A study on endocrine disrupters in the environment through the microarray technology

Dissertation for the attainment of the academic degree of *Doctor rerum naturalium* given by the Faculty of Mathematic and Natural Sciences of the Technical University of Dresden

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....Zu meine kleine Dicktator

"Da steh ich nun, ich armer Tor!
Und bin so klug als wie zuvor."

Introduction

1. Outline of the project

Due to the current rise of exposure to natural and synthetic compounds in our daily life, the debate concerning the safety of many substances is becoming increasingly relevant.

The estrogenic activity of various compounds, described as xenoestrogens, is the major part of this debate. Humans beings are exposed to these substances from different environmental contaminations ranging from conscious intake of estrogenic substances, as in contraception or in hormone replace therapy (HRT), to unconscious exposure, from food, the use of synthetic material in daily life and air and water pollution. At this point the need for methods to investigate the activity and the safety of these substances is becoming increasingly important.

Classical methods for the analysis of the estrogenic activity of substances, like batteries of *in vivo* test systems on the rat uterotrophic assay are not able to describe the different pathways of action of recently discovered estrogenic substances. This evidence was already shown by the Organization for Economic Cooperation and Development (OECD), introducing new test guidelines for the investigation of effects of endocrine disruptors (according to enhanced Test Guideline 407). As reviewed by Nilsson (Nilsson *et al.,* 2001), after the interaction of the estrogens with the Estrogen Receptor (ER) in the cells, the mechanism of activation possible is not only via direct binding of the ER to the Estrogen Responsive Elements (EREs) present in the promoter region of the target gene, very well described for many target genes, but that also other mechanisms are used: the interaction of the ER with the AP-1, Sp-1 and NFkB modes, that are discovered but not yet comprehensively described.

The aim of my work is to produce a microarray DNA chip for the investigation of the estrogenic activity of different compounds present in the environment. The chip will consist of a selection of 100 genes that are estrogen responsive and it will cover the spectrum of activities of estrogenic compounds in various organs of the body. In the gene selection, genes were chosen that are estrogen responsive in the classical target tissues of estrogens, linked to reproduction, like uterus and mammary gland, and also in tissues not related to reproduction like liver, bones and capillars. In addition, other genes are included to monitor different pathways that are related to disease states; control of cell proliferation, apoptosis or cancer related genes. Currently these kinds of investigations are already in process, but by other methods which are more time consuming and with a lower throughput e.g. the gene expression profiling using the real time RT-PCR. The use of microarray's satisfies the need for a less time consuming, high throughput method, to obtain a fast characterization of the gene expression finger print of the candidate substances and their mechanism of action in the organism.

2. Overview on estrogens

The number of xenoestrogens, substances with estrogenic activity not derived from any human source, is constantly increasing. Some studies like the "million women study" (1999) concerning the use of classical methods in HRT did not deliver good results about the safety of the classical methods in HRT like estrogen and progesterone. It is already been observed that, in western society, the causes of several cancers including breast, endometrium, ovary, liver, and prostate cancers, have been linked to inappropriate and/or prolonged exposure to synthetic steroidal hormones (Yager *et al.,* 1996).

Currently, xenoestrogens are in contact with human beings constantly during everyday life from many different sources. They can be related to the health care as with HRT, treatment of hormonal dysfunction, or in the use of contraceptives. Xenoestrogens could also come in contact with us unintentionally: consuming foods containing substances with estrogenic activity, like phytoestrogens in soy beans and hop, as well as by the consumption of meat derived from animals treated by hormones to enhance their growth. There are also many other possibilities of contamination from the use of synthetic material in daily life. These range from the wearing of synthetic clothes to the use of plastic in the alimentary industry, including bottles and cutlery. There is also other evidences of a contamination of xenoestrogens by the pollution of the environment through air and water, for instance there are studies on the estrogenic potential of the Venice lagoon waters too (Pojana *et al.,* 2004).

The question of the real activity and safety of these substances is still open. The three days uterotrophic assay alone, consisting in the assessment of uterus wet weight after three days treatment with a specific substance in ovariectomised (ovx) or juvenile rats, is not sufficient to elucidate the molecular mechanism of action of these "new" compounds. New methods are needed to investigate their effects in detail.

One possible method to investigate the activity of these substances in depth is the gene expression profiling of selected genes, already described in literature, that are estrogen regulated according to the four different mechanisms of action reviewed by Nilsson (Nilsson *et al.,* 2001) described in paragraph 2.3 of this section: the activation of estrogenic mechanism by ERE, the first to be discovered, classical mechanism of action of estrogen after diffusion into the cell, and the three alternative mechanisms of action through the AP-1, Sp-1 and NFκB pathways. For the classical mechanism of action, through ERE, a comprehensive set of genes are already described, but for the other three major mechanisms there are insufficient genes described as potential molecular markers.

The gene expression analysis of these target genes is mostly performed by real time RT-PCR. This method allows a really sensitive study of the gene expression but is limited by the low number of genes to be assessed in a reasonable amount of time.

2.1 Estrogen target tissues

The main targets of sexual hormones are tissues related to reproduction like the uterus mammary gland, vagina, ovary or urogenital tract. Other tissues, which were not previously included in the list of sex hormone target organs, as with the liver, central nervous system (CNS) skin and many other organs, represent sensitive novel targets of estrogen exposure (fig. 1). Other organs like bones, even though not related to reproduction present since a long time, a high sensitivity to estrogenic substances. It's then possible to talk about a pleiotropic effect of estrogen in the body (Huber *et al.,* 2002; Muller, 2004).

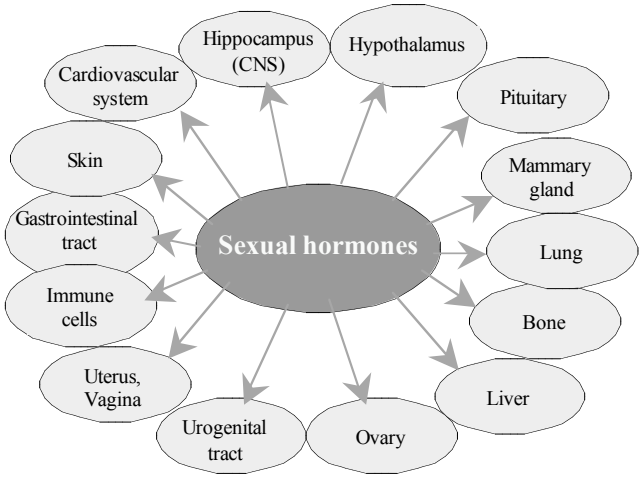

Fig. 1: Target tissues of the sexual hormones (Vollmer *et al.,* 2002).

