Genomic and genetic alterations in endometrial adenocarcinoma
To my beloved family
for their support throughout my PhD Studies
Genomic and genetic alterations in endometrial adenocarcinoma
Abstract


The most frequently diagnosed cancer of the female genital tract is cancer of the endometrium (endometrial cancer), ranking fourth among the invasive tumors that affect women in Europe and North America. As most other cancer types, endometrial cancer is a complex genetic disease influenced by both genetic and environmental factors.

The human population is genetically heterogeneous and studies of complex diseases in human are proven to be difficult. By using a model system such as the BDII rat, some of the obstacles related to the study of complex diseases can be avoided. The BDII rat strain is prone to spontaneously develop endometrial adenocarcinoma (EAC) and more than 90% of the virgin females develop EAC during their lifetime. Development of EAC tumors in BDII rats is comparable in pathogenesis and histopathological properties to that of human.

The aims of this thesis were i/ to characterize EAC in the BDII rat experimental model system by analyzing structural and numerical chromosome aberrations, ii/ to evaluate the importance of the genetic set-up in EAC development, and iii/ to determine the impact of genomic and genetic alterations on the functionality of candidate genes in rat EAC and in human endometrial tumors of different FIGO grades.

Non-random numerical and structural aberrations that could contribute to tumor formation were identified, and evidence that the genetic background had a significant influence on the genome make-up of tumor cells was provided. Certain genes (Gpx3/GPX3, Met/MET, Phf5a/PHF5A, and Gja1/GJA1) were selected for further analysis and aberrant expression of some of them were found in both rat and human EACs. By separating EAC cell lines according to the genetic cross background, for two of the genes (Phf5 and Met), we showed that the expression pattern differed significantly between different cross backgrounds, which clearly pinpoint the importance of using animal models as a complement to clinical studies in identification of cancer-related genes.

Keywords: Endometrial cancer, Genetic background, BDII rat model, SKY, Chromosomal aberrations, Gene expression.

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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BDII</td>
<td>BDII/Han inbred rat strain</td>
</tr>
<tr>
<td>BN</td>
<td>BN/Han inbred rat strain</td>
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<tr>
<td>BRCA1</td>
<td>Breast cancer susceptibility gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer susceptibility gene 2</td>
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<tr>
<td>CCD</td>
<td>Charge-coupled device</td>
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<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
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<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
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<td>CSI</td>
<td>Chromosome structure instability</td>
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<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>EAC</td>
<td>Endometrial adenocarcinoma</td>
</tr>
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<td>EC</td>
<td>Endometrial cancer</td>
</tr>
<tr>
<td>F1</td>
<td>First generation</td>
</tr>
<tr>
<td>F2</td>
<td>Second generation</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence In Situ Hybridization</td>
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<tr>
<td>Gja1</td>
<td>Gap junction protein, alpha 1 gene</td>
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<tr>
<td>Gpx3</td>
<td>Glutathione peroxidase 3 gene</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non-polyposis colorectal carcinoma</td>
</tr>
<tr>
<td>ISCN</td>
<td>International System for Human Cytogenetic Nomenclature</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<tr>
<td>M-FISH</td>
<td>Multi-fluorochrome FISH</td>
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<tr>
<td>Met</td>
<td>Hepatocyte growth factor receptor gene</td>
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<td>MSP</td>
<td>Methylated specific PCR</td>
</tr>
<tr>
<td>N1</td>
<td>Backcross generation</td>
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<tr>
<td>NME</td>
<td>Non malignant endometrium</td>
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<tr>
<td>NUT</td>
<td>Backcross uterine tumor</td>
</tr>
<tr>
<td>Phf5a</td>
<td>PHD finger-like domain-containing protein 5A gene</td>
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<td>qPCR</td>
<td>Quantitative Real-Time PCR</td>
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<td>RB1</td>
<td>Retinoblastoma susceptibility gene</td>
</tr>
<tr>
<td>REF</td>
<td>Rat embryo fibroblast</td>
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<tr>
<td>RGSPC</td>
<td>Rat Genome Sequencing Project Consortium</td>
</tr>
<tr>
<td>RNO</td>
<td>Rattus Norvegicus</td>
</tr>
<tr>
<td>RUT</td>
<td>Rat uterine tumor</td>
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<tr>
<td>SKY</td>
<td>Spectral karyotyping</td>
</tr>
<tr>
<td>SPRD</td>
<td>SPRD-Cu3/Han inbred rat strain</td>
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<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumor suppressor gene</td>
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</table>
Papers included in the thesis

Paper I

Paper II

Paper III

Paper IV
Papers included in the thesis

Paper I

Paper II

Paper III

Paper IV
Falck, E., & Klinga-Levan, K. Expression patterns of Phf5a/PHF5A and Gja1/GJA1 in rat and human endometrial cancer. Manuscript
Background

The central dogma of biology

Transcription of DNA to RNA to protein, the central dogma that forms the backbone of biology (Figure 1), is represented by three major stages.

Replication: the process in which the DNA is duplicated. The chromosomes are prepared for replication in G1 phase of the cell cycle. The tightly packed chromatin will unwind to let the replication machinery get access to the DNA. The cell replicates all its information in the S phase of the cell cycle in a process that involves many enzymes. At the replication fork, DNA polymerases copy the template DNA strands into new complementary strands. Duplication of chromosomal material occurs in two separate steps, initiation of replication origin and elongation of the new DNA.

