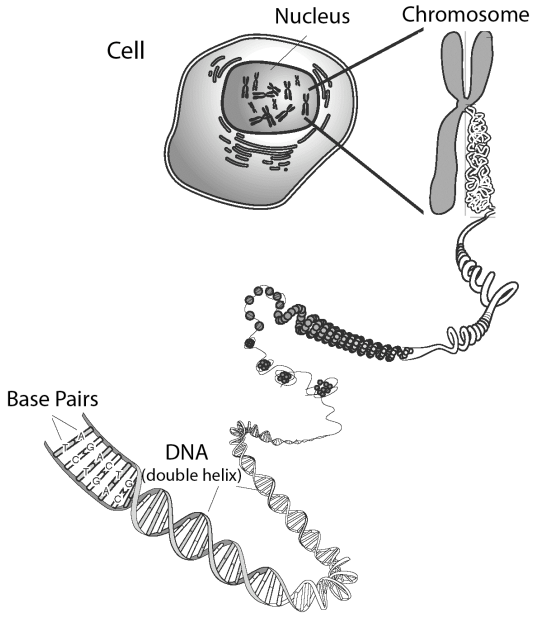


Figure 1. Schematic presentation of chromosomes within the cell nucleus. In the figure, a simplified image of a chromosome and its structure in relation to the DNA sequence is shown. Image modified from National Institutes of Health, National Human Genome Research Institute.

The central dogma of molecular biology: the flow of genetic information from DNA → RNA → Protein was postulated by Francis Crick and is called the "central dogma" (Crick 1970). To transmit genetic information from the DNA molecule to the polypeptide chain of the protein, DNA first needs to be translated into a RNA molecule through a process called transcription. The RNA (ribonucleic acid) structure is similar to that of DNA, except in one base: thymine (T) in DNA is substituted by uracil (U) in RNA. An additional difference between RNA and DNA molecules is that RNA generally exists as a single-stranded molecule. The primary RNA transcript is modified at the ends and spliced so that portions of non-coding segments of the gene, the "introns", are removed. The remaining RNA is now called mRNA (messenger RNA) and contains all the information in coding sequences, i.e. the "exons". It is transported from the nucleus to the cytoplasm, where mRNA is translated into the amino acid sequence of the encoded polypeptide and folded to the correct structure of the protein (Fig. 2). However, there are RNA molecules with other roles – for example, the genome of retroviruses and the end products of some genes that function as regulatory elements, e.g. miRNA involved in different cell activities, including gene expression regulation.

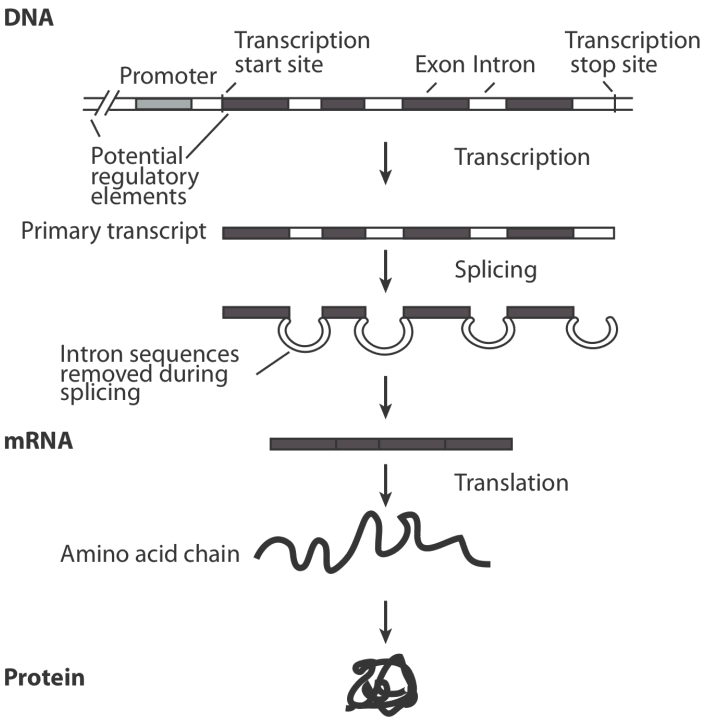


Figure 2. Flow of information from DNA to RNA to protein.

Genetic basis of cancer

Cancer is a complex disease characterized by abnormal cell proliferation. It is caused by the accumulation of genetic alterations resulting in the loss of control of cellular growth. Cancer comprises several hundred different forms that can arise in almost every tissue. Each cancer type has a unique feature, but the basic processes of the transformation of normal cells into cancer cells appear to be essentially the same. It has been suggested that, during tumor development and progression, tumor cells in general exhibit six acquired capabilities through different mechanisms (Hanahan and Weinberg 2000).

1. Self-sufficiency in growth signals
2. Insensitivity to anti-growth signals
3. Evading apoptosis
4. Sustained angiogenesis
5. Limitless replicative potential
6. Tissue invasion and metastasis

In other words, tumor development can be described as a stepwise evolutionary process involving multiple genetic events.

Models of carcinogenesis: The "stochastic models" of carcinogenesis hold that transformation results from random mutation and subsequent clonal selection. The process starts in a single ancestral cell, in which the first mutation occurs, either inherited or produced in the specific tissue itself, providing a growth advantage for the cell and thus yielding a particular clone of more rapidly growing cells. Further accumulation of mutations among these cells drives the progression from normal tissue to tumor development (Nowell 1976; Vogelstein and Kinzler 2004). The "stem cell model" of carcinogenesis suggests that cancer originate in tissue stem or pro-genitor cells probably through dysregulation of self-renewal pathways. This leads to expansion of this cell population that may then undergo further genetic or epigenetic changes to become fully transformed to a cancer cell (Ashkenazi, et al. 2008; Wicha, et al. 2006). The primary tumor is usually not invasive or metastatic; these properties arise only after the additional collection of genetic alterations in the tumor cells. Since the same alterations do not occur in every cell of the tumor, there is usually a development of sub-clones with different altered genes within the tumor (Fig. 3). Consequently, the tumor is biologically heterogeneous (Yokota 2000).

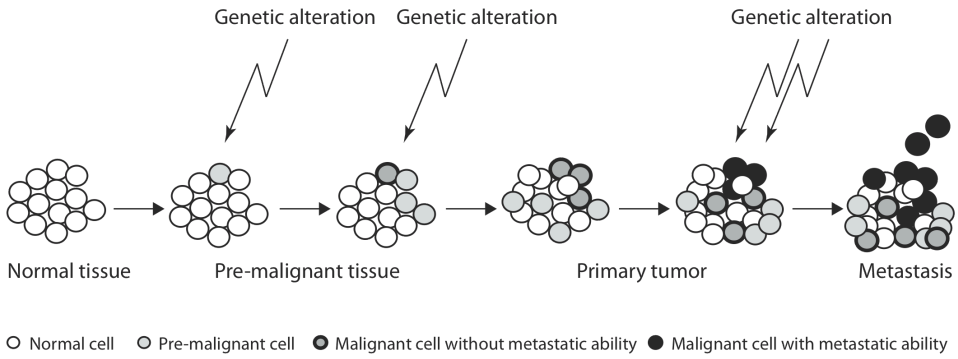


Figure 3. By the sequential accumulation of genetic alterations, normal cell is transformed into tumor mass. Cancer develops through a multi-step process of genetic changes. In fact, it is estimated that four to six essential alterations are required to overcome the normal defense mechanisms of the organism against cancer and thus enable a tumor to develop. Image modified from Yokota 2000.

Genes involved in cancer

The genetic alterations leading to cancer are often found in certain types of genes with specific activities known as proto-oncogenes and tumor suppressor genes (TSG). These genes are involved in the gatekeeper machinery of the cell that regularly controls the balance between cell growth and cell death. An additional group of genes frequently reported to be involved in genetic changes during tumorigenesis are known as DNA repair genes. This latter group of genes is responsible for keeping the genome intact. Whenever certain combinations of these genes are mutated, the normal cell can escape growth control, start to proliferate in an uncontrolled manner and transform to a cancer cell.

Proto-oncogenes

Proto-oncogenes are groups of genes that, in normal conditions, are involved in the control of essential functions of cell growth, such as cell proliferation and differentiation. Proto-oncogenes include genes encoding growth factors, growth factor receptors, signal transducers and transcription factors (Haber and Fearon 1998; Nebert 2002). The abnormal activation of a proto-oncogene (thereafter an oncogene) can be caused by point mutation, chromosomal rearrangement and/or amplification, all resulting in the increased and/or sustainable expression of these genes. For example, during a chromosomal rearrangement, the proto-oncogene may move to a new position in the genome, where it may come under the control of a highly active promoter belonging to another gene and thus be over-expressed. Gene amplification manifests cytogenetically as homogeneously staining regions (HSR) or double minutes (DM) and results in an increased copy number of the gene and thereby increased gene expression (Fig. 4 f, g) (Albertson, et al. 2003).

Tumor suppressor genes

Tumor suppressor genes (TSG) are normally involved in the control of cell proliferation and differentiation and loss or inactivation of both alleles of this group of genes is suggested to be associated with malignancy (Haber and Harlow 1997; Hinds and Weinberg 1994; Weinberg 1994). According to Knudson's "two-hit" theory of TSG inactivation, the first hit is usually a point mutation or submicroscopic deletion in the first allele, followed by the second hit often in somatic cells that inactivates the intact allele (Fig. 4) (Hino 2003; Knudson Jr 1971). The second hit may result from different genetic mechanisms, such as the loss of the whole chromosome by mitotic non-disjunction, chromosomal translocation followed by the loss of a part of the chromosome harboring the TSG, mitotic recombination and subsequent selection, or deletion of the segment that harbors the TSG (Fig. 4 a-e) (Albertson, et al. 2003; Devilee, et al. 2001). Consequently, in these examples, the second hit generates the loss of heterozygosity (LOH) in a chromosomal segment spanning the TSG, which can be used as a tool for mapping TSGs by using polymorphic loci in the region. Recently, implication of epigenetic silencing of one or both copies of a TSG has been shown in cancer and thus has been included in a modified version of the Knudson model (Jones and Baylin 2007). Moreover, it has been suggested for certain TSGs, such as $p27^{Kip1}$, the function of the gene might be in a haploinsufficient mode (Cook and McCaw 2000; Quon and Berns 2001). Haploinsufficiency is defined as a situation in which the protein produced by a single copy of an otherwise normal gene is not sufficient to assure normal function and tumor progression may thus occur (Paige 2003; Payne and Kemp 2005).

DNA repair genes

To ensure genome integrity, a complex network of a DNA repair system has evolved, including several types of "DNA repair" or "caretaking" genes. These genes are involved in the machinery responsible for correcting DNA sequence errors generated by polymerase mistakes or by mutagens. Two major repair systems have been described as nucleotide-excision repair (NER) and mismatch repair (MMR) systems (Lengauer, et al. 1998; Rajagopalan and Lengauer 2004). It is suggested that any defect in the caretaking machinery may indirectly promote tumor development by at least increasing the rate of mutations (Kinzler and Vogelstein 1997).

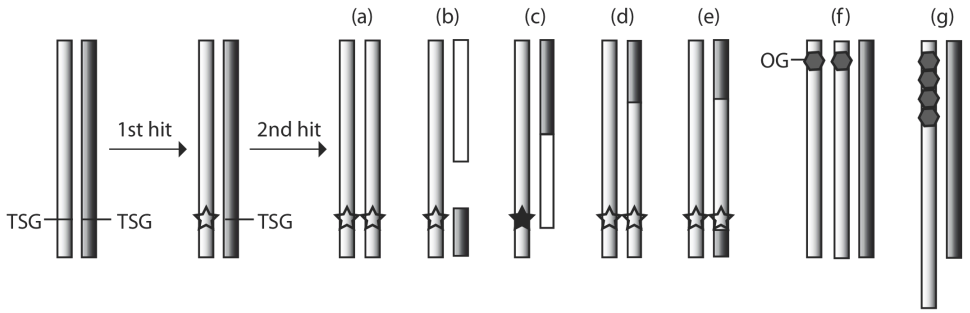


Figure 4. Examples of chromosomal changes leading to AI. According to Knudson's classical model for the inactivation or removal of a TSG, the "first hit" is usually in form of a mutation in the DNA sequence, followed by the "second hit" that results in the omission of the other normal allele, leading to LOH. The "second hit" may lead to LOH in a chromosome region across the location of the TSG through: (a) the non-disjunction and reduplication of the mutated chromosome, (b) subchromosomal deletion, (c) unbalanced translocation and (d,e) mitotic recombination. Another mechanism potentially resulting in AI in a chromosomal region is increase in the copy number or amplification of an activated oncogene through (f) aneuploidy or (g) HSR, for example.

Genetic instability in cancer

Cancer cells frequently exhibit genetic instability in the form of chromosomal instability (CIN) and/or microsatellite instability (MIN) (Rajagopalan and Lengauer 2004).

Chromosomal instability

CIN refers to the occurrence of gross chromosomal changes in the form of losses and gains of whole chromosomes (aneuploidy) or large chromosomal segments. Aneuploidy is when there is an aberrant chromosome number in the cell deviating from a multiple of the haploid genome. There are several genes that can make a potential contribution to CIN, including those involved in the formation of mitotic spindles (e.g. *BUB1*, *MAD2*), those encoding centromeric protein and cell cycle checkpoint genes (e.g. *CYCLIN E, CDC4*), as well as genes involved in the mitotic process (e.g. *AURORA A*, *APC*) (Lengauer, et al. 1998; Rajagopalan and Lengauer 2004).

Microsatellite instability

MIN is characterized by alterations in the sequence of microsatellite markers, resulting in new microsatellite allele(s) in the tumor material compared with its corresponding normal DNA. MIN arises due to mutations in mismatch repair genes (e.g. *MLH1*, *MSH2*). These genes are responsible for the post-replicative DNA repair and act to correct mismatches that arise through the incorrect incorporation of nucleotides or the slippage of DNA polymerases during DNA replication (Lengauer, et al. 1998).

Epigenetic changes in cancer

Epigenetic mechanisms describe heritable states, which do not depend on DNA sequence. In cancerogenesis, it has become evident that the epigenetic silencing of TSGs may be functionally equivalent to genetic alterations, such as mutation and deletion (Delcuve, et al. 2009; Esteller 2008; Grønbaek, et al. 2007). It is suggested that epigenetic alteration involves two major mechanisms: DNA methylation and the covalent modification of histones. In addition to these two, a third mechanism referred to as RNA interference has recently been suggested to be also involved in the regulation of gene expression (Downward 2004; Novina and Sharp 2004; Siomi and Siomi 2009).

DNA methylation is the methylation of cytosine bases within CpG islands. The CpG islands are unevenly distributed in the genome and are defined as stretches of DNA, over 0.5 kb in length, with a G+C content equal to or more than 55%. Many CpGs are located near or within the promoter of a gene and hypo- or hyper-methylation at these sites may contribute to gene activation or silencing, respectively.