2.2 Estrogen receptors

Since 1962, it has been clear that the biological activity of estrogen is mediated by a receptor protein (Jensen & Jacobsen 1962). In 1986 two groups reported, independently, the cloning of the first Estrogen Receptor (ER) gene (Green *et al.,* 1986; Greene *et al.,* 1986). A second type of ER had been discovered in 1996, it was identified in a rat prostate cDNA library (Kuiper *et al.,* 1996). The first identified ER is now called ERα and the second one ERß, since then many isoforms of both subtypes have been described, consisting of ablation of some exonic regions (Shupnik *et al.,* 2002) or in the case of the ERß2 the insertion of short sequences, 54 nucleotides, between the 5^{th} and 6^{th} exon (Chu & Fuller, 1997).

Structurally the two receptors are characterized by six individual domains with those functional important domains (fig. 2): the NH2-terminal (A/B domain), the DNA-binding domain (DBD) (C domain) and the ligand binding domain (LBD) (D/E/F domain). The N-terminal domain encode for an independent activation function (AF1) (Berry *et al.,* 1990), which is involved in the protein-protein interaction and transcriptional activation of the target-gene (Onate *et al.,* 1998). The DBD plays an important role in the dimerization and the binding of the receptor to specific DNA sequences (Schwabe *et al.,* 1993). The LBD mediates ligand binding, receptor dimerization and transactivation of the target gene (Tsai *et al.,* 1994).

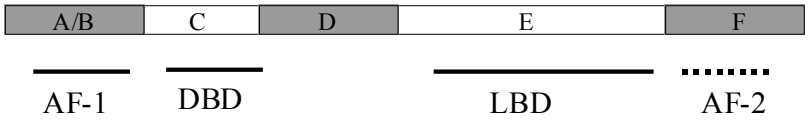

Fig. 2: Domain structure of an estrogen receptor, with division by domain. AF: activation function, DBD: DNA binding domain, LBD ligand binding domain (according to Nilsson *et al.,* 2001).

The two receptors present a high level of amino acid sequence homology (fig. 3). In human beings the homology of the DBD (C domain) is 97 %, the LBD (E domain) presents also the 60% of homology, for the other region the level is lower, between 20 and 30% (Saunders, 1998).

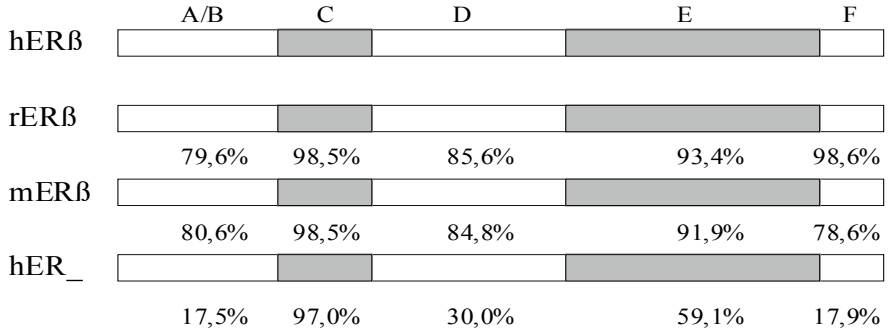

Fig. 3: Homology of estrogen receptors (ER), reported in percentage, in comparison to the human ERß (hERß). Rat ERß (rERß), mouse ERß (mERß) and hERα, according to the different domains (Saunders, 1998).

The receptors are also phylogenetic conserved. The ERß, for example, presents a high degree of homology between human, rat and mouse (fig. 3) (Kuiper *et al.,* 1996; Tremblay *et al.,* 1997). A homologue of the mammalian ERß is present also in fish (Tchoudakova *et al.,* 1999; Chang *et al.,* 1999). The phylogenetic tree of the cloned estrogen receptors is shown in fig. 4 (Nilsson *et al.,* 2001).

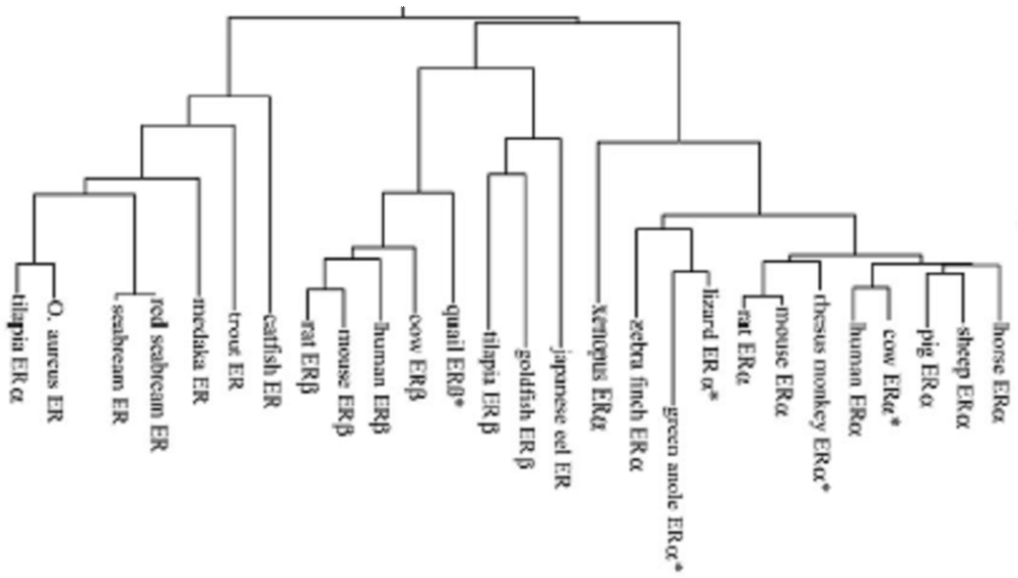

Fig. 4: Phylogenetic tree of the cloned ER (Nilsson *et al.,* 2001).

The two types of ER are not distributed equally in the body. Both receptors are present in breast, CNS, cardiovascular system, bone tissue and the urogenital tract. In the liver, only the presence of ERα is reported and ERß is predominantly present in the gastrointestinal tract, as shown in fig. 5 (reviewed by Gustafsson, 1999).