Transcription: the process in which the genetic information is transcribed from DNA to RNA (mRNA). RNA is synthesized by RNA polymerase in three steps: initiation, elongation and termination. (I) RNA polymerase initiates transcription by locating and binding specific to the transcription start, the promoter, of the gene (II) and then begins to transcribe one nucleotide at the time until the whole gene has been transcribed. The transcription is regulated by pauses of the RNA polymerase in certain DNA sequences to correct mismatched base pairs and correct it. (III) The transcription is terminated when the polymerase reaches a signal, terminator sequence, on the DNA.

Translation: the mature mRNA carries coded information from the cell nucleus to the ribosomes. The ribosomes "read" this information and use it for protein synthesis. A ribosomal complex binds to the initiation codon on the mRNA (AUG). The tRNAs bring amino acids to the ribosome in the order given by the codon on the mRNA. The ribosomes function as a catalyst and bind the amino acids together by the formation of peptide bonds. Termination occurs when the ribosome encounters a stop codon.

During the replication and transcription process information is copied and transferred with minimal errors. There are many control mechanisms to repair mutations during the replication process. However, there may be some not repaired...
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During the replication and transcription process information is copied and transferred with minimal errors. There are many control mechanisms to repair mutations during the replication process. However, there may be some not repaired
replication errors during the process that will lead to mutations such as base substitutions, codon change or a frame shift mutation.

![Diagram of the central dogma of biology](image)

*Figure 1. The central dogma of biology is represented by three major stages. Replication: The DNA copies its information. Transcription: The DNA codes for the production of messenger RNA (mRNA). Translation: Messenger RNA carries coded information to ribosomes. The ribosomes "read" this information and use it for protein synthesis.*

Cancer

Cancer is a complex disease involving genetic factors, inherited or acquired, and environmental factors. During cancer development a genetic change in one cell is followed by new alterations in the cells. One of the main characteristics of cancer is uncontrolled growth of the cells in a tissue. The cells start to divide out of control and can eventually end up in a malignant tumor. Seemingly, the transition from a normal cell to a cancer cell is a multistep process, during which genetic alterations are accumulated in the cell (Figure 2) [4, 5]. Once the cells have acquired a set of critical genetic alterations, the cancer cells proliferate in an uncontrolled manner and further random alterations will then accumulate in the daughter cells [6, 7].
For that to occur cells have to overcome many of the regulatory mechanisms that normally controls cell growth (Table 1). To remain in an active proliferative state and not to be dependent on surrounding tissue for growth factors, tumor cells have to become self-sufficient of growth signals. Tumor cells also have to become insensitive to surrounding anti-growth factors, and acquire the capability to avoid apoptosis and the checkpoints in the cell cycle. Furthermore, tumor cells can maintain the replicative potential by an up-regulation of the telomerase enzyme, which adds hexanucleotides to the telomeric ends to prevent them from shortening. By acquiring the capability to sustain angiogenesis, and the ability to invade surrounding tissue tumor survival is further maintained [6, 7].

Table 1. Acquired characteristics of cancer cells (Hanahan et al 2000, 2011).

<table>
<thead>
<tr>
<th>Acquired characteristics</th>
<th>Mechanism</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome Instability and mutation</td>
<td>Enabling characteristics, crucial for acquiring the six capabilities</td>
<td></td>
</tr>
<tr>
<td>Tumor-Promoting inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self-Sufficiency in growth signals</td>
<td>Activation of Oncogenes</td>
<td></td>
</tr>
<tr>
<td>Insensitivity to Antigrowth Signals</td>
<td>Loss of tumor suppressor activity</td>
<td></td>
</tr>
<tr>
<td>Evading Apoptosis</td>
<td>Production of survival factors</td>
<td>Acquired functional capabilities, crucial for survival</td>
</tr>
<tr>
<td>Limitless Replicative Potential</td>
<td>Turn on telomerase</td>
<td></td>
</tr>
<tr>
<td>Sustained Angiogenesis</td>
<td>Production of VEGF inducer</td>
<td></td>
</tr>
<tr>
<td>Tissue Invasion and Metastasis</td>
<td>Inactivation of E-cadherin</td>
<td></td>
</tr>
<tr>
<td>Deregulate cellular energetics</td>
<td>Emerging hallmarks, crucial for cancer phenotypes</td>
<td></td>
</tr>
<tr>
<td>Avoiding immune destruction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Endometrial cancer

Endometrial cancer (EC) is the most common diagnosed cancer among women in the western world, ranking fourth among the invasive tumors that affect women in Europe and North America with a ten times higher rate in the developed countries. One reason may be longer lifespan in the Western countries and therefore increased number of diagnosed EC cases. The uterus consists of two layers, the myometrium (the outer muscular layer) and the inner lining, endometrium (Figure 3). Endometrial cancer develops when cells in the endometrium begin to grow out of control. In later stages the cancer cells can invade nearby tissues or spread throughout the body [9]. Endometrial cancer can be classified into two types. Type I occurs most in pre- and peri-menopausal women and are often the effect of unopposed estrogen, which may cause genetic alterations, and do not often invade the surrounding tissues. The estrogen related Type I tumors, also known as low-grade endometrioid (endometrial-like) cancer, represent 80-85% of endometrial cancers. Endometrial adenocarcinoma (EAC) is the predominant subtype of Type I tumors and in the Western world approximately 80-85% of the patients diagnosis with EAC are over 50 years of age [10]. About 20% of endometrial cancers are diagnosed as Type II, which is a more aggressive high-grade carcinomas with non-endometroid pathology and not estrogen dependent. Type II tumors usually occur in post-menopausal women where serous carcinoma is the most common pathology [9-13].