Histone modification involves the covalent modification of amino acid residues in the histone proteins around which the DNA is wrapped. The most common of these alterations are acetylation, methylation and phosphorylation, all of which are post-translational modifications that regulate the structure of the chromatin and thereby gene expression. For instance, the removal of acetyl groups will result in a closed chromatin structure that cannot be accessed by the transcriptional machinery and thereby inhibits gene expression.

RNA interference involves two types of small RNA molecules that are involved in gene expression regulation, i.e. micro RNA (miRNA, single-stranded RNA molecules, 21-23 nucleotides in length) and small interfering RNA (siRNA, double-stranded RNA molecules of 20-25 nucleotides). These small RNAs can bind to specific RNA molecules and increase or decrease their activity and/or lifetime. For instance, it has been shown that siRNA can bind to their specific mRNA molecules and prevent them from being translated to a functionally active protein.

Complex diseases and animal models

The detection of genetic changes responsible for a complex disease can be difficult, due mainly to the genetic heterogeneity of individuals, as well as the diversity of lifestyle and environmental factors influencing the human population (Consortium 2004a). In human cancer studies, it is therefore difficult to determine which of the observed genetic changes play a significant part in the development of the tumor. Experimental model systems may help to minimize this complication, as, by using isogenic inbred animal strains, for example, the background genetic diversity can be substantially reduced and the environmental factors can be reasonably controlled. In an inbred strain, all animals have the same genetic

composition and each member of the strain is therefore an "identical twin" to other members of the strain. Consequently, it is reasonable to suggest that the detection and characterization of important genetic alterations might be easier when model systems for the disease are used. The results of studies in the model systems have been shown to be readily transferable to the human situation by using maps comparing humans with the model (Aitman, et al. 2008).

The laboratory rat as a potent model organism

The laboratory rat (*Rattus norvegicus*) has been widely used as the model system of choice in biomedical research, namely in analysis of complex traits within the fields of physiology, toxicology, neurobiology and cancer (Abbott 2004; Aitman, et al. 2008; Gill III, et al. 1989; Hedrich 1990; Szpirer, et al. 1996). Hundreds of inbred rat strains have been developed by selective breeding, most of them to model complex human diseases, such as hypertension, diabetes and cancer (Aitman, et al. 2008). There is a great similarity in physiology and hormone responsiveness pattern between humans and rats at organ level, making the rat an excellent model for human cancer genetic investigations (Gould, et al. 1989).

The rat genome contains about the same number of genes as the human and mouse genomes. "Disease-related genes" appear to be highly conserved through mammalian evolution, as almost all human genes known to be associated with diseases have counterparts in the rat genome (Lindblad-Toh 2004). The rat genome, in its diploid form, consists of approximately 2.5 billion base pairs of DNA organized into 21 pairs of chromosomes, compared with the human genome with 3.3 billion base pairs organized into 23 chromosome pairs. The human and rat genomes are estimated to contain approximately 21,000-25,000 known and predicted protein-coding genes encoding for 40,000-60,000 transcripts (Bourque, et al. 2004; Gibbs, et al. 2004; Worley, et al. 2008).

Endometrial carcinoma and a powerful rat model for this malignancy

Endometrial carcinoma (EC) is the most common gynecological malignancy in the western world (Esteller, et al. 1999; Ryan, et al. 2005) and its incidence is increasing (Amant, et al. 2005). In Sweden, approximately 1,300 new cases of this cancer type are diagnosed every year, accounting for 5.7% of all diagnosed cancers among Swedish women (Socialstyrelsen 2002).

Endometrial carcinoma arises from the endometrium, the lining layer inside the uterus. Normal endometrium consists of epithelial glandular and stromal components (Fig. 5) that undergo structural alterations in response to fluctuations in estrogen and progesterone during the menstrual cycle. It has been suggested that endometrial carcinoma (also referred to as uterine cancer) occurs when the cycle of hormones is disturbed by hormone replacement therapies, genetic alterations, or both. Like many other cancer types, the transition from normal endometrium to a malignant tumor is thought to involve a stepwise accumulation of alterations in cellular mechanisms leading to dysfunctional cell growth.

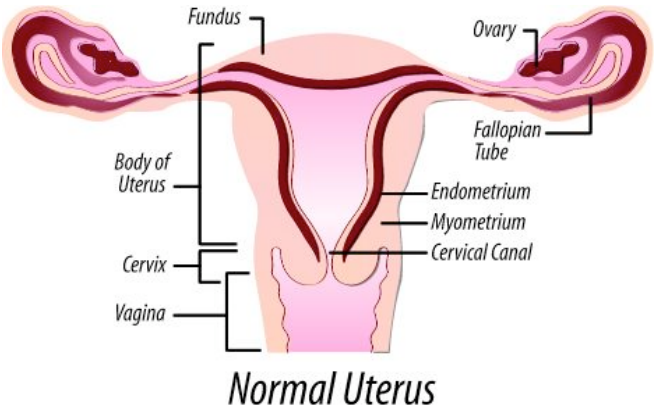


Figure 5. Schematic picture of the female reproductive organ. Tumors arising in the cells lining the uterus (endometrium) are referred to as endometrial cancer.

EC is composed of several tumor types each with its specific clinico-pathological and molecular features. The majority of cases are roughly divided into two main subtypes: type I, estrogen-related tumors, and type II, non-estrogen-related tumors. The estrogen-related type I tumors are of endometrioid histology, generally associated with endometrial hyperplasia, and comprise about 75-80% of all endometrial carcinomas. These tumors are associated with risk factors such as estrogen replacement therapy and/or obesity, whereas the risk decreases with each pregnancy. The non-estrogen-related type II tumors comprise about 20-25% of all endometrial carcinomas, are of non-endometrioid histology (serous papillary or clear cell morphology) and usually associated with a poor prognosis.

Molecular basis of distinction between types I and II tumors

In addition to clinico-pathological features, it appears that types I and II tumors differ markedly in their molecular mechanisms of transformation. The molecular basis of the distinction between types I and II tumors is only partially understood. These molecular alterations are of prognostic value, but they have not provided a basis for improved therapy (Salvesen, et al. 2009). The genetic alterations described for type I tumors can be illustrated in a signaling pathway that is initiated by estrogen stimulation (Fig. 6) (Di Cristofano and Ellenson 2007).

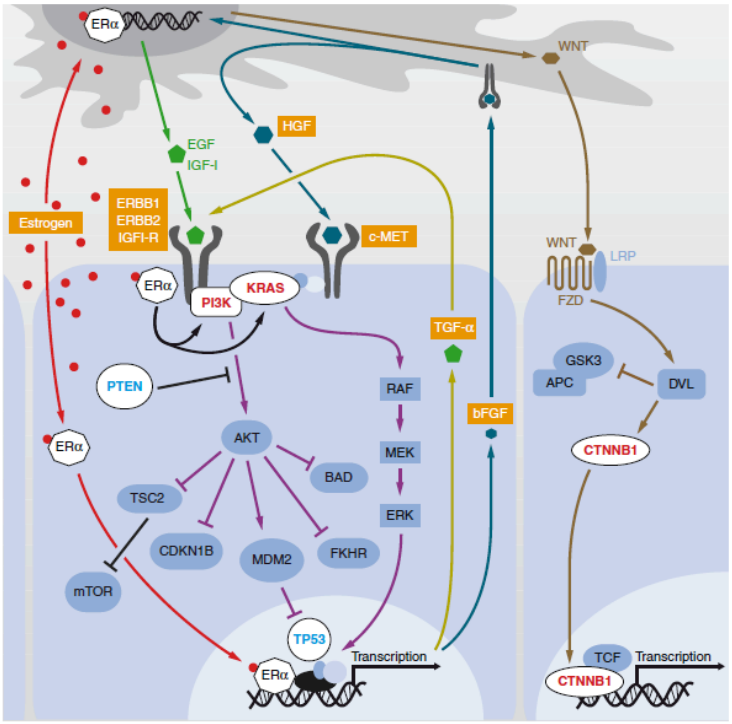


Figure 6. Signaling pathway initiated by estrogen stimulation in the endometrium, including the most commonly reported alterations in endometrial cancer. The white boxes show the most commonly altered genes reported in this cancer type: inactivating targets in blue and activating targets in red text. The genes presented in orange boxes are those for which the up-regulation of gene expression has been reported. Image adapted from Di Cristofano et al., 2007 and "Reprinted, with permission, from the Annual Review of Pathology, Volume 2 © 2007 by Annual Reviews: www.annualreviews.org".

One of the most common genetic alterations reported in type I tumors is the mutation/inactivation of the *PTEN* tumor suppressor gene, which is detected in 50-80% of cases. It has been suggested that *PTEN* inactivation, along with *MLH1* mutations (reported in 20-45% of type I tumors), acts as the deriving force for the transformation of the normal endometrium to endometrial hyperplasia. Several additional genetic changes are required to transform hyperplasic lesions to a full-blown carcinoma; they include MSI (resulting from *MLH1* mutations) and mutation of *KRAS* (in 10-30% of cases), as well as *CTNNB1* (in about 20% of type I tumors). *TP53* mutations are mainly reported in high-grade endometrial carcinomas and they have therefore been suggested to be involved in the transformation of low-grade to high-grade type I tumors (Fig. 7).

The two major genetic alterations described in type II tumors are *TP53* (early event reported in 90% of the tumors) and *CDH1* mutations (a later event detected in 80-90% of type II tumors). However, *ERBB2* over-expression and *CDKN2A/P16* inactivation are also described as two other common genetic aberrations in type II tumors (Fig. 7) (Boyd 1996; Esteller, et al. 1999; Lax 2004; Purdie 2003; Salvesen, et al. 2009; Sherman 2000).

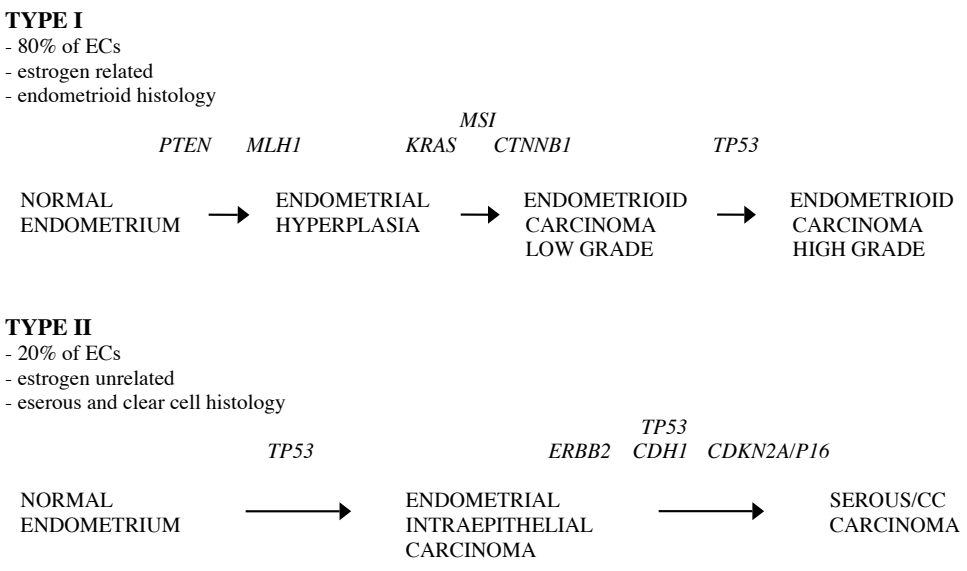


Figure 7. Endometrial cancer progression model. Major genetic alterations during the carcinogenesis process are compared between type I and type II endometrial carcinomas.

Treatment and prognosis of endometrial carcinoma

When a woman is diagnosed with EC, the most common procedure is to surgically remove her uterus and ovaries (Alvarez Secord, et al. 2007; Wang, et al. 2007). Patients with a high risk of relapse are given adjuvant treatment and, in some more aggressive cases, the lymph nodes are surgically removed and examined for signs of metastasis (Jadoul and Donnez 2003). Approximately 75% of cases are diagnosed with the tumor confined to the uterine corpus, but, after primary surgery, 15-20% of these tumors recur and show a limited response to systemic therapy (Salvesen, et al. 2009). In the light of these recurrences, along with the fact that the prevalence and mortality of EC is constantly increasing, there is a major need for the development of reliable prognostic markers, as well as new and effective intervention strategies for this malignancy. The development of these intervention approaches has been hampered by limitations in the understanding of the mechanisms involved in this tumor type.

The inbred BDII rat tumor model

Female rats of the BDII rat strain are highly prone to develop spontaneous endometrial cancer; more than 90% of virgin females develop EC before the age of 24 months (Deerberg and Kaspareit 1987; Kaspareit-Rittinghausen, et al. 1987). The inbred BDII rat model for spontaneous endometrial carcinogenesis provides a powerful tool for detailed analysis of genetic factors contributing to the carcinogenesis and progression of this complex disease

(Vollmer 2003). It has been shown that tumors developed in this model are estrogen related, since, when animals were ovariectomized, tumor incidence was radically reduced (Kaspareit-Rittinghausen, et al. 1990).

Our research group has been working with the genetic and molecular characterization of this tumor model during the last decade and, through our work, this model has become very well characterized in terms of its cytogenetic, genetic and molecular features. A genome-wide screening with microsatellite markers identified susceptibility regions on rat chromosomes (RNO) 1, RNO11 and RNO17 in the BDIIxSPRD genetic crosses and on RNO12 and RNO20 in the BDIIxBN crosses. Interestingly, the chromosome regions affecting susceptibility to endometrial carcinoma were different in the two genetic crosses, suggesting that various genes interact in the different genetic backgrounds (Roshani, et al. 2005; Roshani, et al. 2001). Cytogenetic and comparative genome hybridization (CGH) analysis of tumors revealed recurrent alterations in several chromosomes and chromosomal regions, including amplification/gains in RNO4 and RNO6 and losses in RNO5, RNO10 and RNO15 (Hamta, et al. 2005; Helou, et al. 2001). RNO10 was one of the most frequently altered chromosomes, with recurrent losses in the proximal to middle part, usually combined with gains in the distal part of the chromosome (Behboudi, et al. 2001; Hamta, et al. 2005; Helou, et al. 2001).

Rat chromosomes 10

RNO10 is homologous to segments of human chromosomes (HSA) 5, 12, 16 and the entire HSA17 (Fig. 8A) (Behboudi, et al. 2002; Bourque, et al. 2004; Consortium 2004b). Several cancer-related genes are reported to be located on these chromosomes/chromosome segments, e.g. *TSC2*, *IRF1*, *TP53*, *BRCA1*, *NF1*, *ERBB2* and *GRB2*.