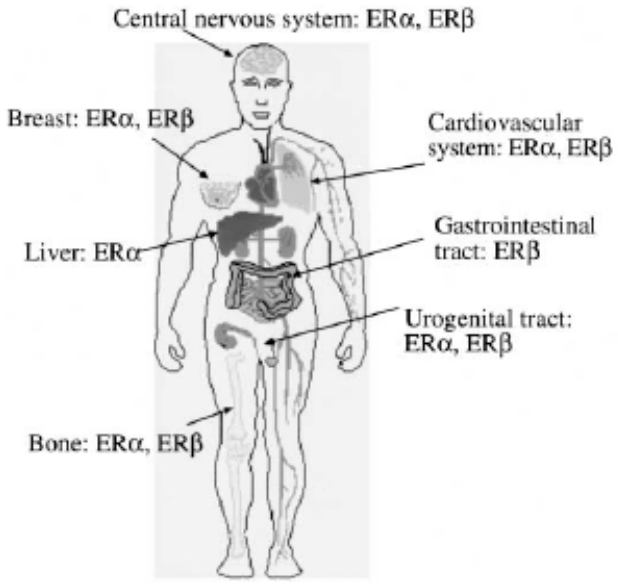

Fig. 5: Distribution of ERα and ERß in the body in the different organs, according to Gustafsson (1999).

2.3 Mechanism of estrogens action

After diffusion into the cell through the membrane, estrogens can operate with or without interaction with their respective receptors. In the first case, the ER mediated response, the activated receptor-ligand complex, with possible interaction with other co-factors, acts through specific regions of target genes to influence their gene expression. Interaction with the respective receptors can also follow another path, commonly described as the non-genomic pathway, in which the complex estrogen-receptor moves not to the nucleus but to the cellular membrane and activates the Src (steroid receptor-coactivator) / Shc (Src-homology/collagen protein) / ERK (extracellular signal-regulated kinase) signaling cascade (Kousteni *et al.,* Science 2002). In the non-genomic pathways the liganded receptor is recruited to the membrane thereby activating signal transduction cascades. In the second case estrogens can affect cellular processes through other mechanisms without interaction with the receptor. In this receptor independent mechanism of action, the estrogens can bind and alter enzymatic activities in membranes isolated from target cells, they can also influence the activities of purified enzymes and change cell permeability and polarization; all these actions are performed excluding transcriptional activities (Weiss *et al.,* 1998). This receptor independent mechanism of action presents a rapid time course and insensitivity to transcriptional and translational inhibitors, confirming the assumption that genomic effects are not involved (Falkenstein *et al.,* 2000).

In this work only the ER mediated and genetic mechanisms of action will be taken into consideration, because it's possible to study them through gene expression analysis.

In the ER mediated mechanism the receptor dimerizes, after binding with the ligand (an estrogenic substance) in three possible ways: as a homodimer of ERα, ERß, or as heterodimer ERα/ERß (Cowley *et al.,* 1997; Pettersson *et al.,* 1997). Thereafter the dimer can bind to the ERE present in the promoter region of the target gene (fig. 6).

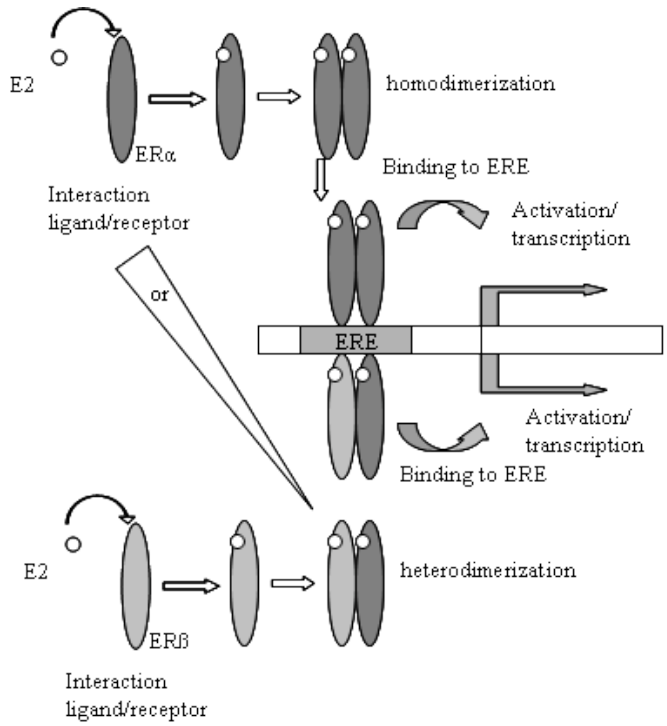

Fig. 6: Model of the homodimerization (upper part of the image) /heterodimerization (lower part of the image) of the estrogen receptor (ER) after the binding with the ligand (E2), and interaction with the estrogen responsive element (ERE) present in the promoter region of the target gene.

The ER can play a role in gene expression regulation also without interacting directly with the genomic DNA sequence (review by Nilsson et al. 2001). Besides the classical mechanism of estrogen action through the ERE sequence, there are alternative pathways via direct protein-protein interactions. There is also the possibility of interaction with other proteins. An example is the Sp-1 activation mechanism, the estrogenic ligand binds the ER, and without the need to dimerize this complex, can interact with the Sp-1 protein and through this bond activate the transcription of the target gene (fig. 7B).

Another case of indirect activation of transcription is the AP-1 mode. The activated ER interacts with the Ap-1 dimer, formed by the sub-units c-Fos and c-Jun, thereafter the whole complex binds the promoter region of the target gene, enhancing its transcription (fig. 7C). The last major mechanism of modulation of gene expression reviewed by Nilsson is the inhibition of activity of the NFκB protein. Under normal conditions, this protein binds the NFκB response element in the promoter of a target gene enhancing its transcription. The ER, after activation by binding with the estrogenic substances, can interact with the NFκB complex detaching it from the promoter and deactivating the target gene (fig. 7D).