Figure 3. An overview of the female reproductive organ and the two layers of the uterus, the myometrium (the outer muscular layer) and the inner lining, endometrium (Copyright antranik.org).
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The rat as a potent animal model system
The human population is genetically heterogeneous and is under influence of various environmental factors. Consequently, studies of complex diseases in human are proven to be difficult to perform. By using inbred model systems, some of the obstacles related to the study of complex diseases can be avoided. Since there is a high degree of conservation among mammals, information gained in an animal model can usually be applied on clinical human material. Inbred rat strains are commonly used as models in cancer studies [19, 20].

The rat was the first mammalian species that was used in science (18th century). The earliest studies focused on the fur color as a Mendelian trait. Inbreeding of the rat for biomedical research purposes first began in 1909 and at almost the same time inbreeding of the mouse as the animal model of choice for genetic studies started [21].

Rat is one of the most used species in research of disease processes in humans, and plays a key role in analysis of a number of complex polygenic diseases such as cancer. Presently, more than 1000 inbred rat strains have been developed by selective breeding of specific disease alleles to match a variety of complex disorders in human, such as hypertension, arthritis and cancer [22-26].

The rat draft sequence was first published in 2004 and was the third mammalian species to be sequenced after mouse and human. Since then, the rat sequence has been continuously updated [27]. Currently, more than 90% of the rat genome has been sequenced (the latest version RGSC 3.4), Rat Genome Sequencing Project.
Consortium (RGSPC) [23, 28, 29]. The majority of the genes associated with diseases has equivalents in the rat genome and are highly conserved among rat, mouse and human. In addition, during the last years a number of inbred strains of the species *Rattus norvegicus* have been sequenced for detection of SNP variations [27, 29, 30].

The human and rat genomes exhibit a high level of conservation and more than 95% of the genes found in rat have orthologs in the human genome. Thus, by using comparative mapping, findings from experiments in a rat model can easily be transferred to the human genome. Accordingly, human complex disease studies may become simpler if the use of animal model systems in combination with studies in corresponding human clinical material is considered.

The BDII rat model

There are four inbred rat strains (BDII/Han, Wistar/Han, Donryu/Han and DA/Han) that spontaneously develop EAC. Female animals of the BDII/Han rat strain (here after BDII) develop hormone dependent tumors with a very high incidence (more than 90% of the virgin females develop EAC during their life time [20]) and therefore represent a powerful endometrial tumor model for spontaneous hormonal carcinogenesis. Females in the other three strains (Wistar, Donroy and DA) also die from endometrial adenocarcinoma, but with lower incidence rates of 39%, 35% and 60%, respectively, if kept to their natural life ends [14].

Development of EAC tumors in BDII rats is comparable in pathogenesis and histopathological properties to that of human. The BDII tumor model has been genetically well characterized, but there is still much important genetic information that remains to be fully understood [14, 31].

Genomic and genetic alterations in cancer

Chromosome instability (CIN) and chromosome structure instability (CSI) are distinct features of most cancer cells. CIN and CSI are believed to contribute to tumor progression and are characterized by an increased rate of chromosome missegregation during mitosis resulting in numerical and structural changes in the daughter cells. Commonly CIN and CSI cause loss of function of tumor suppressor genes or gain of function of oncogenes. CIN is caused by abnormal segregation of chromosomes during mitosis and result in genomic alterations such as aneuploidy, which in some circumstances is regarded as good cytogenetic
markers for specific cancer types [32-34]. CSI is the outcome of chromosomal errors that are inappropriate repaired during cell division and may result in abnormal gene function [35].

A chromosomal translocation is an example of a structural abnormality, where the whole or a part of a chromosome becomes attached to, or exchanged with another chromosome or chromosomal segment. Deletions and duplications of chromosomal segments are also common examples of structural abnormalities. Broken chromosomal segments ends are highly reactive and tend to join other chromosomes with an open end, resulting in translocations. Amplification of chromosomal segments is another example. Sometimes the amplification includes a small segment of a chromosome containing one or a few genes that are amplified or duplicated many times at its/their original location resulting in the formation of homogenously staining region on the chromosome (HSR, Figure 4) [36, 37].

![Figure 4. Shows examples of structural chromosomal aberrations. A) In a translocation, the whole or a part of a chromosome becomes attached to or exchanged with another chromosome. B) Gain of chromosomal segments containing one or a few genes at its/their original location on the chromosome results in the formation of HSR (Courtesy: National Human Genome Research Institute, www.genome.gov).](image)

Although most cancers are sporadic, and always caused by genetic changes, a small fraction (5%) of all cancers are inherited. In such cases specific genetic changes are inherited, which leads to predisposition to develop cancer [38]. Two examples are inherited mutations of the BRCA1 and RB genes, involved in the
development of breast cancer, and, retinoblastoma, respectively. Women with one inherited mutation in one of the BRCA1 gene alleles will almost certainly develop breast cancer due to the higher risk of acquiring additional changes, the same as for the children who inherit a defected copy of the RB gene and will almost certainly develop cancer in the retina [39, 40].

Genetic changes associated with malignant transformation of cells are often related to two types of genes, tumor suppressor genes (TSG) and oncogenes. Both types of genes are involved in fundamental processes in the cell, such as cell division and apoptosis, but when mutated they are involved in progression and development of tumors. Tumor suppressors (ex. BRCA1, BRCA2 and TP53) usually act in a recessive manner, which means that both gene copies must be altered in order to silence the gene.