In the present work, we chose specifically to focus on the changes observed in the middle to distal part of RNO10, which is homologous to HSA17. Although the entire HSA17 is homologous to the distal part of RNO10, the gene order was shown not to be entirely conserved between these two chromosomes, most likely due to evolutionary breaks and inversions that have occurred in at least seven places along the chromosome (Fig. 8B) (Behboudi, et al. 2002).

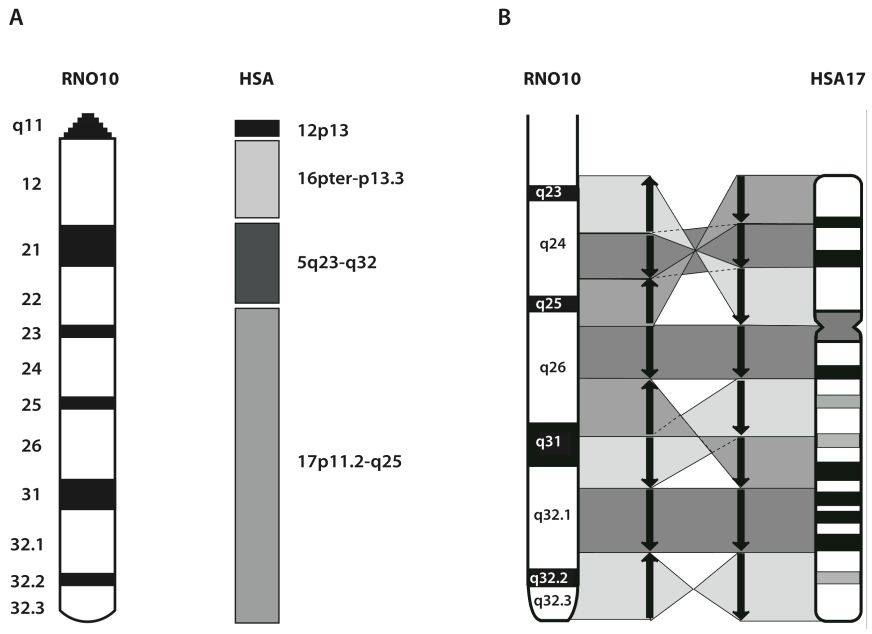


Figure 8. A: Comparative map of RNO10 and the corresponding homologous chromosomal segments in humans (HSA 12, 16, 5 and 17). B: The middle to distal part of RNO10 is homologous to the entire HSA17; however, gene order is not conserved, since chromosome breaks followed by inversions had occurred, probably resulting from evolutionary events taking place in the divergence of the two species. (Adapted and modified from Behboudi et al., 2002)

From whole genome to identification of candidate gene(s)

Different approaches can be used when it comes to finding and defining the potential candidate gene(s) for a disease. One of the most commonly used methods involves first identifying chromosome(s) with recurrent aberrations on a whole genome basis. This can be carried out by using genetic techniques such as conventional cytogenetic analysis (Caspersson, et al. 1970), spectral karyotyping (SKY) (Schrock, et al. 1996), comparative genome hybridization (CGH) and CGH arrays (Kallioniemi, et al. 1992; Pinkel, et al. 1998). The results of these studies usually suggest whole chromosome(s) or large chromosomal segments as candidate targets for further analysis. The next step is to narrow down these regions by defining the smallest regions of overlap (SRO) for aberrations in the whole tumor set. This can be done using fine molecular techniques, namely FISH-based chromosome analysis (Ried, et al. 1998), polymorphic marker allelotyping (Skotheim, et al. 2001), SNP analysis (Sapolsky, et al. 1999) and expression arrays (Bucca, et al. 2004; Luo, et al. 2003) combined with real-time quantitative PCR (Kubista, et al. 2006). Once a candidate gene(s) is identified, advanced molecular techniques such as mutation sequencing, promoter methylation, protein expression assays and functional analysis (e.g. cell migration and

transfection-based analyses) can be used to verify the finding and to find and define the potential function(s) of the candidate gene (Sapolsky, et al. 1999) (Fig. 9).

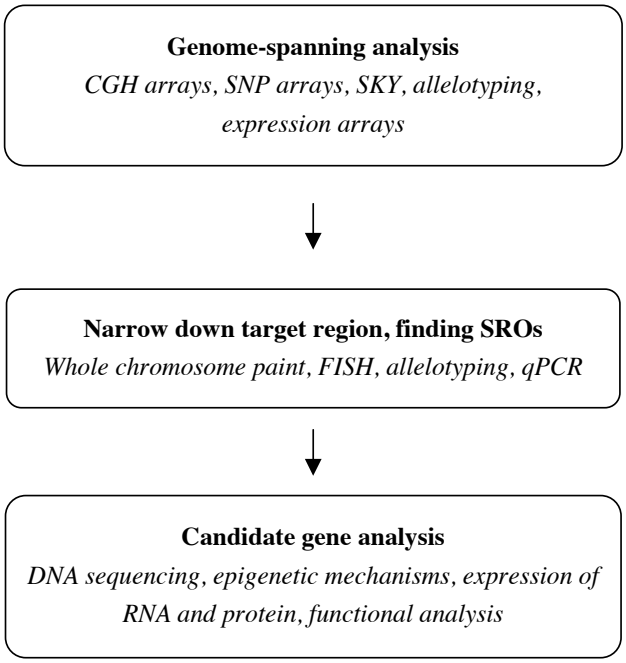


Figure 9. Schematic presentation of workflow from whole genome to finding candidate gene(s).

AIMS OF THE STUDY

The overall objective of the present work was to use the BDII rat tumor model for human endometrial cancer to provide a better understanding of the mechanisms involved in the development and progression of this cancer type. Particular emphasis focused on aberrations affecting RNO10 in this animal model.

The specific aims were

- To compare and contrast molecular findings in the BDII rat EC with those of human EC in order to determine how effectively this model represents the corresponding tumors in humans, *Paper I*

- To define the smallest regions of overlap (SRO) of RNO10 aberrations using microsatellite allelotyping analysis of the chromosome in a panel of EC tumors, *Paper II*

- To examine whether AI/LOH data could be used in mathematical algorithms to determine order of important RNO10-related genetic events in this model by deriving evolutionary tree models, *Paper III*

- To characterize the frequently deleted chromosomal segment at the mid-proximal part of RNO10. This chromosomal segment was identified to harbor early and important event(s) implicated in EC tumorigenesis by the derived tree models, *Paper IV*

- To find the best candidate(s) in a minimal segment of AI/deletion in the neighborhood of *Tp53* and define their potential contribution to EC tumorigenesis, *Paper V*

EXPERIMENTAL BACKGROUND

Materials and methods are explained in detail in Papers I-V and are only briefly described in this section.

Material

Animal crosses

Females of the inbred BDII/Han rat strain are genetically prone spontaneously to develop endometrial cancer, particularly of the EAC subtype. More than 90% of virgin females develop this tumor type before the age of 24 months.

F1 progeny was produced in crosses between BDII female rats and male rats from two EC non-susceptible strains, BN/Han and SPRD-Cu3/Han. In order to produce F2 progeny, brother-sister mating was performed among F1 animals. Back-cross progeny (N1) was generated by crossing F1 male rats with BDII female rats (Fig. 10).

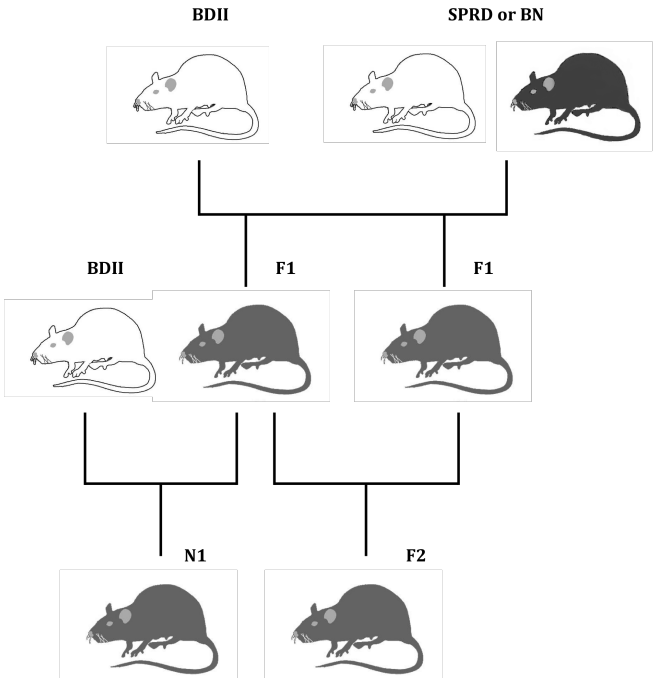


Figure 10. Female rats of the EC-susceptible BDII strain were crossed with male rats from two EC non-susceptible strains to produce F1 animals. F2 progeny was produced by brother-sister mating of F1 animals. A back-cross progeny (N1) was produced by crossing F1 males with BDII females.

Tumor material

The rats were kept in a specific pathogen-free (SPF) environment to ensure that infectious diseases did not interfere with the experiment. Tumors developed spontaneously in a fraction of F1, F2 and N1 females from all crosses and in most cases they were classified as EC. In addition, a few other types of malignant tumors and benign neoplasms were found in the animals (Table 1).

Table 1. Animal material and tumors developed in F1, F2 and back-cross (N1) offspring.

Cross	**Offspring**	**Female progeny**	**Tumors developed**	
			EAC	**Others***
BDII x BN	F1	18	10	2
	F2	59	11	12
	N1	105	26	14
BDII x SPRD	F1	17	2	5
	F2	54	9	22
	N1	103	32	11

* Benign neoplasms, such as cystic endometrial hyperplasia, endometrial cell polyps and mammary fibroadenomas, as well as malignant tumors, such as squamous cell carcinoma and sarcomas

The animals were palpated regularly and, when a tumor was suspected, the animal was sacrificed. At necropsy, tumors were collected and subjected to pathological analysis and DNA extraction. Cell cultures were set up from a number of tumors, DNA and RNA were extracted and cDNA and metaphase chromosomes were made. In addition, normal DNA from all animals was extracted from liver or spleen tissue (Table 2). In some cases no malignant cells were detected in the removed cell mass from animals when pathologically characterized. We believe that these tissues represent normal or pre-malignant endometrium and are therefore of great importance in the present study. Herein, these cell lines are referred to as non-malignant endometrium (NME) (Table 2).

Table 2. Material used in this study (Papers I-V): RUT, tumors derived from F1 and F2 inter-cross animals; NUT, tumors derived from back-cross animals; ST, solid tumor; TC, tumor cell culture. Age refers to the time at which the animal was sacrificed.

Tumor designation	Cross progeny	ST	TC	Age	Pathology	Paper
RUT5	BN (F2)		√	518	ESCC	III, IV
RUT7	BN (FI)		√	662	EAC	I, III, IV, V
RUT8	BN (F1)	√		652	EAC	III
RUT10	BN (F1)	√		669	EAC	III
RUT12	BN (F1)	√	√	677	EAC	I, IV, V
RUT18	BN (F2)	√		623	EAC	III
RUT21	BN (F2)	√		637	EAC	III
RUT24	BN (F2)	√		662	EAC	III
RUT25	BN (F2)		√	670	EAC	I, III, IV, V
RUT27	BN (F2)	√		668	EAC	III
RUT29	BN (F2)	√	√	679	MPM	III, IV
RUT30	BN (F2)		√	689	EAC	I, IV, V
RUT32	BN (F1)	√		634	EAC	III
NUT5	BN (N1)	√		511	EAC	II, III
NUT6	BN (N1)	√	√	471	EAC	I, II, III, IV, V
NUT9	BN (N1)	√		543	EAC	II, III
NUT16	BN (N1)	√	√	612	EAC	I, II, III, V
NUT26	BN (N1)	√		651	EAC	II, III
NUT27	BN (N1)	√		766	EAC	II, III
NUT31	BN (N1)	√	√	640	EAC	I, IV, V
NUT43	BN (N1)	√	√	670	EAC	II, III
NUT46	BN (N1)		√	666	EAC	I, V
NUT50	BN (N1)	√	√	702	EAC	I, II, III, IV, V
NUT51	BN (N1)	√	√	709	EAC	I, II, III, IV, V
NUT52	BN (N1)	√	√	673	EAC	I, II, III, IV, V
NUT76	BN (N1)	√	√	735	EAC	I, II, III, V
NUT81	BN (N1)	√	√	738	EAC	I, II, III, IV, V
NUT82	BN (N1)	√	√	738	EAC	I, II, III, V
NUT97	BN (N1)	√	√	738	EAC	I, II, III, IV, V
NUT98	BN (N1)	√	√	738	EAC	I, II, III, V
NUT99	BN (N1)	√	√	738	EAC	I, II, III, IV, V
NUT100	BN (N1)	√	√	738	EAC	I, II, III, IV, V
NUT103	BN (N1)	√		739	EAC	II, III
NUT127	BN (N1)	√	√	742	EAC	I, II, III, IV, V
NUT128	BN (N1)		√	748	EAC	I, IV, V
NUT130	BN (N1)	√		748	EAC	II, III
NUT209	BN (N1)	√		652	EAC	II, III
NUT118	BN (N1)		√	738	NME	I, V
NUT122	BN (N1)		√	742	NME	I, V
NUT123	BN (N1)		√	742	NME	I, V
NUT129	BN (N1)		√	748	NME	I, V

Table 2, cont.