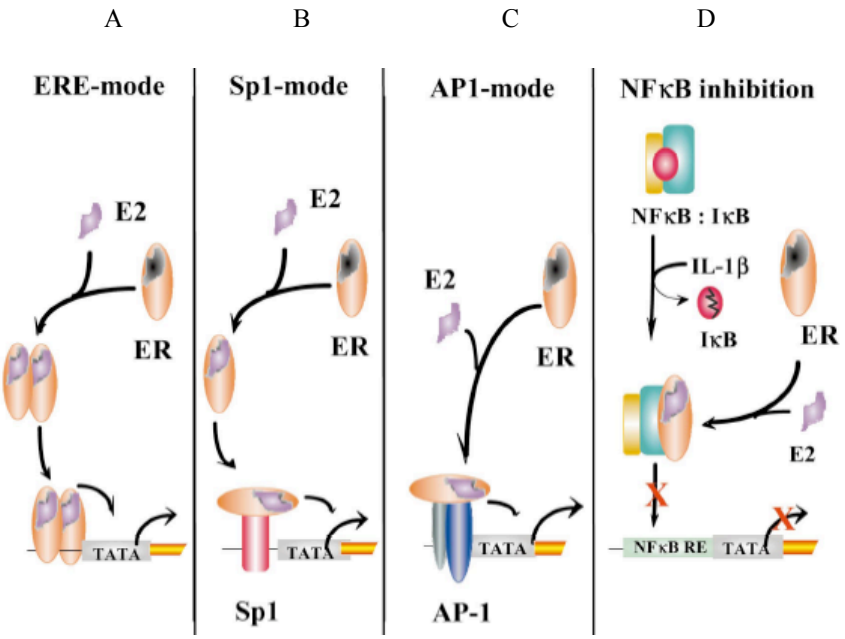

Fig. 7: Model representing the four different mechanisms of activity of estrogen (E2) or estrogenic substances through estrogen receptors (ERs). Panel "A" represents the classical interaction between the activated receptor with estrogen response elements (EREs) and DNA. In the other three panels the indirect effects of ER on transcription interaction are reported. This occurs through protein-protein interaction with the Sp-1 (panel "B"), AP1 (panel "C"), and NFκB proteins (panel "D") (Nilsson *et al.,* 2001).

2.4 Xenoestrogens

Xenoestrogens are compounds which possess estrogenic proprietis but their chemical structure does not necessarily resemble that of female sex hormones. This definition includes all substances that present estrogenic activity, for simplicity the xenoestrogens can be grouped according their source in the environment: phytoestrogens (produced by plants) and synthetic estrogens (synthesized in laboratory), which are mostly environmental pollutants.

2.4.1 Phytoestrogens

Phytoestrogens are members of the class of polyphenolic compounds synthesized by plants. They may act as fungicides, repel the herbivores (for their "bad taste"), regulate plant hormones, and protect plants against ultraviolet radiation (Barrett, 1996). Their abiltiy to mimic the estrogen activity was described more than 20 years ago (Martin *et al.,* 1978). The phytoestrogens can be subdivided into three classes according to their molecular structure: isoflavons, coumestans and lignans. In this work only 4 phytoestrogens will be described and used, those that belong to the group of isoflavonoids, two isoflavones, Genistein (Gen) and Daidzein (Dai), and two flavones, 8-Prenylnaringenin (8PN) and 6-(1,1-dimethylallyl)naringenin (6DMAN).

Exposure to Gen is mainly from the consumption of soy and soy products like soymilk and tofu. It is one of the best described phytoestrogens due to many years of investigation and its capacity to bind the ER (Mathieson *et al.,* 1980). Gen can stimulate osteoblastic bone formation, inhibit osteoclastic bone resorption and prevent bone loss in ovariectomised rats (Fanti *et al.,* 1998). It can also induce, although with lower efficiency, the same effects of 17-ßEstradiol (E2) in rat tissue and cell culture (Dopp *et al.,* 1999; Diel *et al.,* 2001).

The consumption of Dai, like Gen, occurs mainly by the consumption of soy. Its weak agonistic and antagonistic activity was already tested in different *in vitro* and *in vivo* models (Kuiper *et al.,* 1998; Diel *et al.,* 2001; Wober *et al.,* 2002).

The flavone 8PN, described for the first time in *Anaxagorea luzonensis* (Kitaoka *et al.,* 1998) is consumed principally from the female flower of hops, used in the beer brewery. The estrogenic activity of 8PN was detected for the first time in 1999 (Milligan *et al.,* 1999) and further characterized thereafter (Zierau *et al.,* 2002, 2004; Diel *et al.,* 2004). However the estrogenic activity of hops was known from evidence such as the menstrual disturbance of female hops hand pickers, or the use of hop baths for the treatment of gynecological disorder.

6DMAN isolated from the African tree *Monotes engleri* (Seo *et al.,* 1997), has been characterized due to interesting estrogenic activity in *in vitro* systems (Zierau *et al.,* 2002).

2.4.2 Synthetic estrogens

Synthetic substances with an estrogenic activity can be divided in two main groups, one consisting of substances created for pharmaceutical applications; for example, oral contraceptives or the control of disease as in HRT. The other group of substances disturbs the production or function of sex hormones. To this group of substances belong compounds which are synthesized for other purposes, and which also act as environmental pollutants, as with pesticides. Other compounds present in everyday life have been shown to have estrogenic activity such as in plastic containers for food and beverages.

This second group of xenoestrogens, described as environmental pollutants, comprises of many other substances. For instance the DDT mixture, a famous pesticide with estrogenic activity, particularly the o,p'-DDT (Robison *et al.,* 1982). Bisphenol A is an industrial compound (plasticizer) that showed estrogenic activity in *in vitro* systems (Krishnan *et al.,* 1993; Sumpter & Jobling 1995; Wober *et al.,* 2002). Alkylphenols, like Nonylphenol (NP) and Octylphenol (OP), that are derived from the degradation of alkylphenol polyethoxylates, non-ionic surfactant, widely used in detergents, paints, pesticides cosmetics and other formulated products (Jobling, 1994).

In my study one representative of each group will be described and used, 17α-ethinylestradiol (EE) will represent compounds created with the purpose of pharmacological application, and NP as an exponent of the synthetic substances with unintended estrogenicity.
EE is the result of modification of the natural estrogen E2, and it is commonly used in oral contraceptives.
NP, as already described, belongs to the alkylphenol family, its estrogenic activity was already described in several *in vitro* systems (Shelby *et al.,* 1996; Wober *et al.,* 2002).

3. Gene selection to study the estrogenic activity of different compounds

3.1 Genes used in the real time RT-PCR experiment

Cytochrome c Oxydase Subunit 1 (1A):

1A was use as reference for the gene expression analysis in all real time RT-PCR experiment it is an established reference gene in many experiment of gene expression analysis performed by real time RT-PCR in different rat tissues treated by E2 (Diel *et al.,* 2001, 2004; Zierau *et al.,* 2004).