Except for inactivation by genetic/genomic alterations, TSGs can be inactivated by epigenetic mechanisms, like methylation (Table 2) [8, 41, 42]. Methylation occurs in CpG-rich areas, only at cytosine residues, and has an important regulatory effect on gene expression. These CpG-rich areas, also known as CpG islands, are located in the promoter regions of many genes. When CpG islands become methylated, the result will be a decreased gene expression or even total inactivation of a TSG [43].

### Table 2. Mechanisms of inactivation of tumor suppressor genes

<table>
<thead>
<tr>
<th>First hit (can be inherited)</th>
<th>Second hit LOH (loss of heterozygosity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small deletion</td>
<td>Nondisjunctional loss</td>
</tr>
<tr>
<td>Base substitution</td>
<td>Sub deletion</td>
</tr>
<tr>
<td></td>
<td>Unbalanced translocation</td>
</tr>
<tr>
<td></td>
<td>Mitotic recombination</td>
</tr>
<tr>
<td></td>
<td>Methylation</td>
</tr>
</tbody>
</table>

Cytogenetic and molecular techniques

In studies of chromosomal abnormalities, cytogenetic methods are used to detect chromosome aberrations and rearrangements through direct examination of chromosomes. Karyotyping is an appropriate method to detect chromosomal aberrations like translocations, inversions and deletions. G-banding, fluorescence in situ hybridization (FISH), and spectral karyotyping (SKY) are the most common techniques used for cytogenetic analysis [34]. The most suitable method to detect numerical changes and large structural aberrations through visualization in
FISH is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH is based on hybridization of a fluorescently labeled probe sequence to a specific gene and only those parts of the chromosome with sequence complementarity will be recognized and bound to the fluorescent probes. With the introduction of the FISH method in cytogenetic analyzes, a big change in how to analyze and interpret cytogenetic results was made, and for a long time FISH was the most widely used method to detect genomic changes. However, translocations or the origin of so-called marker chromosomes cannot be detected. The FISH technique was later developed into a multi-fluorochrome FISH (M-FISH), where all the chromosomes are labeled with a series of different dyes making detection of different forms of chromosomal alterations more feasible. There are two forms of M-FISH techniques, one is based on the use of a specific filter for each fluorochrome and the other is SKY, which is based on the specific signature of fluorochrome mixtures in a probe cocktail for all chromosomes [45, 46].

SKY

SKY is a method used to paint all chromosomes in one experiment by using a cocktail of probes for all chromosomes in the cell. SKY makes it possible to feasibly detect chromosomal aberrations like deletions, amplifications and translocations. Chromosome-specific painting probes for all chromosomes are hybridized to the metaphase preparations of tumor cells or cell lines in a single experiment. The chromosome specific painting probes are obtained by flow sorting of the chromosomes followed by two rounds of DOP-PCR. DNA from each chromosome is then labeled separately by different fluorescent dyes. Combinatorial labeling is used to give each chromosomal probe a specific fluorescent color by labeling with one or more of the fluorescent dyes (Table 3). SKY probes for rat has been available since 2003 and Buwe et al. performed the SKY technique on rat chromosomes for the first time [47]. In the SkyPaint kit for rat (Applied Spectral Imaging, ASI, Israel) the probes are labeled with three fluorochromes (Spectrum Green, Spectrum Orange and TexasRed) and two haptens (Cy5 and Cy5.5) or a mixture of them (Table 3). Chromosomal DNA is directly labeled with the fluorochromes, while labeling of the haptens is performed indirectly through biotin and Digoxin antibody chains [48].
Table 3. Combinatorial labeling scheme of the probe for the rat chromosomes for the SKY experiment

<table>
<thead>
<tr>
<th>Dye</th>
<th>Rat Chromosomes</th>
</tr>
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<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>x</td>
</tr>
<tr>
<td>B</td>
<td>x</td>
</tr>
<tr>
<td>C</td>
<td>x</td>
</tr>
<tr>
<td>D</td>
<td>x</td>
</tr>
<tr>
<td>E</td>
<td>x</td>
</tr>
</tbody>
</table>

A = Rhodamine (orange), B = Texas Red (red), C = Cy5 (infrared 1), D = FITC (green) and E = (infrared 2)

Following hybridization of the probe cocktail to metaphase chromosomes and subsequent immunodetection, a spectral image is acquired by using a conventional fluorescence light microscope equipped with a triple-bandpass filter. Signals from Texas Red, Rhodamine, FITC, and the SpectraCube (Applied Spectral Imaging, Israel) are detected, which enables retrieval of spectral information for every pixel in a digital CCD image. The light passes through an interferometer and the generation of a spectral image is achieved by acquiring approximately 100 frames of the same image. Each frame differs in the optical path. An emission curve is created for each pixel of the raw spectral image that shows the wavelength of the fluorescent intensity (Figure 5). The DAPI image is acquired separately. A software-viewing program is then used to display the images. The software SkyView® spectral imaging system (Applied Spectral Imaging, Israel) shows three different images of the chromosomes:

- One in red-green-blue (RGB), used for the monitoring of the hybridization quality and the signal strength.
- The second image shows the pseudo-color after the classification of the spectral signatures, where the whole range of colors can be seen. The pseudo-colors can be seen as a painted image.
- The third image is an inverted DAPI that shows the chromosome bands and is used as a complement to the other two images for banding control of the chromosomes [45].
Table 3. Combinatorial labeling scheme of the probe for the rat chromosomes for the SKY experiment