Tumor designation	Cross progeny	ST	TC	Age	Pathology	Paper
RUT1	SPRD (F2)	√		610	US	III
RUT2	SPRD (F1)	√	√	565	EAC	I, III, IV, V
RUT3	SPRD (F2)	√	√	624	EAC	I, III, IV
RUT6	SPRD (F2)	√	√	638	EAC	I, III, IV, V
RUT9	SPRD (F2)	√		631	EAC	III
RUT13	SPRD (F2)		√	666	EAC	I, III, IV, V
RUT16	SPRD (F2)	√	√	688	EAC	III
RUT17	SPRD (F1)	√		776	EAC	III
RUT22	SPRD (F2)	√		729	EAC	III
RUT26	SPRD (F2)	√		780	EAC	III
NUT4	SPRD (N1)	√	√	560	EAC	I, II, III, IV, V
NUT7	SPRD (N1)	√	√	590	EAC	I, II, III, IV, V
NUT8	SPRD (N1)	√		604	ESP	II, III
NUT12	SPRD (N1)	√	√	692	EAC	I, II, III, IV, V
NUT14	SPRD (N1)	√	√	653	EAC	I, II, III, V
NUT15	SPRD (N1)	√		653	EPA	II, III
NUT17	SPRD (N1)	√		654	EAC	II, III
NUT19	SPRD (N1)	√	√	720	EAC	II, III
NUT29	SPRD (N1)	√		747	EAC	II, III
NUT33	SPRD (N1)	√		724	EAC	II, III
NUT35	SPRD (N1)	√		707	EAC	II, III
NUT39	SPRD (N1)	√	√	714	EAC	I, II, III, IV, V
NUT42	SPRD (N1)	√	√	741	EAC	I, II, III, IV, V
NUT47	SPRD (N1)	√	√	711	EAC	I, II, III, IV, V
NUT49	SPRD (N1)	√		712	EAC	II, III
NUT55	SPRD (N1)	√	√	780	EAC	I, II, III, IV, V
NUT59	SPRD (N1)	√		704	EAC	II, III
NUT70	SPRD (N1)	√		728	EAC	II, III
NUT84	SPRD (N1)	√	√	735	EAC	I, II, III, IV, V
NUT201	SPRD (N1)	√		511	EAC	II, III
NUT202	SPRD (N1)	√		745	EAC	II, III
NUT203	SPRD (N1)	√		662	EAC	II, III
NUT204	SPRD (N1)	√		765	ESP	II, III
NUT205	SPRD (N1)	√		732	EAC	II, III
NUT18	SPRD (N1)		√	644	NME	I, V
NUT58	SPRD (N1)		√	771	NME	I, V
NUT89	SPRD (N1)		√	738	NME	I, V

EAC: Endometrial adenocarcinoma; NME: Non-malignant endometrium; ESP: Endometrial stromal polyp; EPA: Endometrial papillary adenoma; ESCC: Endometrial squamous cell carcinoma; MPM: Malignant peritoneal mesothelioma; US: Uterine sarcoma

Methods

A summary of the methods used in this project is presented in Figure 11 and are short described in the text.

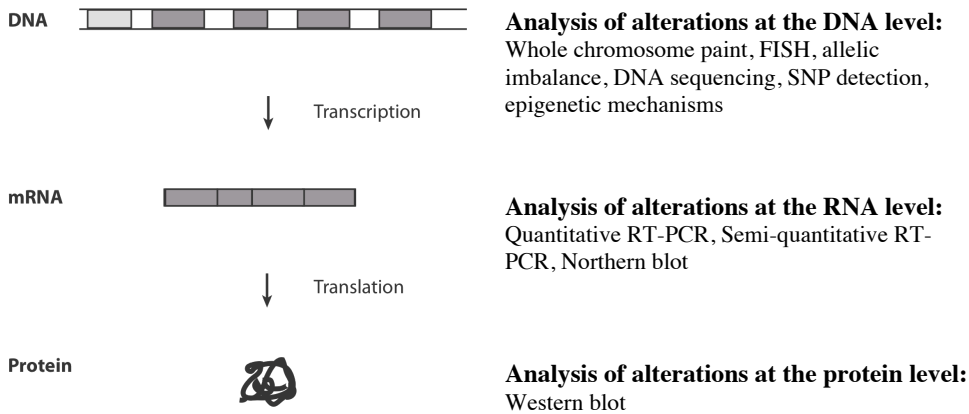


Figure 11. Summary of methods used in the present work.

Polymerase chain reaction (Papers I, II, IV and V)

The polymerase chain reaction (PCR), developed by Kary Mullis in 1984, represents a breakthrough in medical and biological research. Using this technique, a DNA template is amplified from a few copies to millions in a short time (Bartlett and Stirling 2003; Saiki, et al. 1988). The reaction is accomplished in a number of cycles of DNA amplification, each cycle comprising three steps. First, double-stranded DNA is denatured at a temperature of 94-96°C to produce a single-stranded template. The second step is to allow the primers to anneal to the single-stranded template DNA by reducing the temperature to 50-60°C. This is followed by the elongation step during which the synthesis of a new DNA strand based on the sequence in the template strand is performed by the enzyme DNA polymerase at 72°C (Fig. 12).

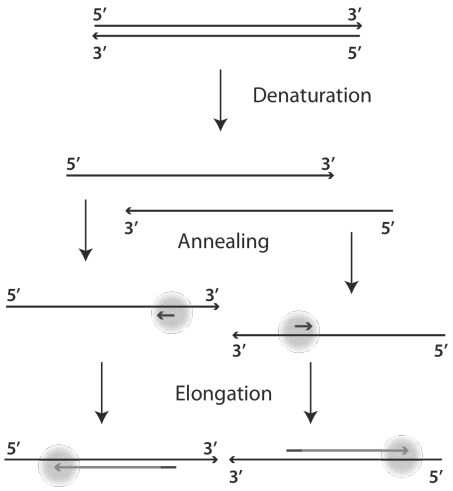


Figure 12. A PCR cycle, including denaturation, annealing and elongation steps.

Allelic imbalance/loss of heterozygosity (Papers I-III)

Microsatellites are a group of markers, which are tandem repetitions of di- tri- or tetranucleotide sequences and are randomly distributed across mammalian genomes (Goldstein and Schlotterer 1999). The number of repeats for a given marker may differ from one chromosome to another, making these markers appropriate for screening the genome for genetic changes and the genotyping of individuals. Genotyping of polymorphic markers is a technique in which size differences in markers caused by variations in the number of repeat units between individuals are recorded and can be used as a genetic signature for the individual (Fig. 13).

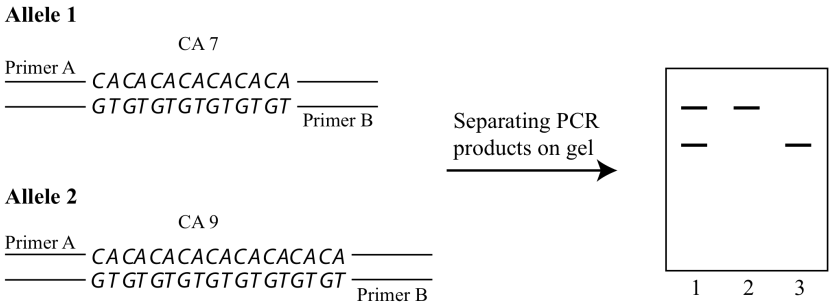


Figure 13. Microsatellite allelotyping using PCR. A marker is PCR amplified using primer pairs flanking the repeat, followed by the separation of the PCR products on the gel for visualization. Since every individual has two copies (alleles) at each locus, the person can be homozygous (equal number of repeats in both alleles) or heterozygous (different numbers of repeats in the two alleles) at the marker. In the figure, the DNA sample loaded in lane 1 is referred to as an "informative" sample, since the individual is heterozygous (two different alleles) for the marker. The DNA samples loaded in lanes 2 and 3 are examples of "uninformative" samples, since they are homozygous for the marker (the two alleles in each individual are identical).

Microsatellite markers are additionally used for allelotyping analysis of tumor cells (AI/LOH analysis) to detect chromosomal segments that are deleted and/or amplified. AI is defined when, in an allelotyping analysis, tumor DNA shows a significant deviation from the expected 1:1 ratio between the two parental alleles at a polymorphic locus (Devilee, et al. 2001; Skotheim, et al. 2001). When there is a complete absence of one allele in the whole tumor material, the condition is termed LOH (loss of heterozygosity). So, in microsatellite allelotyping analysis, if the tumor DNA shows hemi- or homozygosity for a certain marker, while the corresponding normal DNA is heterozygous, it is concluded that there is LOH at the marker site in the tumor sample. This might be a sign that a TSG located in the vicinity of the marker is deleted and thus, microsatellite allelotyping can be a useful tool for finding the approximate position of as yet unidentified TSGs.

In this work, a RNO10 marker set was selected from available microsatellite marker databases (RatMap, RGD, Wellcome Trust-Rat Mapping Resources). To determine markers polymorphic among the parental strains, PCR reactions on genomic DNA from the BDII, BN and SPRD rats, as templates, were performed with marker-specific primers. Based on the results, we selected a dense panel of polymorphic markers to screen informative tumors for AI/LOH at RNO10. The PCR reactions were performed with fluorescent dye-labeled dUTPs, followed by the separation of products in polyacrylamide gel electrophoresis in an automated ABI Prism™377 Genescan Analyzer (PE Applied Biosystems) (Cawkwell, et al. 1993). In this instrument, a laser beam scans the fluorescent bands and the software records the size and intensity of each allele as a peak for which peak height and area represent the quantity of each allele in the PCR product (Fig. 14). AI was determined by calculating the allelic imbalance ratio (AIR) formulas as follows:

$$AIR=(T1/T2)/(N1/N2)$$

where T1/T2 is the ratio of the areas of the two alleles in each marker in the tumor and N1/N2 is the corresponding ratio in the normal liver sample from the same animal. When the AIR was greater than 1, the inverted values were used to give AIR values in a range between 0 and 1. In our analysis, we used the cut-off value of 0.60 as an indicator of allelic imbalance and 0.15 as an indicator of the loss of heterozygosity (Skotheim, et al. 2001).

It is important to point out that AI may equally represent a chromosomal deletion as well as a chromosomal gain. It has been suggested that AIR values between 0.35-0.75 indicate a moderate gain of one allele, whereas very low AIR values (close to 0) be result of total loss or high amplification of one allele in an amplified region (Skotheim, et al. 2001). Accordingly, the accurate interpretation of AI data is possible if only cytogenetic data for the samples are available.

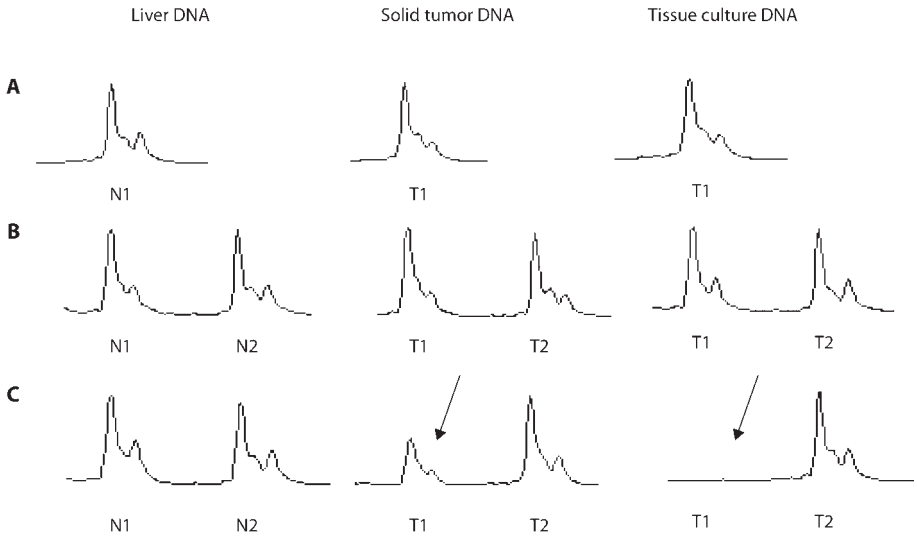


Figure 14. Examples of allele profiles resulted from an allelotyping analysis of one microsatellite marker in three different sample sets. Each sample set included three DNA samples: normal liver DNA, DNA extracted from solid tumors and the corresponding tumor tissue culture. In each sample set, N1 and N2 represent the peak areas in the normal control sample, whereas T1 and T2 are peak areas in the corresponding tumors. The allelic imbalance ratio (AIR) was calculated as AIR=(T1/T2)/(N1/N2). A: Normal DNA is homozygous for the marker and the sample is therefore uninformative, B: Normal DNA is heterozygous for the marker and the sample is therefore informative. However, the corresponding solid tumor and tumor cell culture show no AI and C: Another example of an informative sample set that shows AI (AIR less than 0.6) in the solid tumor DNA and LOH (AIR close to 0, i.e. total loss of one allele) in the tumor tissue culture DNA.

Mutation screening by DNA sequencing (Papers I, IV-V)

In DNA sequencing the order and content of nucleotides in a DNA molecule is determined. In this work, we used the Sanger (dideoxy) direct DNA sequencing method to sequence purified PCR products of interest amplified from genomic and/or cDNA sequences (Sanger 2004; Sanger and Coulson 1975). The purified PCR product was amplified in a cycle sequencing reaction, with a mixture of normal deoxynucleotide triphosphates (dNTPs) in excess, together with modified fluorescence-labeled dideoxynucleotide triphosphates (ddNTPs), using the BigDye sequencing reaction kit (Perkin Elmer, Foster City, CA) according to the protocol provided by the manufacturer. In this method, the ddNTPs are the chain-terminating nucleotides lacking a 3'-OH group required for the formation of a phospodiester bond between two nucleotides. After incorporation of ddNTPs, the DNA strand extension is terminated, producing fragments of various lengths. The sequencing products were separated on polyacrylamide gels by electrophoresis in an automated ABI Prism™377 Genescan Analyzer (PE Applied Biosystems). The obtained DNA sequences were compared with reference sequence in order to detect the potential alterations/mutations (Fig. 15). Bi-directional sequencing was used in the experiments.

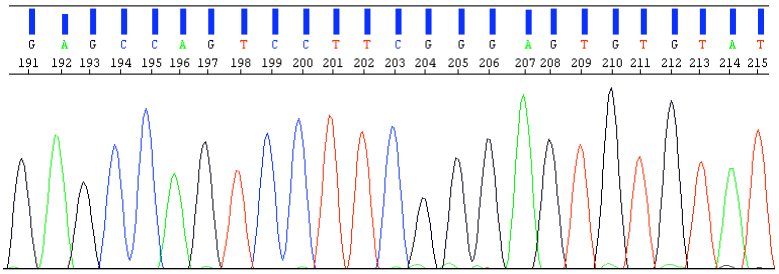


Figure 15. Example of a DNA fragment sequence obtained from an automatic sequencer using fluorescent dyes with Sanger's direct sequencing method.

Real-time quantitative PCR (qPCR) and analysis of data (Papers I and V)

Real-time quantitative PCR was performed to quantify the mRNA expression of a number of genes in the material. In this technique both detection and quantification of a specific sequence in a cDNA sample is made possible, as an absolute number of copies of a PCR product during each cycle of PCR amplification is measured (Kubista, et al. 2006; Overbergh, et al. 2003). The Taqman assay is a modification of this technique, in which the accumulation of a product is measured during exponential stages of the PCR using a fluorophore. This system is 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 (Fig. 16).

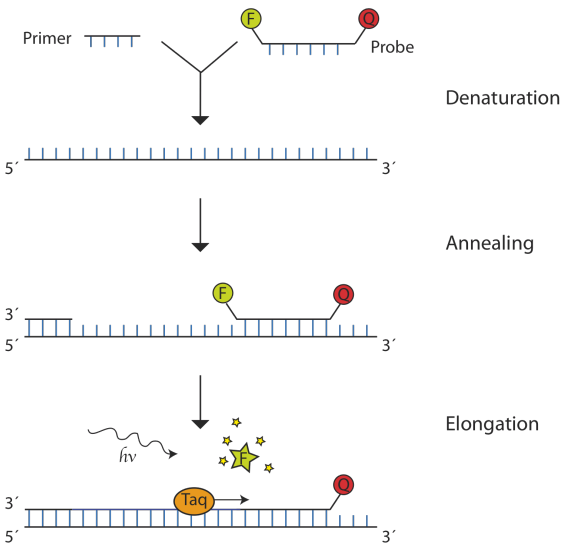


Figure 16. Schematic presentation of the TaqMan principle. The procedure starts with the denaturation of the DNA, followed by the annealing of the primers and probe to the target sequence of the DNA. During the elongation, Taq polymerase cleaves the probe by 5´-3´-exonuclease activity and fluorescence emission occurs. F, fluorophore; Q, quencher.