Complement C3:

Complement C3 is the major estrogen responsive gene in the rat uterus (Sundstrom *et al.,* 1989) used since many years as positive control gene for the estrogenic activity of different compounds in rat uterus (Rivera-Gonzalez *et al.,* 1998; Diel *et al.,* 2001, 2004; Zierau *et al.,* 2004)

Clusterin (Clu):

This gene was isolated for the first time as Clusterin in 1983 (Blaschuk *et al.,* 1983) and is also reported in literature as Testosterone Repressed Protein Message 2 (TRPM-2) (Leger *et al.,* 1987), or Sulfated GlycoProtein 2 (SGP-2) or Apolipoprotein J, isolated from different tissues. It is easy to understand that this protein plays different roles in different tissues. This gene produces an anti-apoptotic protein regulated by androgen in prostate (July *et al.,* 2002). It is repressed by E2 treatment in liver and uterus of ovariectomised rats (Diel *et al.,* 2003). It is multifunctional and involved in apoptosis, spermatogenesis, promotion of cell-cell interaction, modulation of complement systems and tissue regeneration and remodeling (Joseph *et al.,* 1994).

Proliferating Cell Nuclear Antigen (PCNA):

PCNA is an auxiliary protein present during late G1 phase and S phase of the cell cycle. It is an established proliferation marker (Sullivan *et al.,* 1993; Lorz *et al.,* 1994).

Insulin-like Growth Factor Binding Protein 1 (IGFBP-1):

The proteins of IGFBP family can bind IGF with high affinity, for this reason they are used as IGF depot, to collect IGF in a tissue, and release it when necessary. The estrogen responsiveness of IGFBP-1 in liver of ovariectomised rat has already been shown by Diel (Diel *et al.,* 2004).

Calcium Binding Protein 9 kDalton (CaBP9k):

CaBP9k is involved in Ca^{2+} ion transport, it is extremely estrogen responsive in rat liver, liver cell line, (Diel *et al.,* 1995; Geis *et al.,* 2005) and in rat uterus (Rivera-Gonzalez, 1998).

Carbonic Anhydrase II (CAII):

CAII enzyme catalyzes the dissolution of CO_2 in water ($CO_2 + H_2O \leftrightarrow HCO^-_3 + H^+$). It is necessary for osteoclastic activity, and presents an interesting regulation profile to estrogens given by the presence of an AP-1 binding site in the promoter, hence one example of the non classical mechanisms of estrogen activity (David *et al.,* 2001). It is down regulated by E2 in osteoblast and osteoclast of ovariectomised rats (Williams *et al.,* 1999). It plays an important role in the osteoclast differentiation, and bone resorption. It is also involved in the regulation of intracellular pH and Ca^{2+} concentration (Lehenkari *et al.,* 1998). Up-regulation of CA II brings an increase in the osteoporotic process and bone resorption in ovariectomised rats. Estrogen loss results in an up-regulation of CA II and E2 supplementation reduces gene expression of CA II after 18 h (Zheng *et al.,* 1995). CA II is involved in CO_2 exchange, fluid secretion and pH regulation (Fleming *et al.,* 1994). CA II is also an essential metabolic enzyme of the CNS, it is key in the production and regulation of cerebral fluids (Nogradi *et al.,* 1993). It plays a role in the degradation of lurcher Purkinje cells and its related protein (CARP) has an important activity in the development and maturation of the celebrellar cortex (Nogradi *et al.,* 1997). It is insufficient to consider this gene as a simple representative of the estrogen responsive genes in bone only.

3.2 List of the 100 genes to include in the final microarray chip

In tab. 1 100 genes are listed which have been chosen to be included on the final DNA chip for the detection of estrogenic effects of different substances in the environment. They are classified according to the tissues where they show their major estrogenic response (bone, vessel, uterus, breast and liver) or as representatives of a specific biological pathway (apoptosis, pathophysiological conditions, cancer and heat shock proteins) or as marker for cell cycle progression. The list also includes control genes which are constitutively expressed

following exposure to estrogenic substances, commonly referred as housekeeping genes. The housekeeping genes are constitutively expressed, because their function is vital for the organism, for this reason they are generally independent from external influence. They were used as control genes, for the normalization of the signals stemming from differentially treated samples for gene expression analysis. The list also contains a newly identified sequence that presents an interesting regulatory pattern in the uterus of ovariectomised rats following treatment by E2.

Tab. 1: List of 100 genes chosen for the detection of estrogenic activity of substances in the environment.

Target	Gene
Each tissue	Estrogen Receptor α
	Estrogen Receptor ß
	Clusterin
	Progesterone Receptor
	Calcium Binding Protein 9 k Dalton
Bone	Macrophage Colony Stimulating Factor
	Osteoprotegerin
	Alkaline Phosphatase
	Type 1 Collagen
	Tartrate-Resistant Acid Phosphatase
	Carbonic Anhydrase II
	Matrix Metalloproteinase (2-9-13)
Vessel	Nitric Oxide Synthase (inducible & endothelial)
	Vascular Endothelial Growth Factor
	VEGF Receptor (1 & 2)
	Cyclooxygenase 2
Liver	Apolipoprotein A1
	Cholesterol 7a-Hydroxylase
	Insulin-like Growth Factor (1 & 2)
	IGF Binding Protein family (1-7)
	C-fos
	C-jun
	Major Acute Phase protein
Uterus	Complement C3
	Interleukin 4 Receptor
	Metallothionein
	Tumor Necrosis Factor regulated protein 6
	Inositol 1-Monophosphate Synthase
	Creatine Kinase B
	Aquaporin-water channel gene
	Uterine Ovarian Specific Gene 44
	Syndecan-3
	Telomerase-Associated protein 1
	Secretin
	Wilms' tumor gene
Breast	Msx-2
	Cathepsin D
	Transferrin

Target	Gene
Cell cycle progression	Inhibitor of Differentiation
	Cyclin D1 and E
	Proliferating Cell Nuclear Antigen
	Ki-67
HSP	Heat Shock Proteins (27 & 70)
Cancer	ER Binding Fragment Associated Antigen 9
	Transforming Growth Factor-ß
	Wnt-1 Inducible Signaling Pathway protein 2
	pS2
	High Mobility Group 1
	CD-22
	Breast Cancer 1
	Angiopoietin 2
	Early Growth Response (1 & 3)
	Thrombospondin 1
Apoptose	Bcl-2; Bcl-XL; Bcl-w; Mcl-1
	Bax; Bad; Bak; Bik; Bid
	Death Associated Protein 3
	C-Myc
	Interleukin (4 & 6)
	Fas ligand
	Embigin
	Programmed Cell Death 9
	Caspase family (2-3-6-8)
	Caspase Activated DNAse
	Inhibitor of Caspase Activated DNAse
	Death associated protein kinase
	Tumor Necrosis Factors
	TNF receptors (1 & 2)
Control	Cytochrome c Oxydase Subunit 1
	Cyclophilin
	ß-Actin
	Hexokinase1
	Ubiquitin
Novel	Fragment r52

3.3 List of the 16 genes used during establishment of the method

The different genes represented on the DNA chip for establishment of the microarray technique are reported and described according to the enclosure of the proposed chip (tab. 2). These genes are also used in the first gene expression experiment reported in paragraph 3.5 of

the result section. Some of these genes have been chosen because of their proven sensitivity to estrogenic treatment in different target tissues e.g. bone, liver and uterus. Other genes have been chosen because of their interesting role in different metabolic pathways like: apoptosis, cell cycle, marker of stress and cancer related genes. In some cases the same genes may be described in different parts of the list.