<table>
<thead>
<tr>
<th>Rat Chromosomes</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
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<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
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A = Rhodamine (orange), B = Texas Red (red), C = Cy5 (infrared 1), D = FITC (green) and E = (infrared 2)

Following hybridization of the probe cocktail to metaphase chromosomes and subsequent immunodetection, a spectral image is acquired by using a conventional fluorescence light microscope equipped with a triple-bandpass filter. Signals from Texas Red, Rhodamine, FITC, and the SpectraCube (Applied Spectral Imaging, Israel) are detected, which enables retrieval of spectral information for every pixel in a digital CCD image. The light passes through an interferometer and the generation of a spectral image is achieved by acquiring approximately 100 frames of the same image. Each frame differs in the optical path. An emission curve is created for each pixel of the raw spectral image that shows the wavelength of the fluorescent intensity (Figure 5). The DAPI image is acquired separately. A software-viewing program is then used to display the images. The software SkyView® spectral imaging system (Applied Spectral Imaging, Israel) shows three different images of the chromosomes:

- One in red-green-blue (RGB), used for the monitoring of the hybridization quality and the signal strength.
- The second image shows the pseudo-color after the classification of the spectral signatures, where the whole range of colors can be seen. The pseudo-colors can be seen as a painted image.
- The third image is an inverted DAPI that shows the chromosome bands and is used as a complement to the other two images for banding control [45].

Figure 5. Spectral imaging acquiring and analysis. Hybridized metaphase chromosomes on the slide are visualized by fluorescence microscopy ([49], By permission of Thomas Read laboratory).

The color display and the chromosome classification are based on the unique emission spectrum of the chromosomes. Together with the chromosome banding information from an inverted DAPI, a comprehensive overview of chromosomal aberrations can be obtained [48]. The SKY images of the chromosome look like a painted image and the breakpoint of the translocation has to be compared to the inverted DAPI banding to find out the approximate translocation point. For further analysis, probes for specific arms or chromosomal bands or FISH technique can be used as a complement.

SKY analysis may provide new markers for early diagnosis and therapeutic purposes as genomic approaches including SKY have proven to be effective in detecting recurrent chromosomal alterations and pinpointing candidate genes that are involved in the cancer development [34].

PCR and Methylated Specific PCR (MSP)

The polymerase chain reaction (PCR) is a widely used method to amplify DNA for different purposes, for example to detect epigenetic modifications in the DNA. For that purpose, the DNA template is treated with sodium bisulfite, which modifies non-methylated cytosines to uracils. PCR is based on the hybridization of the primer sequence to the DNA, and in MSP two sets of primer pairs is used to distinguish methylated from un-methylated cytosine. This is made
possible due to the sequence differences from the bisulfite treatment of the DNA template [43].

Real-Time PCR
Real-Time PCR or as it also called quantitative PCR (qPCR) enables to determine the exact concentration of amplified DNA in a sample by quantification of the number of specific targets in the sample. In qPCR a fluorescent reporter that binds to the PCR product is used. The reporter generates a fluorescence signal that mirrors the level of product formed during the run. The signal initially increases exponentially until it reaches the plateau phase. The cycle threshold (Ct) is defined as the number of cycles required for the amplified signal to cross the threshold, the level background signal. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. Therefore, Real-time PCR allows for monitoring of the accumulation of PCR product at any time point during the amplification reaction, thereby enabling the investigator to identify the cycles of near-logarithmic PCR product generation. The absolute number of mRNA target copies in the sample can then be compared with the Ct values of the endogenous controls and samples [50, 51].
Previous work on EAC in the BDII rat model

In order to study the genetic aspects of EAC development in the BDII rat model, intercross (F1, F2) and backcross (N1) populations were set up by breeding female rats of the BDII strain to males from two different strains with low EAC incidence, BN/Han and SPRD-Cu3/Han (hereafter BN and SPRD). The females were examined weekly and in cases of suspected tumor, the animals were submitted to necropsy. The tumors were surgically removed and pathologically examined, and cell cultures were established whenever possible (Figure 6) [52, 53].

Association studies between certain marker alleles and tumor incidence were performed in the female cross progenies by means of genome-wide screening with microsatellites [53, 54]. From these studies it became clear that the onset of tumors depends not only on the presence of susceptibility alleles from the EAC-prone BDII strain, but is also affected by the contribution of a genetic component from the non-susceptible strains (Table 4).

Characteristic chromosomal changes were scanned for in the tumors that appeared in the progeny by comparative genomic hybridization (CGH) and cytogenetic studies [55, 56]. The conclusion from these studies was that certain chromosome regions are recurrently engaged in chromosomal copy number changes, including both increases (e.g. ploidy changes, duplications of parts of or whole chromosomes, amplifications) and decreases (e.g. deletion of parts of or whole chromosomes). In five of the chromosomes with recurrent chromosomal aberrations detected by CGH, specific regions of allelic imbalance were identified (Table 4) [57-64]. Certain genes located within regions with chromosomal aberrations were shown to be affected [65-68].