To analyze the real-time quantitative PCR data, fluorescence emissions corresponding to each cycle of PCR amplification were measured and plotted against the number of PCR cycles. In the diagram, the threshold line was set at the exponential phase of the amplification curve. From the plot, the C_T value was determined and corresponded to the number of PCR cycles at which a significant exponential increase in fluorescence (i.e. exponential increase of the PCR product) was detected (Fig. 17). The lower the C_T value, the higher the level of the expression of the target gene. Each assay was amplified in triplicate to minimize the effect of technical errors in the analysis and the average C_T value for each sample was calculated.

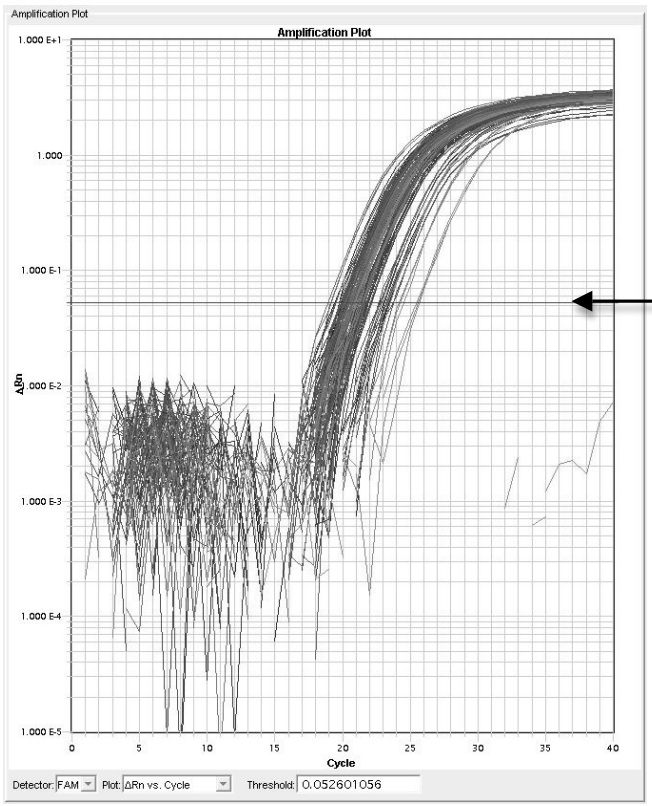


Figure 17. Example of a real-time qPCR amplification plot. The cycle number on the X-axis is plotted against the fluorescence emission on the Y-axis. The threshold is presented as the vertical red line (arrow) and is set to the exponential phase of the fluorescence emission curves.

To calculate the relative quantification of gene expression, we used the Relative Standard Curve Methods. The standard curve is used to determine the efficiency of the PCR amplification and 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 product are then 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, 1:18, 1:36 and 1:72) using cDNA from of one of the tumor samples (RUT30) and/or a commercially available rat RNA mix (Stratagene, La Jolla, CA, USA). For each gene, the mean C_T-value for triplicates was calculated, and the gene concentration 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 differences in mRNA expressions between NME samples and EAC tumor samples were evaluated using Welch's t-test and a value of $P < 0.05$ was considered to be significant. 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 of the tumor samples and the mean expression levels in the NME group.
The Welch's t-test, is similar to an ordinary student's two samples t-test in that sense it compares the mean of two samples, but the Welch t-test allows unequal variances between the samples (Welch 1947).

Derivation of evolutionary tree models using AI/LOH data to select candidate chromosomal segments harboring early important events (Paper III)

By using evolutionary tree algorithms on genetic data, the order of genetic events during oncogenesis can be determined (Desper, et al. 1999; Fitch and Margoliash 1967; Radmacher, et al. 2001). In this approach, we used AI data for RNO10 to determine early important changes along the chromosome in this material. To this end, the informative markers along RNO10 used for AI analysis were grouped into 24 chromosome segments (Table 3). To identify the non-random genetic events, distribution frequencies in each region were set up using a Monte Carlo simulation under the null hypothesis (i.e. all events occur randomly). Three different tree algorithms were applied, all resulting in a similar order of events. A statistical analysis was then performed to validate the degree of reliability of the models.

Table 3. Position of markers in the rat linkage map and central physical position of the segments defined by the 59 informative markers, determined from the Ensembl Genome Browser (v 35, Oct 2005).

Segment	Linkage position	Central physical position (kb)	Markers
1	9	5,202	D10Rat67, D10Rat96
2	14	9,762	D10Rat91, D10Rat103
3	24	13,118	D10Got25
4	26	15,676	D10Rat183, D10Rat50, D10Rat257, D10Rat47
5	27	17,544	D10Rat121, D10Rat42, D10Rat64, D10Rat182, D10Rat181, D10Got35
6	31	19,692	D10Rat180, D10Rat118, D10Rat113, D10Rat45
7	37	24,081	D10Rat43, D10Rat44
8	42	27,186	D10Mit10
9	46	30,738	D10Rat71, D10Mgh11, D10Rat39, D10Got46, D10Got49, D10Rat72, D10Mit9
10	52	35,694	D10Rat37, D10Mgh10
11	54	39,088	D10Mgh9
12	56	26,978	D10Rat165
13	60	53,787	D10Rat31, D10Rat32, D10Wox11
14	63	56,947	D10Wox12, D10Mgh7
15	66	65,686	D10Rat69, D10Mit2, D10Wox24, D10Got80
16	68	70,705	D10Rat98
17	70	79,229	D10Rat26
18	73	79,229	D10Rat24
19	77	83,528	D10Rat93, D10Rat86, D10Rat21
20	83	87,273	D10Rat106, D10Got120, D10Got128, D10Rat127
21	83	89,167	D10Rat55
22	88	93,903	D10Got133
23	94	96,800	D10Got144, D10Mgh4
24	98	109,525	D10Rat137, D10Rat11, D10Rat105

Chromosome painting and dual-color fluorescence in situ hybridization (FISH, Paper IV)

The question was then to determine the extent to which gross chromosomal aberrations such as amplifications, deletions or translocations affected RNO10 in the tumors. To address this question, we applied RNO10 whole chromosome paint analysis to the tumor material (Ried, et al. 1998). The biotin-labeled paint probe was hybridized to denatured chromosome preparations from the tumors and the signals were detected by adding FITC-conjugated avidin (Fig. 18). To visualize the banding pattern, the chromosomes were counterstained with DAPI. Detected aberrations were then confirmed and verified using dual-color gene-specific fluorescence *in situ* hybridization (FISH). We used ten biotin- or digoxigenin-labeled BACs and PACs probes, evenly spread from the middle to the distal part of the chromosome, and were able to determine the approximate position of chromosome breaks and/or deletions along RNO10 in the tumor material. The detection procedure was carried out by adding rhodamine-conjugated antidigoxigenin (for the digoxigenin-labeled probe) and FITC-conjugated avidin (for the biotin-labeled probe). The results were visualized using a Leica DM RXA fluorescence microscope (Leica, Wetzlar, Germany).

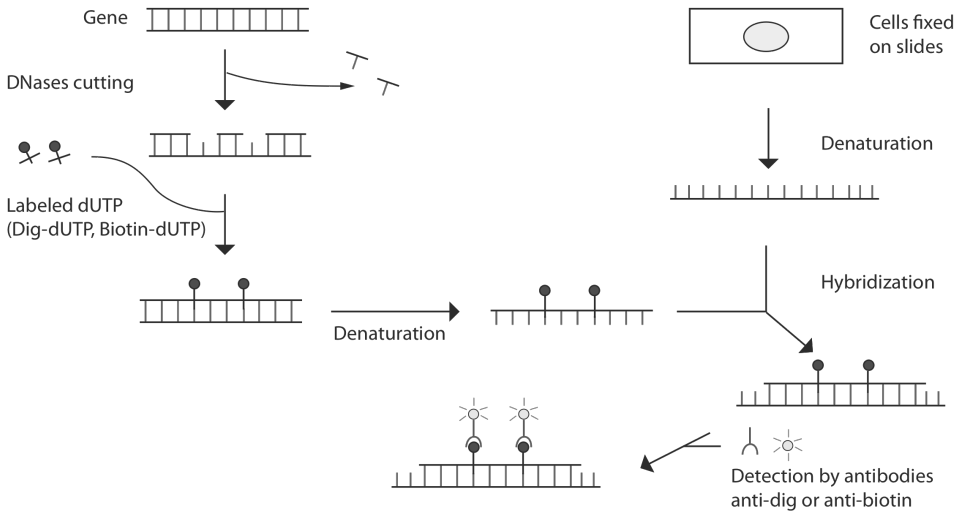


Figure 18. Schematic presentation of the FISH technique. A DNA probe is labeled with fluorescent dye, denatured, hybridized on metaphase chromosome fixed on slides and detected.

Northern blot (Paper IV)

Northern blot is a technique developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University (Alwine, et al. 1977) and it is used to examine the expression of a gene by analyzing gene-specific RNA. Using this technique, the degree of expression of the gene *Tp53* compared with the endogenous control *ß-actin* in tumor samples was analyzed. Total RNA was extracted from tumor samples, loaded and separated by electrophoresis in agarose gel using the Northern-Max-Gly kit and transferred to BrightStar-Plus nylon membrane according to the manufacturer's instructions (Ambion, Austin, TX). The fragments of interest were detected by hybridization with radioactively labeled gene-specific probes.

DNA methylation analysis (Paper V)

This analysis was used to investigate whether the observed lowered expression of candidate genes (*Hic1*, *Skip* and/or *Myo1c*) was due to the hypermethylation of the promoter of these genes. The CpG islands were predicted for each of the genes using the web server CpG Island Searcher (URL: http://www.uscnorris.com/cpgislands2/cpg.aspx). Primers were designed using the Meth-Primer software (URL: http://urogene.org/methprimer/) and/or BiSearch web server (URL: http://bisearch.enzim.hu/) and were synthesized by a commercial supplier (SIGMA-Genosystem, Cambridge, UK). For the analysis, DNA was denatured, treated with sodium bisulphite to convert all the unmethylated cytosines to uracil and then purified. Subsequently, the methylation status of the sequence was analyzed by bisulphite DNA sequencing or MSP (methylation specific PCR) using gene-specific primers designed to amplify the promoter regions.

5-aza-2´-deoxycytidine (5-Aza-dC) and/or trichostatin A (TSA) treatment (Paper V)

5-Aza-dC is a demethylating agent that inhibits DNA methylation by causing a covalent complex between active methyltransferases (DNMT) isoforms and DNA, thereby depleting the cell of methylase activity. TSA is a histone deacetylase inhibitor that leads to the acetylation of histones. When cells are treated with either or both of these reagents, the expression of those groups of genes that are under the control of epigenetic gene silencing is expected to be restored. Following the treatment of the cells with these agents, the expression of *Hic1*, *Skip and Myo1c* was examined using semi-quantitative RT-PCR at the RNA level and/or Western blot at the protein level.

Semi-quantitative RT-PCR (Paper V)

RNA was converted to cDNA using reverse transcriptase and cDNA was then used as a template in PCR reactions, using primers corresponding to the genes of interest. To determine the relative gene expression of *Hic1*, *Skip and Myo1c* after the 5-Aza-dC and/or TSA treatments, the genes were co-amplified with a reference housekeeping gene (*ß-actin*) in a multiplex PCR reaction using cDNA from treated cells as a template. The PCR products were loaded on an agarose gel in such a way that the intensity of the band corresponding to *ß-actin* expression was uniform in all samples and the relative expression of *Hic1*, *Skip* and *Myo1c* could therefore be compared among the samples.

Western blot (Paper V)

The Western blot technique is used to detect the expression of specific proteins in a sample (Towbin, et al. 1979). Using this technique, cell lysates are prepared and, when analyzing several samples, equal amounts of protein extracts for all samples are denatured and separated by electrophoresis. The separated proteins are then transferred to membrane and probed with a gene-specific primary antibody, followed by a secondary antibody for visualization. We performed Western blot in order to confirm the results from the qPCR for *Hic1* and *Myo1c* and to analyze the level of Hic1 and Myo1c protein subsequent to 5-aza-dC and/or TSA treatment in tumor samples. Detection was carried out by using a Western blotting detection system according to the manufacturer's recommendations. To estimate the relative expression of Hic1 and Myo1c, the membranes were also probed with Gapdh as an endogenous control. Expression of Skip protein was not successful, most likely due to problem with the specificity of the Skip antibody.

RESULTS AND DISCUSSION

Paper I: Molecular classification of spontaneous endometrial adenocarcinomas in BDII rats

The molecular mechanisms behind endometrial carcinogenesis are poorly understood and a better understanding of these mechanisms will certainly provide new tools for prognosis, diagnosis and therapy. Prognosis of this malignancy in humans is based on the categorization of endometrial cancers into two major subtypes: type I and type II. Type I endometrial cancer accounts for more than 80% of all cases, is associated with a good prognosis and of endometrioid histology. These tumors are coupled with hyperestrogenic risk factors and they are therefore more frequently estrogen and progesterone receptor positive. Type I tumors usually display a diploid karyotype, microsatellite instability and *KRAS*, *PTEN* and *CTNNBI* mutations. Type II tumors are associated with a poor prognosis and are characterized by non-endometrioid histology. These tumors often display aneuploidy, as well as aberrations in *CDKN2A*, *TP53* and *ERBB2* genes (Table 4). Although the molecular basis of the distinction between type I and II tumors is of prognostic value, it has not provided a basis for improved therapy (Salvesen, et al. 2009).

Table 4. Summary of molecular findings of ECs developed in the BDII rat tumor model compared with different subgroups of human ECs.