Tab. 2: The 16 genes present in the microarray chip during establishment of the method and used in the first experiment of gene expression analysis. The "*" indicates genes which are not allocated to a single target.

Target	Gene
Each tissue	Estrogen Receptor α Estrogen Receptor ß Clusterin Calcium Binding Protein 9 k Dalton
Bone	Carbonic Anhydrase II
Vessel	Cyclooxygenase 2
Liver	Insulin-like Growth Factor 1 IGF binding protein 1
Uterus	Complement C3
Breast	Insulin-like Growth Factor 1*
Cell cycle progression	Proliferating Cell Nuclear Antigen Ki-67
HSP	Heat Shock Protein 70
Cancer	Cyclooxygenase 2*
Apoptose	Insulin-like Growth Factor 1*
Control	Cytochrome c Oxydase Subunit 1 Cyclophilin ß-Actin Hexokinase1

The genes 1A, C3, CLU, PCNA, IGFBP1, CaBP9k and CAII were already described in the paragraph above.

Estrogen Receptor α (ERα):

ERα was the first receptor that binds the natural estrogen E2 to be discovered. It is expressed in many tissues and organs as reported by Gustafsson in 1999: CNS, breast, cardiovascular system, liver, urogenital tract and bone. In uterine tissue of ovariectomised rats the expression of ERα is repressed after 72 h exposure to E2 (Green *et al.,* 2001; Diel *et al.,* 2003).

Estrogen Receptor ß (ERß):

ERß was the second estrogen receptor to been discovered, cloned by Kuiper (Kuiper *et al.,* 1996). It is expressed in the following tissues: CNS, breast, cardiovascular system gastrointestinal tract, bone and urogenital tract (Gustafsson, 1999). ERß is down regulated in

the uterus by E2 treatment as already described for ERα (Green *et al.,* 2001; Diel *et al.,* 2004).

Cyclooxygenase 2 (Cox-2):

This gene presents the peculiarity, in opposite to its relative isoform Cox-1, that is not affected by E2 exposure, to be highly responsive to estrogens. Cox-2 is involved in regulation of gastrointestinal motility, vessel dilatation and immune response (Eberhart & Dubois, 1995). It plays a role in tumor genesis, it is an enzyme that catalyzes the synthesis of prostaglandins.

Insulin-like Growth Factor 1 (IGF-1):

IGFs are growth factors in many cell lines and tumors, sensitive to estrogenic administration in liver, uterus (Sahlin, 1995) and breast (Chan *et al.,* 2001). IGF-1 gene expression is repressed after a 48 hours exposure to the pure antiestrogen ICI 182,780 in MCF-7 (human breast cancer cell line) (Huynh *et al.,* 1996). The IGF-1 is stimulated by E2 and treatment in ovariectomised rats (Ghahary *et al.,* 1990).

Ki-61:

This proliferation marker encodes for the protein MIB-1 used to assess cell cycle progression (Bruno *et al.,* 1992; Sullivan *et al.,* 1993).

Heat Shock Protein 70 (HSP70):

HSPs are an important family of endogenous protective proteins, their over expression is protective against cardiac injury. They are also generally used as a control for general types of stress in a cell, an up regulation of their expression is an indication of a stress response of the organism. Exposure to E2 enhances the protein level of HSP70 in brain of male and female rats (Lu *et al.,* 2002). The sensitivity of HSP70 to estrogen is already shown in uterine tissue of ovariectomised rat treated by E2 for 4 h (Rivera-Gonzalez *et al.,* 1998).

Controls:

It is difficult to find information about genes which are constitutively expressed also during the estrogen exposure. For three of four genes reported here evidence is provided for the independence of expression to estrogens treatment, the last one is a good candidate because of its pivotal role in the basal cell metabolism.

Cyclophilin (Ppia):

Cyclophilin was also isolated with the name of Peptidyl Propyl Isomerase A (Ppia), and is an ubiquitous protein. This gene seems not to be influenced by estrogenic treatment in the uterus of ovariectomised rats (Weisinger *et al.,* 1999). It has been already used as a control in low-

density cDNA array analysis by Choi (Choi *et al.,* 2001), or in a rat renal model treated by E2 (Michel and Famworth, 1992).

ß-Actin (ß-Act):

ß-Act gene expression seems not to be influenced by estrogenic treatment in the uterus of ovariectomised rats (Weisinger *et al.,* 1999), it is also used as control gene in many commercial microarray chips.

Hexokinase1 (HK1):

HK1 catalyzes the phosphorylation of glucose to glucose-6-phosphate, it is the first enzyme of the catabolic pathway of glucose. For its position in this metabolic pathway its gene expression may be considered independent from exposure to estrogenic substances. HK1 is also included in many commercial microarray chips as a housekeeping control gene.

4. Tools

A first series of experiments was conducted in cell culture to identify new molecular markers of estrogenic action in response to different compounds and to improve the characterization of the already known markers. Thereafter, attention was moved to the *in vivo* experiment, to verify the estrogenic sensitivity in an experimental *in vivo* model.

4.1 *In vitro* models

For the study on the influence of the test compounds on the expression of target genes two cell lines are used.

4.1.1 RUCA-I cell line

This cell line derives from rat endometrial adenocarcinoma cells. RUCA-I is already used as tool for the investigation of estrogenic substances with both estrogens (Vollmer et al. 1995; Tueshaus *et al.,* 2003) and phytoestrogens (Hopert *et al.,* 1998; Dopp *et al.,* 1999). There is evidence, deriving from analysis of the protein level of complement C3 and fibronectin, that RUCA-I is estrogen/antiestrogen sensitive only by culture on an extra cellular matrix (ECM), particularly on a layer of reconstituted basement membrane (Vollmer *et al.,* 1995, Hopert *et al.,* 1998).