Specific expression patterns involved in EAC tumourigenesis were studied by global gene expression profiling. Hierarchical clustering in combination with statistical methods revealed several genes belonging to different cancer-associated pathways/systems to be differentially expressed between EAC samples and normal controls (Table 4) [66, 69, 70].
Table 4. Previous studies of genetic aspects of EAC development in the BDII rat model

<table>
<thead>
<tr>
<th>Type of study</th>
<th>Publications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Association studies</td>
<td>Roshani et al 2001, 2005</td>
</tr>
<tr>
<td>Cytogenetics and CGH</td>
<td>Hamta et al 2005; Walentinsson et al. 2001; Helou et al 2000, 2001,</td>
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</table>
Aim of the thesis

The overall aims of this thesis were to

- Characterize EAC tumors from the BDII rat model
- Evaluate the importance of the genetic set-up in the experimental model.
- Determine the impact of genomic and genetic alterations on the functionality of the genes in rat EAC and in human endometrial tumors of different FIGO grades

Paper I

The aim was to characterize structural and numerical aberrations in the BDII rat model of EAC.

Paper II

The aim was to clarify the impact of the genetic setup of the two non-susceptible parental strains (BN and SPRD) in EAC tumourgenesis and to identify aberrant chromosomal regions independent of cross background.

Paper III

The aim was to elucidate the mechanisms underlying down regulation of Glutathione peroxidase 3 (Gpx3) and the oncogene Hepatocyte growth factor receptor (Met) in EAC from the BDII rat model as well as of the corresponding genes in human EAC tumor samples of FIGO grades I-III.

Paper IV

The aim was to determine the expression of Phf5a and its effect on Gja1 expression in tumors from the BDII rat model from different genetic backgrounds, and in human EAC tumors of FIGO grades I-III.
Ieva Falck  
Genomic and Genetic Alterations in EAC

Materials and methods  
The rat material  
Crosses had previously been set up between BDII females (genetically predisposed to EAC) and BN or SPRD males as described in Roshani et al 2001 (Figure 6). The primary cell cultures from the suspected tissues were grown only for a few passages to retain the genetic status in the cell lines to that of the original tumors. The tumors that developed in the N1, F1 and F2 progeny were pathologically classified as EAC, or other uterine tumors. At necropsy in some of the suspected tissues no cancer cells were detected when pathologically analyzed. These tissues and cell lines derived from them were referred to as non-malignant endometrium (NME), and cell lines derived from malignant tissues were referred to as EAC (Table 5) [66]. RUT cell lines represent EAC/NME developed in F1 and F2 progeny and the NUT tumors represent EAC/NME developed in the backcross progeny [52, 53].  
A total of 41 rat EAC tumor cell cultures were used in the entire study. Eighteen (12 EAC and 6 NME) of the samples came from the SPRD and 23 (18 EAC and 5 NME) from the BN background (Table 5). The material was organized according to the origin of the cell lines. Thus, the four groups represent cell lines derived from tumors or non-malignant tissues developed in the female cross progeny between BDII and SPRD, and between BDII and BN.  

Figure 6. An overview of the crosses performed in the BDII rat model (Illustration C. Torudd).
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The rat material

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Figure 6. An overview of the crosses performed in the BDII rat model (Illustration C. Torudd).
Table 5. Cell lines in the four papers included in the thesis

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Human tumor material

The human material consisted of totally 30 EACs with FIGO grade I-III and 26 benign samples. Ten tumors from each grade embedded in archival formalin fixed paraffin (FFPE) were used in the study. The control samples consisted of 26 benign endometrial tissues (12 of secretory phase and 14 of proliferative phase), and as reference, material from lung and normal endometrium was used (Table 6). All samples were anonymous. A pathologist marked the tumor area in samples in the hematoxylin and eosin stained slide. Using a Tissue Micro Array equipment (Pathology Devices), 3-4 cores (⌀0.6mm) of tumor tissue was punched out from the paraffin block. After standard tissue sample de-paraffinization using xylene and alcohols, samples were lyzed in a Tris-chloride, EDTA, sodium dodecyl sulfate (SDS) and proteinase K containing buffer.

Table 6. Overview of the human tumor material used in the expression study of GPX3, MET, PHF5A and GJA1.

<table>
<thead>
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</table>

Statistical methods

Data on chromosome number and ploidy grades obtained from paper I was used for further statistical analysis in Paper II but with the main focus on the different backgrounds. The ploidy distribution among the tumors was analyzed with a $\chi^2$-test with the null hypothesis that there were no differences between the backgrounds. The independent sample t-test and $\chi^2$ test were applied to analyze the influence of different cross backgrounds on the occurrence and amount of numerical and structural changes in the tumor material. A $\chi^2$ test was applied to check the influence of ploidy grade on incidence of structural changes in the different backgrounds. Pearson’s correlation coefficient was calculated for the relation between the chromosome size and the number of changes per chromosome.

For statistical evaluations of gene expression data in Paper III and IV, Ct values for differences among replicates was analyzed by ANOVA. For comparisons of
expression differences between normal and malignant tissues, independent sam-ple t-test was applied on the log 2-fold change of ΔΔCt values. In both tests the null hypotheses were assuming no differences between replicates, and no differences between tissue types respectively. In addition the Pearson correlation test was performed on log 2-fold ΔΔCt values to check for correlation between expressions of the genes. The significance levels were set to P<0.05 in all statistical tests. Furthermore, the tumor material from the BDII rat model was analyzed for differences in gene expression between the two backgrounds, BN and SPRD.
Results and Discussion

Summary of Paper I

In Paper I we determined the most common cytogenetic changes among the tumors (Table 6). The International System for Human Cytogenetic Nomenclature (ISCN 1995) and literature on nomenclature for G-bands in rat chromosome were used for classification and localization of the cytogenetic aberrations in the tumor material [57, 71]. The majority of tumors displayed a very complex pattern of numerical and structural aberrations.