		Human endometrial carcinoma				
Gene alteration	**Pathway**	**Type I**	**Type II**	**Arisen from HNPCC**	**Familial site specific**	**Endometrial carcinoma in BDII rats[d]**
Microsatellite instability	MMR	20–45%	0–5%	90–95%	8–9%	0-5%
PTEN mutations	Akt	50–80%	10%	60–85%	N	No mutation, but 2.3 fold-down regulation[a]
K-RAS mutations	Akt	10–30%	0–5%	10%	N	No
ERBB2 over-expression	Akt	10–30%	45–80%	N	N	No, on the contrary, 4.5 fold-down regulation[a]
TP53 mutations	P53	10–20%	90%	Low	N	67% mutations[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 [a]
CTNNB1 mutations	Wnt	20%	0–5%	N	N	No, on the contrary, 2.2 fold-down regulation[a]

[a]Present work; [b]After removing one outlier from the NME samples
[c](Kariola, et al. 2005; Lax 2007; Liu 2007; Ollikainen, et al. 2007); [d](Adamovic, et al. 2008; Behboudi, et al. 2001; Helou, et al. 2001; Nordlander, et al. 2005); N, not known or no information available

In this paper, we wanted to investigate the degree of similarity at the molecular level of tumors developed in the inbred BDII rat strain to those developed in humans. From the available molecular data for human types I and II ECs, we selected seven genes (*Pten*, *Tp53*, *Irf1*, *Ctnnb1* (*beta-catenin*), *Cdkn2a* (P16), *Cdh1* and *Erbb2*) (Lax 2007) for analyzing a panel of 29 EC tumors. For comparison we also analyzed seven non-malignant endometrial samples (NME samples). Gene expression analysis revealed that *Pten*, *Ctnnb1*, *Cdh1*, *P16* and *Erbb2* were expressed at significantly lower levels (nominal *P*-value < 0.05) in the EC compared with the NME samples (Fig. 19). We also performed mutation analysis for the *Pten*, *Ctnnb1* and *Irf1* genes by direct DNA sequencing of the coding regions. We detected sequence aberrations in the *Irf1* gene in three EC samples, whereas no significant difference in the expression level of this gene was detected between EC and the NME samples. These findings suggested that *Irf1* was of less importance in the development of EC tumors, at least in this rat model. No mutation in *Pten* and *Ctnnb1* was found, but allelic imbalance analysis of the *Pten* locus revealed AI in three of seven informative tumors. It therefore appears that, like human type I EC tumors, the inactivation and/or lower expression of *Pten* and *Ctnnb1* are important in endometrial carcinogenesis in the BDII rat tumors. On the other hand, we found frequent *Tp53* mutations and significant *Cdh1* down-regulation in the BDII rat tumors. Although both these molecular features are known as characteristics of human type II tumors, it has been shown that higher-grade type I tumors also frequently display these molecular changes. Our data therefore suggest that endometrial carcinogenesis in BDII rats at molecular level can be related to higher-grade human type I tumors.

To summarize, like human type I tumors, endometrial carcinomas in BDII rats have an endometriod histology and the development of tumors was shown to be hormone dependent (Deerberg and Kaspareit 1987). In this work, we were able to provide additional molecular evidence supporting this similarity, suggesting that this model represents an excellent experimental tool for research on this malignancy in humans.

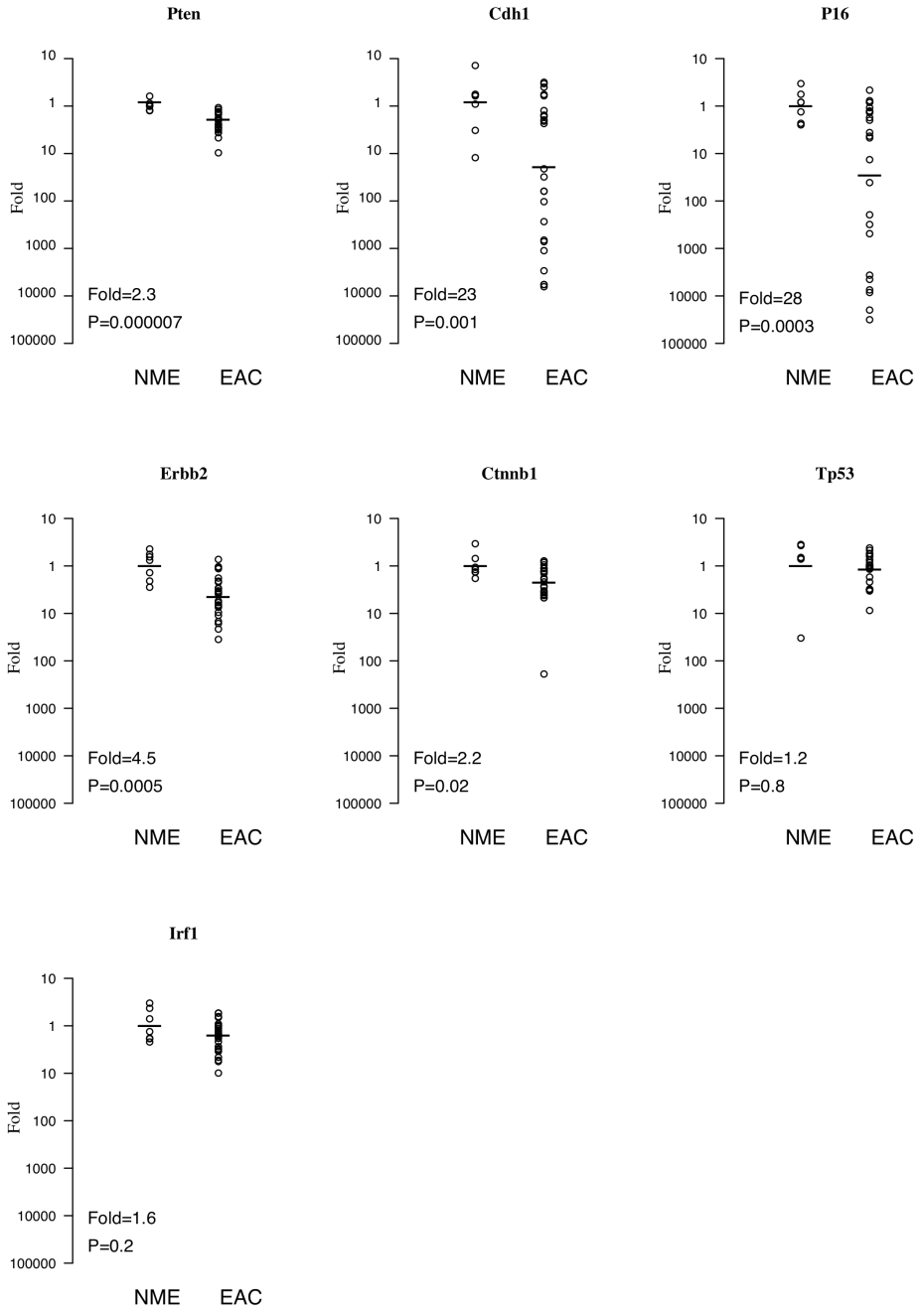
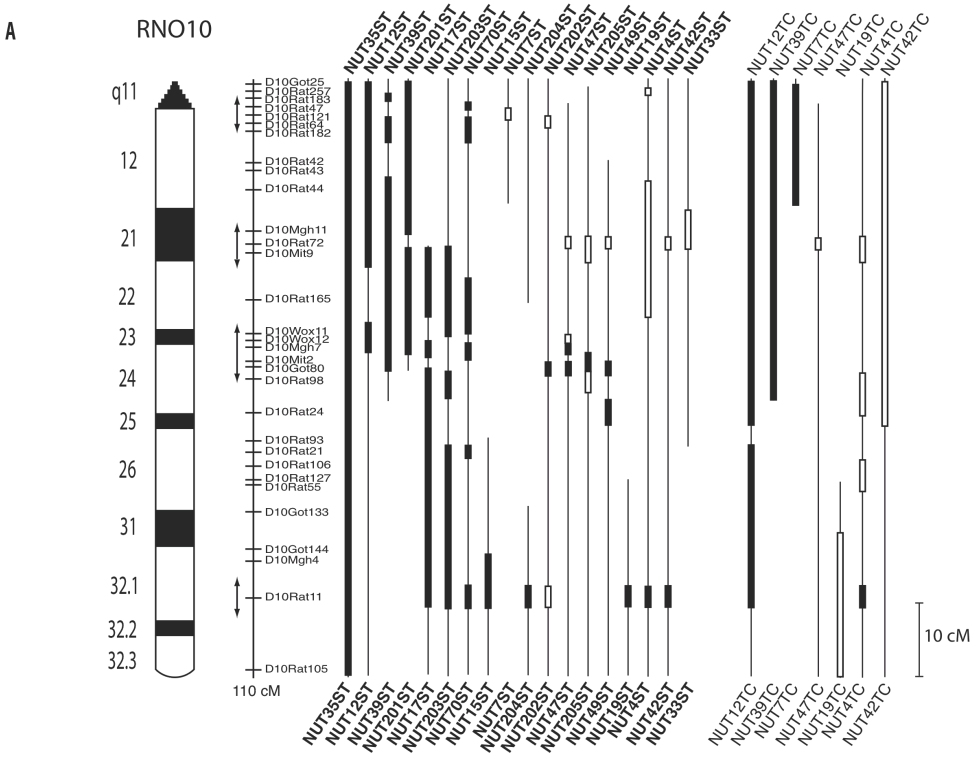


Figure 19. Real-time RT-PCR analysis of seven genes in a panel of 29 rat endometrial adenocarcinomas (EAC) compared with seven non-malignant (NME) samples. The expression level of each gene in each sample is presented as a fold change. The significant down-regulation of *Pten*, *Cdh1*, *P16*, *Erbb2* and *Ctnnb1* was observed among EAC tumors, while no significant changes were detected for *Tp53* and *Irf1*. Nominal *P*-value < 0.05 (Welch's *t*-test).

Paper II: Four segments show allelic imbalance on chromosome 10 in rat endometrial adenocarcinomas

We used a panel of 45 polymorphic markers along RNO10 to investigate the occurrence of AI in this chromosome among tumors developed in animals from the BDIIx(BDIIxBN) and BDIIx(BDIIxSPRD) back-crosses. Four different segments of recurrent AI were identified, two in the proximal part, one in the middle and the fourth in the distal part of RNO10. For each region, we defined the smallest region of overlap (SRO) of AI among tumors (Fig. 20). It appeared that these regions stood out more clearly in the SPRD-derived than in the BN-derived back-cross tumors, but there were no clear indications that patterns of RNO10 changes were distinct between the two crosses. Since the two non-susceptible strains (SPRD and BN) had clearly contributed in a different way due to their dissimilar genetic constitution, it is reasonable to suggest that there might be some differences in the paths towards tumor development between the two crosses.



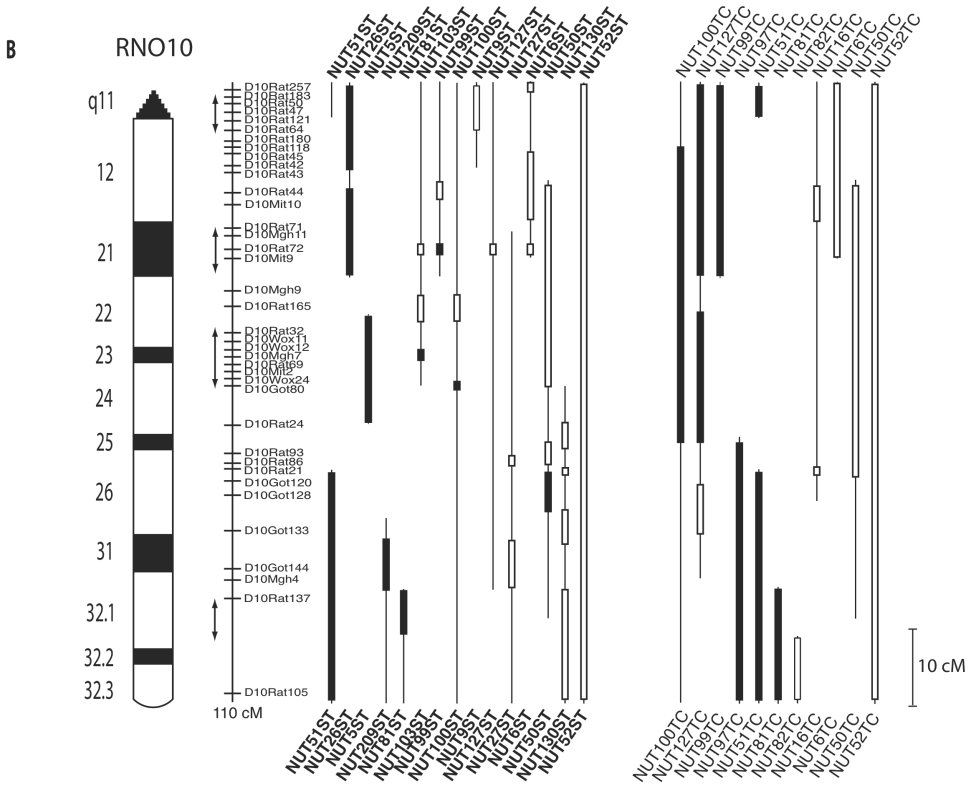


Figure 20. Allelic imbalance in rat tumors from back-cross animals. Open boxes: allelic imbalance, BDII strain allele dominating, non-BDII allele reduced. Filled boxes: allelic imbalance, non-BDII allele dominating, BDII allele reduced. Thin line: retention of heterozygosity. No line: constitutive BDII allele homozygosity (uninformative). A: Tumors from back-cross BDIIx(BDIIxSPRD). B: Tumors from back-cross BDIIx(BDIIxBN). In each figure, the group to the left represents solid tumors (ST), whereas the group to the right represents tumor tissue cultures (TC).

To determine SROs, average AIR values were calculated for every marker and all tumors that exhibited AI in at least one marker. The values were then plotted against marker positions for ST and TC samples independently (Fig. 21). As shown in Figure 21, the curves made dips at positions corresponding to the SROs, giving a fairly precise position for each recurrent AI region among all tumors. Interestingly, three of the regions of recurrent AI appeared in both ST and TC material, whereas the fourth (closest to the centromer) could only be detected in the TC samples. This finding suggested that aberrations resulting in AI in this region might be related to changes that occurred during the establishment of the tumor cells in tissue culture and thus represented an *in vitro* effect. Accordingly, aberrations in the other three regions represented *in vivo* effects and may therefore correspond to important genetic changes during endometrial tumorigenesis.

We found that AIR values were generally lower in TC (close to 0.00) compared with the corresponding ST sample (ranged between 0.15-0.60), suggesting that DNA extracted from ST samples perhaps contained a different degree of normal cell infiltrations (lymphocytes, blood vessels and so on). It was additionally clear that dips were much more distinct and narrower in the ST material for SRO regions 2-4. This observation can be explained by the subclonal heterogeneity of the ST samples. It is known that subclonal heterogeneity can occur during tumor development, resulting in a number of clones of cancerous cells, each with slightly different genetic aberrations than the others (Fig. 3) (Yokota 2000).