4.1.2 Fe33 cell line

Fe33 cell line derives from rat hepatoma cells stably transfected with human ERα. This cell line is proven as a tool to study the effects of EE, an increase of angiotensinogen expression is reported after 24 hours of EE treatment (Krattenmacher *et al.,* 1994), the same substance for the same time course stimulates the expression of IGFBP-1, CaBP9k and Major Acute Phase protein (MAP) (Diel *et al.,* 1995).

4.2 *In vivo* model

Wistar rats were chosen as the animal model for the *in vivo* studies. The three day uterotrophic assay is a classical method used for the study of the activity of estrogenic substances (Watanabe *et al.,* 1992; Diel *et al.,* 2002; 2004; Zierau *et al.,* 2004). The Organization for Economic Co-operation and Development (OECD) directive requires this test for each drug with estrogenic activity before it appears on the market. It consists of ablation of the ovary, ovariectomy, the natural source of sex hormones (that result as first consequence in atrophy of the uterus), following treatment with the test substance for three days, the wet weight of the uterus is measured. This procedure allows for the study of substance's estrogenicity without the interference of the internal production of steroidal hormones. After the ovariectomy the rats are treated for three days with the substances. The carrier of the substances is used as negative control and E2 as positive control of the treatment.

The tissues studied are uterus and liver, which are the tissues of origin of the two cell lines chosen for this work. It would be interesting to investigate the effects of the different substances in other targets of estrogens e.g. bone, breast or blood vessel. The aim of my work is focused on the establishment of a new technology in our laboratory rather than to perform a complete and comprehensive analysis of the activity of the respective test compounds in the entire organism.

4.2.1 Uterus

The uterus is considered as the principal target organ for the investigation of estrogenicity. As already reported the uterus is not only routinely investigated for the estrogenic activity of different substances in the organism, it is also a required test of drug with estrogenic potency according to the OECD guide lines. The uterine tissue shows also a strong responsiveness to estrogen treatment in the regulation of gene expression (Kang *et al.,* 2003; Wu *et al.,* 2003; Diel *et al.,* 2004; Zierau *et al.,* 2004).

4.2.2 Liver

The choice to investigate the liver derives from the fact that a key role of this tissue is estrogen metabolism. Yager and Liehr reviewed the role of estrogens in tumorogenesis in rat liver (Yager and Liehr, 1996). The oral consumption of hormones in HRT has an interaction with cardiovascular diseases due to induction of C-reactive protein release from liver (Miller *et al.,* 2003). There is evidence that indicates that estrogen depresses hepatic lipid oxidation and promotes triglyceride synthesis (Giwer *et al.,* 2002) along with that estrogen treatment also has an influence on risk reduction in cardiovascular disease by lowering the production of blood cholesterol (Parini *et al.,* 2000). The responsiveness of some genes in the liver to estrogens has already been reported (Diel *et al.,* 2004; Geis *et al.,* 2005). After this short overview on the multiple effects which are triggered by estrogen treatment in the liver, it has to be emphasized that liver tissue was selected because this project was a collaborative project and the data obtained should be comparable to that obtained in the rainbow trout, the experimental model of the collaborators.

5. Methods for the gene expression analysis

5.1 Microarray

5.1.1 The "tale" of the microarray

Since 1995, the microarray is a tool used for the analysis of the gene expression. In the beginning, it was created as a high-capacity system to monitor the quantitative expression of multiple genes in parallel. The amazing result for that time, ten years ago, was the possibility to perform a differential expression measurement of 45 genes of *Arabidopsis thaliana* using 2 µg of total cellular mRNA (Schena *et al.,* 1995). The microarray is drawn up as a development of the northern blot technique; it is interesting to note that many reagents, like the wash buffers, are shared by both methods. The innovation is the change of point of view. In northern blot analysis the RNA target is run in a gel to separate it according to its molecular weight. It is then transferred to a membrane where a probe, a marked specific nucleotide sequence, is spread on the membrane, it is washed and where it hybridizes and allows to identify an RNA. In this way a single probe looks for homology in a population of different targets. In the microarray the probes, different DNA sequences corresponding to specific genes, are fixed, in a known configuration, onto a solid matrix. Then they are hybridized with the target RNA-probes, which were previously reverse transcribed into cDNA and labeled. In the microarray the point of view is inverted, the *probes* are affixed on the solid matrix and the *targets* are hybridized to them.

At present it is hard to define the quantity of probes, genes or sequences, which are analyzable in a single experiment. As an example it is reported that the "GeneChip® rat expression set" from Affymetrix® (USA) allows for simultaneous study of the expression of 31.042 transcript sequences.

Multiple companies offer a large choice of ready to use microarray chips, for various studies and analysis, e.g. discovery of new genes, description of genes potentially involved in development, physiological and pathological processes, studies of gene regulation, diagnosis of diseases (via the identification of gene expression patterns that may represent a illness) state, drug discovery and toxicology (review by Murphy 2002).

Usually microarray chips are relatively large, they include more than 5000 genes or EST (Expressed Sequence Target) sequences, and the analysis of the result is entrusted to computer software created specifically for the chip. This type of chip can be really useful for preliminary studies, general application or a clearly developed goal, because it is an inflexible system that does not allow any change or development of the goal. The fact remains that this type microarray chips are very expensive and they relegate the scientist to the position of aq generic user.

The goal of my work is the description of the long way to establish a microarray DNA chip for the detection of the estrogenic potency of different substances in the environment, starting with the selection of the genes on the chip (around 100), until the production and application of the DNA microarray chip. This work places itself in a free area of the research and the market, with the production of a small, user friendly and "intelligent" chip, in which every component can be substituted in order to develop and improve the researcher's needs.

The other advantages of a internally produced small microarray chip are lower costs, constant monitoring of the full procedure, and the possibility to change and improve the performance of every step. On the other hand, the establishment and development of a microarray DNA chip is always complicated and time consuming, independent from the size of the chip itself.

5.1.2 Description of the microarray process

The microarray process can be divided in 3 main steps, the disposition of the probes on the solid matrix, the hybridization of the target to the probes and the analysis of the resulting data (fig. 8). Like every process, the microarray's components are sensitive to the manufacturing process, it is recommended to use component from the same manufacture to avoid interference problems between different reagents and materials.

A microarray experiment starts with the selection of genes (*probes*), usually from literature surveys, to be investigated. These genes are disposed and affixed in an ordered manner onto the solid support (chip). The preparation of the *targets*, derived from differentially treated animals, cell cultures, or biopsies from treated patients, starts with the extraction of the RNA, the labeling of the samples and thereafter they are ready to be hybridized to the probe present on the chip. The resulting chip is scanned with an appropriate reader, elaborated and analyzed. Each step of this process is a source of variability, an attempt is made to review briefly each step of the process and the different methods currently in use. Starting from the precise location and linkage of the probes on the solid matrix, many parameters have to be defined.