Non-random numerical changes were detected in the tumor material (Table 6). By comparing the observed and expected numbers of the chromosomes in the tumor panel, it appeared that chromosome gains were more common than losses (Paper I, Table 4). In the whole material, the most commonly gained chromosome was RNO4 (+23.34%, Figure 2) and the most commonly lost chromosome was RNO15 (-14.19%, Figure 2).

To investigate the frequency of non-random structural changes, amplifications, deletions and translocations were recorded in all metaphases. To distinguish between random and nonrandom changes, a correlation analysis was performed. If the observed changes were random, a correlation between the number of observed structural changes in each chromosome and the size of the chromosome would have been detected. There was no such correlation (Pearson’s coefficient of correlation, P>0.05), and therefore it was concluded that the observed structural changes, at least for some chromosomes, were non-random events, and specific chromosomes were responsible for this lack of correlation. The analysis revealed that the number of structural changes in six chromosomes, RNO3, 6, 10, 11, 12 and RNO20, were higher than would be expected with regards to their sizes. In all of these chromosomes, except for RNO10, more than 80% of the changes were translocations (Paper I, Table 4). For RNO10, translocations and deletions were present to the same extent. Our analysis suggested that structural changes in these chromosomes were selected for during endometrial carcinogenesis, and thus these changes represent non-random events with potential contribution to EAC tumorigenesis in this model.

Summary of Paper II

The aim of the work in Paper II was to analyze the genomic differences between the primary tumor cell lines from the two genetic backgrounds and to identify aberrant chromosomal regions dependent or independent of cross background.
Thus, we analyzed the ploidy state and the total number of numerical and structural changes per chromosome separately in the groups of tumors derived from the BN and SPRD backgrounds (Paper II, Table 1 and 2).

The majority of the tumors comprised a mixed population of clones with different ploidy grades (Paper II, Table 1). Ten of the EAC cell lines were derived from crosses between BDII and SPRD and the other ten from crosses between BDII and BN (Table 5). The majority of tumors derived from the SPRD background displayed a diploid or close to diploid karyotype, whereas tumors from the BN background were, for the most part, triploid (Paper II, Table 1). The distribution of the most common ploidy grades among tumors (i.e. diploids, triploids and tetraploids) differed significantly between tumors from the two genetic backgrounds ($\chi^2=10.33$, df=2, P<0.001).

When the number/type of structural changes and the ploidy state of the tumors was investigated, we found that translocations were the most commonly seen structural aberration in both of the backgrounds, but with a higher frequency in tumors of the SPRD background. The structural changes were more abundant among diploid and triploid EACs developed in the SPRD background (P< 0.001) with translocations as the predominant type (Paper II, Figure 1).

To evaluate whether numerical and structural aberrations of individual chromosomes differed between the BDIIxBN and BDIIxSPRD crosses, we calculated the number of deviations of individual chromosomes. The incidence of numerical changes was clearly dependent on the genetic background as in this analysis highly significant P differences were obtained for certain chromosomes. In addition, distribution and number of structural and numerical changes for certain chromosomes differed significantly between the two groups of tumors derived from the cross progenies (Paper II, Table 2).

There was no correlation between the chromosome size and the number of changes per chromosome when the two backgrounds were analyzed separately (Pearson’s coefficient of correlation, P>0.05) (Paper II, Table 2, Figure 2). The chromosomes responsible for the lack of correlation were RNO3, 6, 9, 10 and RNO13 in the BN background and RNO6, 10, 11 and RNO12 in the SPRD background. We thus identified abundant non-random structural chromosomal changes in the tumor material that in some cases were common to both and in some distinct to the backgrounds.

To address which changes were dictated by BDII, we implemented four criteria to detect aberrations with the same occurrence pattern in the two backgrounds (Paper II, Table 2). We applied a reasonably stringent approach and only when an aberration fulfilled at least three of the four criteria, it was regarded as a common
tribution from the susceptible strain. Five chromosomes fulfilled this requirement: RNO8, RNO9, RNO10, RNO17 and RNO18 (Paper II, Table 3). Identification of chromosomes harboring aberrations independent of the genetic input from the non-susceptible strains provides valuable information about the potential location of EAC susceptibility genes in this model.

Summary of Paper III

In a previous expression microarray study 354 genes were identified as significantly altered compared to normal/pre-malignant tumors [72]. Among these, Gpx3 (Glutathione peroxidase 3) was identified as one of the top three candidate genes with potential implications in EAC development, since the expression was highly down regulated in rat EAC tumors [66]. GPX3 catalyzes the reduction of peroxides and protects the cells against oxidative damage that may lead to an increase in the gene mutation rate.

The mRNA expression of Gpx3/GPX3 and Met/MET (Mesenchymal-epithelial transition factor) were analyzed by qPCR in cell lines from EAC developed in the BDII rat model as well as in 30 human EACs of different FIGO grades and 20 benign endometrial tissues.

The expression of Gpx3/GPX3 was uniformly down regulated in both rat and human EACs regardless of tumor grade or histopathological subtype, whereas the expression in non-malignant or benign tissue displayed a normal to high expression, implying that the down-regulation is an early event in EAC. In the rat cell lines, the expression of the Met gene was slightly higher among the malignant cell lines whereas for the human material the expression of MET was significantly lower among the malign tumors with no differences between FIGO grades. A previous study on human prostate cancer suggested that GPX3 could exhibit tumor suppressor activity by transcriptional regulation of the oncogene MET. We could not confirm tumor suppressor activity of GPX3/Gpx3 or transcriptional regulation of the oncogene Met/MET either in human or rat endometrial tumors [73].