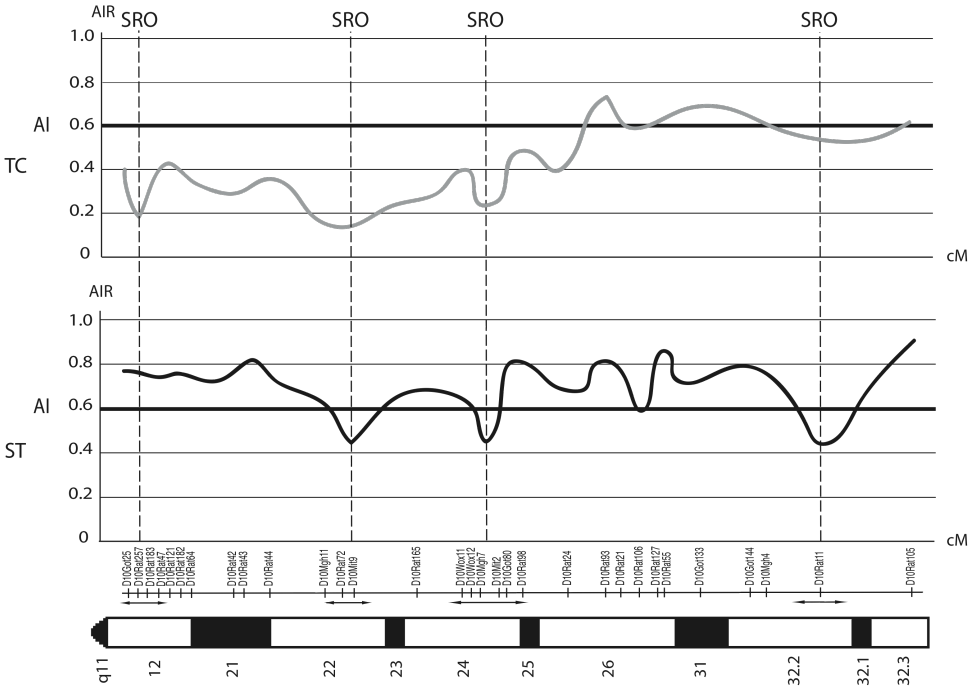


Figure 21. Example of analysis of SRO using AI data. The diagram shows the mean AIR values for all markers in the BDIIx(BDIIxSPRD) cross along RNO10 plotted against their genetic positions. In the solid tumors (ST), the mean AIR values always ranged between 0.45-0.95, whereas, in the tissue cultures (TC), the mean AIR values ranged between 0.10-0.75.

To summarize, we performed AI analysis of RNO10 using 45 microsatellite markers in 45 ST and 23 TC samples. We found three regions of frequent AI in both ST and TC and an additional centromeric AI region only in TC samples. Based on the available cytogenetic data for the tumor set analyzed in the present study (Hamta, et al. 2005), our data indicated that observed AI at regions 1-3 were most likely due to chromosomal losses, whereas AI at region 4 was the result of a chromosomal gain. Accordingly, there were at least three regions of tumor suppressor activity and one region of oncogenic activity along RNO10 with a potential contribution to endometrial carcinogenesis.

Paper III: Deriving evolutionary tree models of the oncogenesis of endometrial adenocarcinomas

By applying tree algorithms on the AI data from the analysis of BDII rat ECs (Paper II and (Behboudi, et al. 2001)) we wanted to determine if this approach was appropriate for defining the most important allelic alterations in RNO10 for using as a basis for the subsequent candidate gene approaches. To this end, we used the integrated linkage map and the available physical map positions (Ensembl v35) to group 59 informative markers along RNO10 in 24 groups, representing 24 chromosomal segments. Since genetic events may appear randomly because of genomic instability during tumor progression, we used the method proposed by Brodeur et al. (Brodeur, et al. 1982)) to select only segments containing non-random events for further analysis in the tree derivation process. For the ST data set, we identified 11 segments (s4, s5, s7, s9, s12, s13, s14, s15, s18, s19 and s24) as being affected by non-random genetic events. Ten of these segments (excluding s19) were also identified as non-random events for the TC data.

We used three different algorithms (maximum weight branching, the distance-based algorithms neighbor joining and Fitch-Margoliash) to derive trees depicting the order of genetic events during oncogenesis using AI data. Since the three algorithms contain different sets of assumptions, the resulting trees can be validated against each other, if they show a certain degree of consistency in the order of events. The two distance-based methods (neighbor joining and Fitch-Margoliash) produced very similar trees and we therefore chose to continue to compare only one of them (neighbor joining) with the result of the third algorithm.

The tree models for ST and TC data sets were derived separately. For each of the ST and TC data sets, the two derived oncogenic tree models were in good agreement with each other, suggesting that the order of events was fairly reliable. Although the derived trees for ST and TC data sets showed slight differences in the order of a number of segments, the four tree models for both ST and TC data taken together indicated that s9 and thereafter s14 are important chromosomal segments, in which the earliest alterations in tumorigenesis had most probably occurred (Fig. 22).

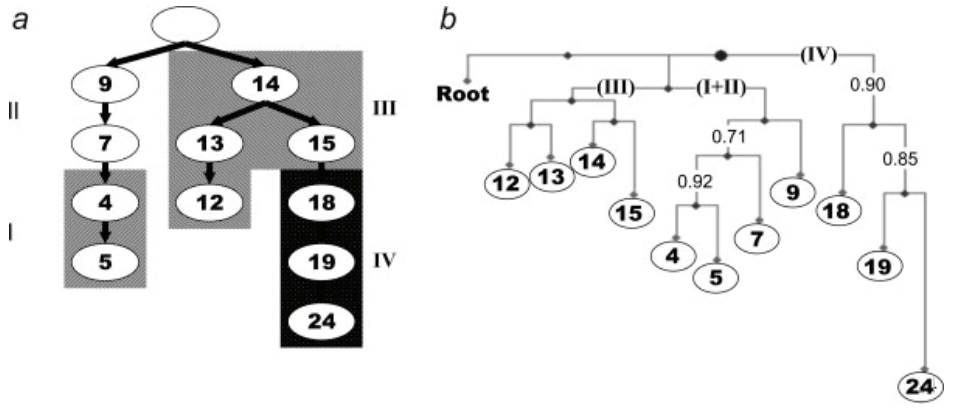


Figure 22. Evolutionary tree models for oncogenesis based on allelic imbalance results derived from ST data. Segments defined by the physical position of the informative markers: (a) branching tree model (b) neighbor-joining distance tree model.

To summarize, we applied onco-tree algorithms to determine the likely order of the observed alterations as well as their relationship in respect to each other during EC tumorigenesis on data from the RNO10 AI analysis of 61 ST and 28 TC. The derived trees indicated that two segments, s9 and s14, represent chromosomal regions where early and important genetic changes with potential implication on endometrial carcinogenesis have occurred.

Paper IV: Analysis of chromosome 10 aberrations in rat endometrial cancer – Evidence for a tumor suppressor locus distal to *Tp53*

Several lines of evidence have pinpointed a small region of the mid-proximal part of RNO10 at cytogenetic band RNO10q24-q25 that was recurrently involved in genetic alterations in BDII rat ECs. The present work was designed to analyze this region in more detail.

We used an RNO10-specific paint to detect chromosomal aberrations, such as translocations and/or deletions, involving this chromosome. In most cases, the rearranged chromosomes appeared to be derived from a break somewhere in the middle part of the chromosome. To investigate this, we performed dual-color FISH using gene-specific PAC probes for two loci, *Tp53* locus at the proximal/central part (10q24-q25) and *Thra1* locus at the distal part of (10q32.1) of RNO10. We calculated the expected number of RNO10 copies per cell from the modal chromosome number (karyotyping data) of each tumor cell line. It appeared that the proximal part of the chromosome (harboring *Tp53*) was often under-represented, whereas the distal part (harboring *Thra1*) was usually over-represented in the tumors. We therefore concluded that the mid-proximal part of RNO10 might confer a tumor inhibitory activity. We performed additional FISH experiments using eight BAC probes evenly distributed between the *Tp53* and *Thra1* genes in 17 tumors that exhibited signs of RNO10 deletions and

developed a detailed deletion map of the region. The results showed that there was at least one breakpoint in RNO10 between *Tp53* and *Thra1* in all the tumors analyzed, but the position of breakpoints varied in different tumors. We were, however, able to define a common minimal region of overlapping deletions among tumors, at the position of 62.3-63.0 Mb.

The well-known tumor suppressor gene *Tp53* is located close to this region and it was therefore selected as the best candidate. Over 90% of the reported *TP53* mutations in human tumors are in the core DNA-binding domain of the p53 protein, which corresponds to exons 5 to 10 of the gene. We therefore sequenced the corresponding rat exons 4-10, in the *Tp53* gene on genomic DNA from 29 rat tumor cell cultures (27 EC tumors and 2 non-EC tumors) and normal liver DNA from the three parental inbred strains (BDII, BN and SPRD). We found mutations in the *Tp53* gene in 18 of 27 EC tumors (Table 5), resulting in truncated or highly abnormal products in 4 tumors and single amino acid substitutions in 14. All the mutations were verified by sequencing of tumor cDNA. We additionally detected a single nucleotide polymorphism (SNP) in intron 7 of the gene, where the BN strain exhibits an A and both BDII and SPRD display a G. This SNP data enabled us to verify our AI data and also to determine which allele was retained in the tumors with RNO10 deletions from the BDII and BN crosses.

Interestingly, there were seven tumors that did not show any mutation in the *Tp53* gene, five of which clearly displayed AI in the region. This finding suggested that, at least in these five samples, the main target for the observed chromosomal deletions/AI was not *Tp53*. In fact, the observed pattern of chromosomal breaks and deletions suggested that the main selection was against a small segment close to, but distal to *Tp53*.

To verify our AI and gene mutation analyses data we screened 19 tumors (for which material was available) for *Tp53* expression using Northern blot analysis. The analysis revealed that the Tp53 transcript was significantly decreased in two tumors, both harboring frame-shift mutations in the *Tp53* gene, which resulted in a truncated Tp53 product.

Table 5. RNO10 deletion map of 17 EC tumors based on FISH and AI data. Two PACs and eight BACs were used as probes in dual-color FISH to investigate the approximate position of chromosome breaks and deletions along RNO10. For reference, *Tp53* mutation data and AI data at RNO10q24-q25 are included. As shown, the AI data are in complete agreement with the resulting deletion map based on the FISH data. D = deletion, * = no deletion, */D = deletion break point inside the BAC probe, U = uninformative in AI analysis, nd = not done. Tumors in bold exhibited allelic imbalance at 10q24, but no mutation in the *Tp53* gene.

Tumor	*Tp53* mutation	AI data at RNO10q24	Chr. number	Expected RNO10	RNO10 paint-positive chr.				FISH signals from BAC/PAC probes (starting position of the insert in the draft sequence, Mb)									
					Total	Normal	del(10)	t(10)	*Tp53* (56.4)	CH230-368I24 (57.5)	CH230-280H23 (62.0)	CH230-8N1 (62.3)	CH230-19F2 (62.8)	CH230-124E19 (72.0)	CH230-524E4 (78.6)	CH230-210I13 (82.7)	CH230-16I9 (86.8)	*Thra* (87.5)
RUT2	**No**	**Yes**	40	2	3	-	1	2	*	*	*	*	*/D	D	D	D	D	*
RUT7	Yes	Yes	43	2	2	1	1	-	D	D	D	D	*	*	*	*	*	*
NUT127	Yes	Yes	70	3	5	2	2	1	D	D	D	D	*	*	*	*	*	*
NUT128	**No**	**U**	59	3	3	2	-	1	D	nd	nd	nd	D	*	*	*	*	*
NUT99	Yes	U	67	3	5	1	2	2	D	nd	nd	nd	D	*	*	*	*	*
NUT12	Yes	Yes	64	3	3	2	-	1	D	nd	nd	nd	D	*	*	*	*	*
RUT25	Yes	Yes	48	2	4	1	2	1	D	nd	nd	nd	D	D	*	*	*	*
RUT30	Yes	U	61	3	4	2	2	-	D	nd	nd	nd	D	D	D	*	*	*
NUT100	**No**	**Yes**	67	3	4	2	2	-	D	nd	nd	nd	D	D	D	*	*	*
RUT6	Yes	Yes	58	3	4	2	2	-	D	nd	nd	nd	D	D	D	*	*	*
NUT7	Yes	U	46	2	5	2	-	3	D	nd	nd	nd	D	D	D	D	*	*
NUT50	**No**	**Yes**	66	3	4	2	-	2	D	nd	nd	nd	D	D	D	D	*	*
NUT31	Yes	U	67	3	3	2	-	1	D	nd	nd	nd	D	D	D	D	*	*
NUT4	**No**	**Yes**	62	3	5	2	2	1	D	nd	nd	nd	D	D	D	D	D	D
NUT6	Yes	U	60	3	4	2	2	-	D	*	*	*	*	*	*	*	*	*
NUT84	Yes	U	42	2	3	1	1	1	D	*	*	*	*	*	*	*	*	*
RUT13	Yes	U	62	3	3	2	-	1	D	*	*	*	*	*	*	*	*	*

To summarize, in this paper, we used different cytogenetic and molecular methods to investigate the extent and nature of RNO10 aberrations in BDII rat ECs. Using "whole chromosome paint" and dual-color FISH, we identified a recurrent deleted region distal to *Tp53* among tumors. Mutation analysis of the tumor suppressor gene *Tp53* showed that there were mutations in 67% of tumors. However, in Northern blot analysis, we showed that Tp53 expression was only decreased in 2 of 19 tumors tested. Both these tumors had frame-shift mutations in the *Tp53* gene, resulting in the production of truncated Tp53. In 12 of the tumors tested, mutation resulting in aa substitution were detected without changes in the mRNA expression. However, in majority of the mutations resulting in amino acid exchange, the hydrophobicity or hydroplilicity was maintained by equivalent substitutions. Moreover, there were at least five tumors with clear AI in the region and without *Tp53* mutation. Taking all the data into consideration, we suggested that *Tp53* was not the main target of the observed RNO deletions and that another candidate(s) should exist in the neighborhood of this gene that would explain the observed findings. Since similar observations of AI at band 17p13.3 unassociated with *Tp53* mutations have been reported in several human tumor types, we used mapping data presented in human studies and in a genome comparative approach and were able to narrow down the candidate region of AI/deletion to a segment 0.7 Mb in size. There are 19 genes located in this chromosomal segment (Table 6), among which there are a few good candidates for further investigation.

Table 6. Genes located within and the ones most closely flanking the tumor suppressor region between 62.3-63.0 Mb on RNO10. For comparison, the corresponding human homologues are included.

Rat			Description	Human	
Start (Mb)	**Symbol**	**Cyto. band**		**Start (Mb)**	**Cyto. band**
62.23	*Est1a*	10q24	Telomerase-binding protein EST1A	1.91	17p13.3
62.47	*Hic1*	10q24	Hypermethylated in cancer 1	1.91	17p13.3
62.48	*Ovca2*	10q24	Ovarian Cancer-Associated Gene 2	1.89	17p13.3
62.49	*Ovca1*	10q24	Ovarian Cancer-Associated Gene 1 (Dph1)	1.89	17p13.3
62.50	*Rtn4rl1*	10q24	Reticulon 4 receptor-like 1	1.79	17p13.3
62.61	*Rpa1*	10q24	Replication protein A1	1.68	17p13.3
62.66	*Smyd4*	10q24	SET and MYND domain containing 4	1.63	17p13.3
62.71	*Serpinf1*	10q24	Serine (or cysteine) peptidase inhibitor, clade F, 1	1.61	17p13.1
62.75	*Serpinf2*	10q24	Serine (or cysteine) peptidase inhibitor, clade F, 2	1.59	17p13
62.76	*Wdr81*	10q24	Wdr81, WD repeat domain 81	1.57	17p13.3
62.79	*Tlcd2*	10q24	TLC domain containing 2	1.56	17p13.3
62.81	*Prpf8*	10q24	Pre-mRNA processing factor 8	1.50	17p13.3
62.83	*Rilp*	10q24	Rab interacting lysosomal protein	1.50	17p13.3
62.84	*Scarf1*	10q24	Scavenger receptor class F, member 1	1.48	17p13.3
62.85	*Slc43a2*	10q24	Solute carrier family 43, member 2	1.42	17p13.3
62.91	*Pitpn*	10q24	Phosphatidylinositol transfer protein, alpha	1.37	17p13.3
62.95	*Skip*	10q24	Skeletal muscle and kidney enriched inositol	1.34	17p13.3
62.99	*Myo1c*	10q24	Myosin IC	1.31	17p13
63.02	*Crk*	10q24	V-crk sarcoma virus CT10 oncogene homolog	1.27	17p13.3

Paper V: Detailed analysis of the independent tumor suppressor loci telomeric to *Tp53* suggests *Skip* and *Myo1c* as novel tumor suppressor gene candidates in this region

In this work, we characterized our previously identified chromosomal segment of 0.7 Mb, which exerted independent tumor suppressor activity in the neighborhood of *Tp53*. There are several reports indicating that the homologous chromosome segment at 17p13.3 has a comparable tumor suppressor characteristic in many human tumor types (Parrella, et al. 2005; Roncuzzi, et al. 2005; Sarkar, et al. 2003; Stöcklein, et al. 2008). Although this region has been the subject of extensive research, no definite candidate gene(s) has as yet been identified. We believe that analysis of the experimental model system on which we are working might be able to shed some light on this search. We selected a panel of well-characterized tumor and control samples and performed real-time RT-PCR analysis on all 19 genes located within this candidate region (Table 6). Using this approach, we were able directly to exclude two attractive genes, *Ovca1* (ovarian cancer associated 1) and *Ovca2,* as potential candidates. Nine genes displayed significantly reduced expression in tumors compared with control samples, but statistical analysis indicated that the expression of four of these genes was exclusively lower in the group of tumors that had deletion/AI in the candidate region (Table 7).

This observation suggested that the reduced expression of these four genes most likely represented a "by-product" of the deletion of genetic material in the tumors and we therefore excluded them as candidates. This left five genes, *Hic1*, *Rpa1*, *Skip*, *Myo1c* and *Crk,* for which reduced expression was detected in both groups of tumors with and without deletion in the region, suggesting that the observed reduced expression of these genes had occurred through different gene-silencing mechanisms during EC tumorigenesis. Among these genes, *Rpa1* and *Crk* were not appealing: *Rpa1* displayed only fairly moderate down-regulation in gene expression analysis and *Crk* is mainly recognized as an oncogene in cancer studies.

Table 7. Changes in relative expression of 19 genes within tumor suppressor region located at 62.3 – 63.0 Mb revealed by Real-time RT-PCR analysis. Nine genes displayed significant decreased expression (nominal *P*-value < 0.05) in EC compared to NME samples. Negative values for the fold change (FC) represent a decreased expression for the gene and are shown only for genes displaying *P*-values < 0.05 between EC vs NME samples. Tumors were then divided into two groups, ECs with and ECs without deletion/AI at RNO10q24, and gene expression data for the nine significantly down regulated genes were compared between these two groups of tumors. Reduced expression of five genes (marked in gray) was found to not to correlate with physical deletion at RNO10q24. *Hic1*, *Skip* and *Myo1c* (marked in bold) with maximum FC as well as minimum *P*-value were selected as candidates.

Transcript	**Start (Mb)**	**Group EC vs NME**			**Group EC with vs EC without loss at RNO10q24**	
		P-value	Significant	Fold change	P-value	Significant
Est1a	62.23	0.002	x	-2.3	0.03	x
Hic1	**62.47**	**0.0004**	**x**	**-4.9**	**0.2**	
Ovca2	62.48	0.760				
Dph1	62.49	0.973				
Rtn4rl1	62.50	0.008	x	-4.5	0.02	x
Rpa1	62.61	0.003	x	-1.9	0.5	
Smyd4	62.66	0.918				
Serpinf1	62.71	0.293				
Serpinf2	62.75	0.324				
Wdr81	62.76	0.00.5	x	-1.7	0.01	x
Tlcd2	62.79	0.218				
Prf8	62.81	0.054				
Rilp	62.83	0.108				
Scarf1	62.84	0.811				
Slc43a2	62.85	0.247				
Pitpn	62.91	0.0003	x	-2.2	0.0004	x
Skip	**62.95**	**0.0003**	**x**	**-6.8**	**0.2**	
Myo1c	**62.99**	**0.000003**	**x**	**-4.8**	**0.1**	
Crk	63.02	0.00001	x	-3.2	0.4	

We therefore focused on *Hic1*, *Skip* and *Myo1c* in the continuation of our analysis. In combined gene mutation sequencing, protein expression analysis, screening of promoter methylation experiments and gene expression restoration assays, we found evidence for *Skip* and *Myo1c* as the best candidates. It is interesting to note that both genes are involved in insulin-induced glucose homeostasis through PI 3-kinase/Akt signaling, which is known to be vital for the survival and growth of cancer cells (Bose, et al. 2002; Holman and Sakamoto 2008; Ijuin and Takenawa 2003; Yip, et al. 2008) (Fig. 23). Neither *Skip* nor *Myo1c* has been reported as a potential cancer-related gene before, but it is intriguing that other members of the two gene families to which *Skip* and *Myo1c* belong have previously been identified as tumor suppressor genes (Maehama and Dixon 1998; Nishioka, et al. 2002; Taki, et al. 2005).

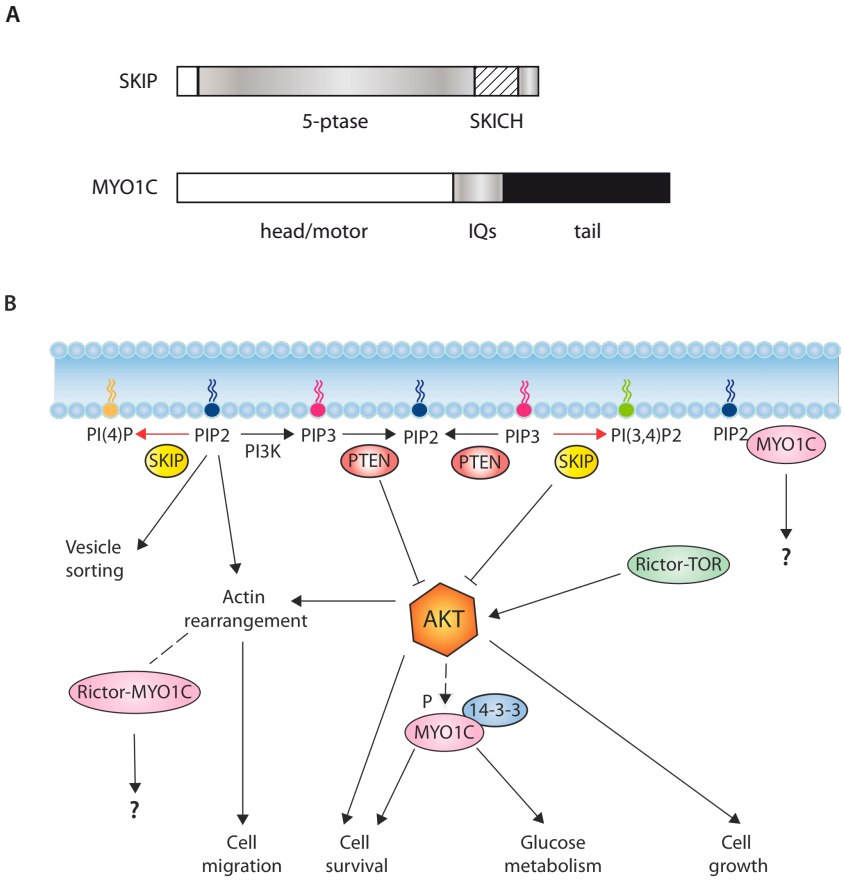


Figure 23. A. Domain organization of the SKIP and MYO1C. The SKIP consists of two major domains: the 5-ptase is the catalytic domain and the SKICH domain that mediates membrane localization of the protein. The MYO1C is a class I myosin and consists of three major domains: the head domain that acts as the motor, IQs that mediate binding to calmodulin, and the tail domain that has been reported to anchor PI(1,4,5)P_3 and PIP_2 regions in the plasma membrane. **B**. Schematic presentation of the phosphoinositide 3 (PI3)-kinase signaling, showing a summary of the literature data on potential locations/roles of SKIP and MYO1C in this pathway.

To summarize, the present work provided evidence that *Skip* and *Myo1c* are two attractive candidate genes located within the reported independent region of tumor suppressor activity distal to *Tp53*, and that they may make a potential contribution to experimental EC tumorigenesis at the very least. Theses two genes are located very close to each other and it is interesting to note that they appear to play an overlapping role in insulin-mediated glucose transport and actin cytoskeletal rearrangement through the PI 3- kinase/Akt pathway, which is known to have implications for the survival and growth of cancer cells. The functional significance of the contribution *Skip* and *Myo1c* make to tumorigenesis processes remains to be investigated.

CONCLUDING REMARKS

In the present work, we took advantage of a powerful experimental model for spontaneous endometrial carcinogenesis and derived new molecular data explaining at least in part, some of the mechanisms involved in this malignancy. We conducted a comparison at molecular level between tumors developed in this model and those in humans in order to determine whether and how this tumor model could represent human endometrial carcinomas (ECs). We also investigated the extent and nature of frequently reported rat chromosome 10 (RNO10) alterations in experimental ECs.

To summarize, the following conclusions have been made:

- The BDII rat ECs can be related to higher grade human type I ECs at molecular level and thus this tumor model represents a potent model for the genetic and molecular investigation of the underlying biological processes in human endometrial carcinoma.

- Four independent regions of recurrent AI were identified along RNO10. Using AI/LOH data in mathematical algorithms and by deriving evolutionary onco-tree models, we concluded that one of these regions at cytogenetic band RNO10q24 most likely represented early and important event(s) during EC tumorigenesis.

- The tumor suppressor gene *Tp53* was the most prominent candidate in this region. In combined mutation screening, gene expression and FISH analysis, however, we found that *Tp53* was not the only target and showed that the major selection was against a 0.7 Mb long region distal to *Tp53*.

- Using a candidate gene approach, we subjected all the genes located in this segment to real-time RT-PCR, followed by mathematical analysis of the results, and identified the *Hic1*, *Skip* and *Myo1c* genes as the best candidates. However, subsequent molecular analysis ruled out *Hic1* and confirmed *Skip* and *Myo1c* as candidates.

- In conclusion, this thesis suggests that *Skip* and *Myo1c* are the most prominent candidates for the reported independent region of tumor suppressor activity distal to *Tp53*.

SWEDISH SUMMARY – SAMMANFATTNING PÅ SVENSKA

Cancer i livmoderslemhinnan (endometriecancer alt. livmoderkroppscancer) är den i västvärlden vanligaste förekommande elakartade tumörtypen i det kvinnliga könsorganet. Nyupptäckta fall av sjukdomen samt dödligheten ökar markant. I Sverige, diagnosticeras cirka 1300 nya fall varje år och dessa svarar för sex procent av all cancer hos kvinnor.

De senaste decenniernas forskning om cancer har påvisat att cancerutveckling kan bero på att ett antal genetiska förändringar (mutationer) ackumuleras i cellernas arvsmassa, vilket i sin tur kan leda till förlust av normal tillväxtkontroll. Förändringarna kan variera i omfång, från enkla defekter i genernas DNA-sekvens till omfattande kromosomala omarrangemang, vilket leder till att den normala sammansättningen är rubbad. Följden av dessa defekter kan ge upphov till förändringar i de proteiner som generna kodar för.

Målsättningen med mitt arbete har varit att identifiera och karakterisera de gener och genmekanismer som är inblandade i uppkomsten av livmoderkroppscancer. Inom vår grupp har vi under ett tjugotal år arbetet med molekylärgenetiska analyser på den inavlade BDII-råttan, som utgör en unik modell för tumöruppkomst i livmoderslemhinnan. Genom vårt arbete har denna modell blivit mycket väl karakteriserad med avseende på genetiska, kromosomala och molekylära egenskaper. Vid tidigare genom-analyser lokaliserades större kromosomregioner som på ett återkommande vis var förändrade. Denna avhandling fokuserar främst på råttans kromosom 10 (RNO10), som vid genom-analyserna visade sig ha förlust av den övre delen följt av uppförökning av den nedre delen av kromosomen. Vi har använt oss av ett antal olika metoder som innebär att vi har kunnat identifiera ett mindre område i mitten på RNO10 som uppvisar förlust av genetiskt material, s.k. deletion. Med anledning av detta har vi dragit slutsats att det i detta gen-område ligger en eller flera tillväxthämmande gen(er) s.k. tumör suppressor gen(er) (TSG). Vi har lokaliserat 19 stycken gener i området och har analyserat uttrycksmönstret för alla generna på material från tumörer samt normal vävnad, och jämfört resultaten mellan grupperna. Från dessa resultat valdes tre gener, *Hic1*, *Skip* och *Myo1c*, ut som de mest troliga kandidaterna för de genetiska händelser vi har identifierat. Dessa tre gener har studerats vidare och *Skip* och *Myo1c* framstår som de starkaste kandidaterna till att utgöra deletionens måltavlor. Båda dessa gener har tillväxt-relaterade funktioner och det är högst troligt att en nedreglering av dem, orsakad av deletion och andra regleringsmekanismer, kan spela en viktig roll vid bildandet av livmodercancer i vårt modellsystem.

Information om specifika genförändringar för experimentella modeller kan överföras från modellen till motsvarande cancerform hos människan. I förlängningen kan kännedom om vilka gener som är involverade i en specifik cancerform även bidra till förbättrade diagnostiska verktyg, samt leda till ökade former för terapi och prevention.

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