In addition to qPCR, the methylation status of Gpx3 was analyzed and Gpx3 promoter methylation was seen in more than 90% of the tumors, where the bi-allelic pattern was the very most common. In two of the tumor cell lines that displayed bi-allelic hypermethylation, the Gpx3 mRNA expression was successfully restored after demethylation treatment.

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**Summary of Paper IV**

PHF5A have previously been suggested to play a very complex role as a general transcriptional activator of different genes. The \textit{PHF5A} orthologous gene in yeast plays a crucial roll for the cell viability and survival, and in \textit{C.elegans} the \textit{Phf5a} orthologoue displays a tissue- and stage-specific pattern of expression during morphogenetic development [75]. In rat myometrium the PHF5A protein has been suggested to function as a transcription factor for the gene \textit{Gja1} in the presence of estrogen by binding to the proximal promoter region and enhance expression of \textit{Gja1} [76]. It has been shown that \textit{GJA1} is down regulated in different human cancer types and that it displays tumor suppressor activity [77].

In association studies in the BDII model of EAC one small genomic region associated to the development of EAC including the \textit{Phf5a} gene was identified (Roshani, Beckman et al. 2000; Roshani, Mallon et al. 2005). As revealed in the expression profiling, the \textit{Phf5a} gene expression was commonly down regulated in EAC cell lines from tumors developed in the F2 and N1 progenies [66, 72].

We performed gene expression analysis for \textit{Phf5a/PHF5A} and \textit{Gja1/GJA1} in a panel of BDII rat EACs and NMEs, as well as in a panel of human EACs of different FIGO grades and benign tumors. The result of the expression study of \textit{Phf5a} in rat could not be verified in the human samples as \textit{PHF5A} was found to be more down regulated in the human benign samples than in the human tumor samples (Paper IV, Table 3, Figure 3A).

The \textit{Phf5a} expression in the rat EAC tumor panel after separating groups of tumors by tissue types EAC and NME as well as by cross backgrounds was significantly different between backgrounds as EACs cell lines derived from the progenies of the (BDIIxBN)xBDII displayed lower expression compared to NMEs (Paper IV, Table 3, Figure 2A). Hence, cross set-ups such as in the BDII rat model, permit findings in relation to the influence of genetic background on gene expression that otherwise is difficult to uncover.
It has been shown that \textit{GJA1} is downregulated under the control by PHF5A in mammary tumors, lung cancer and endometrial adenocarcinoma [78-80]. This could not be validated in this study as the \textit{Gja1/GJA1} gene was not down regulated in the EAC cell lines, or in any of human tumor classes (Table 3, Paper IV).

Not included in paper III, we analyzed the expression of \textit{Gpx3} and \textit{Met} in the different backgrounds and it was revealed that \textit{Met} is differentially expressed between the two backgrounds (P>0.05).
Conclusions

The aim of this thesis was to characterize EAC tumors from the BDII rat model, and to study the impact of the identified genomic and genetic alterations on the functionality of the genes in rat EAC and in human endometrial tumors of different FIGO grades. The overall goal was to identify common changes on chromosomal, DNA and RNA levels that were specifically related to development of endometrial adenocarcinoma in a rat model. The findings can be applied to or taken into consideration in studies of human EAC. This may also help to provide new insights into underlying molecular mechanisms involved in development of this malignancy in humans.

Some of the conclusions that can drawn from the studies are:

- SKY is a useful technique in clinical cytogenetics, especially in analysis of tumor cells in which multiple and complex chromosome aberrations is commonly found.
- Non-random numerical and structural aberrations with potential contribution to tumor formation were identified in experimental EACs.
- Evidence that the genetic background plays a significant role in the influence of the genome make-up of tumor cells was presented as some aberrations were found to be background dependent, i.e. only appeared in one of the crosses.
- Other aberrations were derived from the susceptible strain, as they appeared independent if the backgrounds. Accordingly a detailed cytogenetic analysis of chromosomal aberrations may shed some light on the identification of susceptibility genes involved in EAC development in the BDII model, and potentially in human.
- A consistent down-regulation of Gpx3/GPX3 in both rat and human EACs was demonstrated. From these results important clinical implications of GPX3 expression in EAC can be proposed, both as an important biomarker for EAC and as a potential target for therapeutic interventions.
- PHF5A/Phf5a was found to be differentially expressed both in human and rat EACs, even if the pattern of changes was not completely consistent between the two species. Thus, PHF5A/Phf5a may have an impact on tumor development, but the underlying mechanism(s) remains to be investigated.
In gene expression analysis, by separating EAC cell lines according to the cross backgrounds, we found that Phf5 and Met expression differed significantly between tumors derived from different cross backgrounds.

In summary, by using an experimental model, we showed that certain aberrations in the tumors lead to changes in gene expression. Our analysis also clearly showed that influence of the genetic background is a crucial driving force on the pattern and frequency of genetic changes that are found in tumor samples as well as on the pattern of gene expression. Accordingly, through molecular analysis of recurrent chromosomal, DNA and RNA changes, we might be able to find and define novel cancer-related genes with significance in tumorigenesis pathway(s). Our analysis additionally confirms and extends the importance of using animal models as a complement to clinical studies, in particular for those tumors with very complex patterns of genetic changes.
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References

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