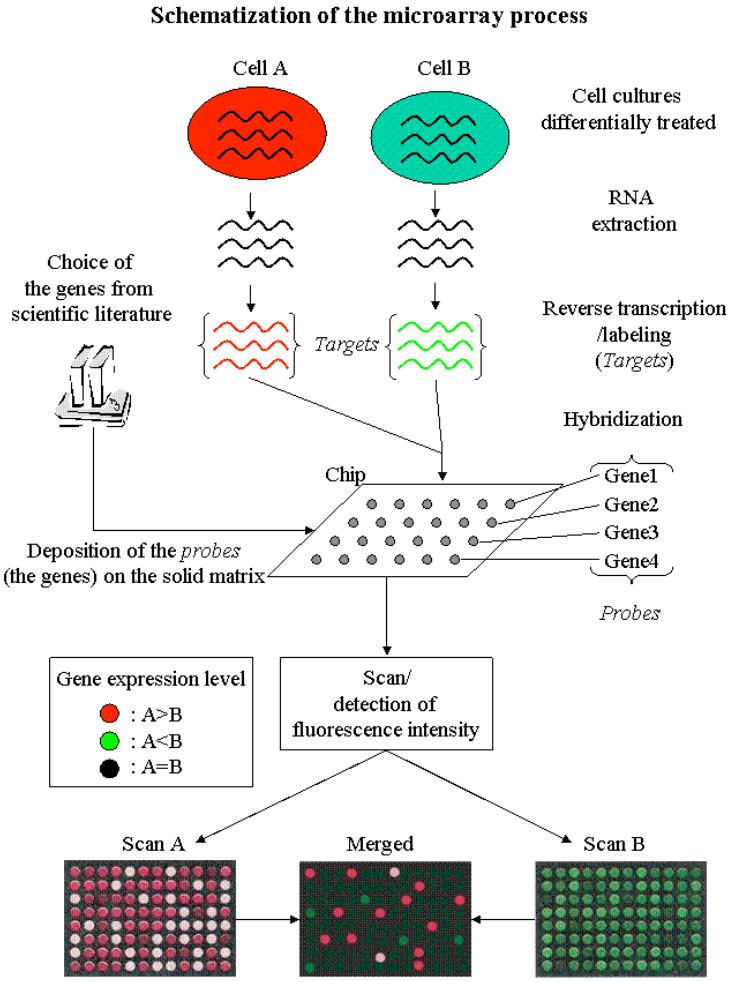

Fig. 8: The microarray process is shown starting from the choice of the probes (genes), proceeding through hybridization to the differentially treated and labeled target and the scan of the derived chip. The last three images of the scanned microarray are from:
http://www.mun.ca/biology/scarr/cDNA_microarray_Assay_of_Gene_ Expression.html.

Probes on the solid matrix

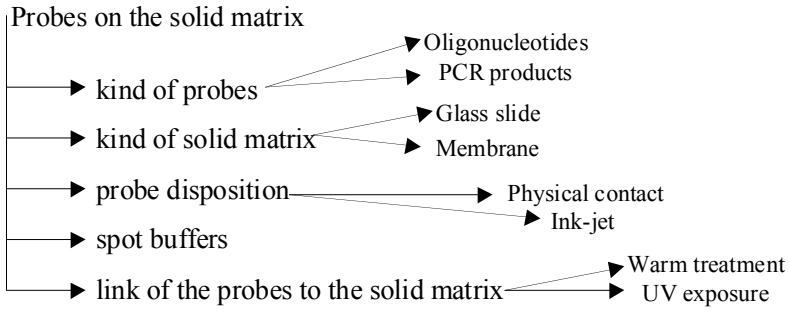

Fig. 9: Schematization of the different steps to produce a microarray chip.

Kinds of probes:

At present two types of probes are in use: oligonucleotides and cDNA. The oligonucleotides are single-strand DNA probes synthesized on the basis of sequence information present in the database. The length of the oligonucleotides plays a central role in the specificity of hybridization with the samples, usually a range from 20 to 80 nucleotides is used. Short oligonucleotides are less specific in their binding between probes and targets, whereas long oligonucleotides might create problems due to its secondary structure hindering the pairing with homologous sequences of the target samples. It is also possible to present a modification at one end, to improve linkage to the solid matrix, or a "spacer" (an unspecific oligonucleotide sequence) to keep a distance between the oligonucleotides and the surface where they are affixed, so to improve the space for hybridization with the target. The cDNA probes are usually PCR products, with a size of 100-2000bp, derived from cDNA clones from a plasmid library. In our laboratory oligonucleotides are in use. They were considered more stable and easier to use.

Kind of solid matrix:

The solid matrix has an influence on the process for the stability of the linkage, the kind of reagents and on the background signal that it can deliver. The slides that are commonly used are glass, like microscope slides, or membranes of different materials, like nylon or cellulose. At present the membranes are not in use anymore due to their instability and porosity. Membranes present the advantage as to allow reuse up to five times, but they deliver a strong background signal because of their own fluorescence (Murphy, 2002).

The glass slides are often covered, coated, with polymers which allow for better linkage between the probes and the slide itself as well as reducing the reflectivity of the slide and therefore delivering a weaker background signal. The market offers many types of slides

ready to use, and it is also possible to coat the slides in the laboratory. The choice of different coats depends on the type of probe used. For example the epoxy silane coated slide allows for use of both oligonucleotides and cDNA (Call *et al.,* 2003; Warsen *et al.,* 2004), in contrast, the poly-L-lysine coating is used for just cDNA probes (DeRisi *et al.,* 1997), like aminosilane coated slides (Hegde *et al.,* 2000). Ready to use epoxy silane coated slides were already present in our laboratory at the beginning of the project.

Probe disposition:

The disposition of the probes onto the slide can be achieved by contact (mechanical spotting) or noncontact (ink jetting). For spotting the probes are loaded by a pin, profiting form the viscosity of fluids, and transferred to the slide surface by physical contact. The pin is then washed after which it is ready to load another probe and dispose it on the solid surface. The inkjet mechanism is similar to an inkjet printer for computers, the probe is loaded into a miniature nozzle and "shot" onto the solid surface, after which the nozzle is washed and loaded again with a new probe. For mechanical spotting the equipment required is cheaper and more durable, in comparison with an ink jetting printer. In our laboratory a spotter device was already present at the start of the project, therefore only the mechanical disposition according to the possibility presents in our laboratory, will be considered.

Spotting buffer:

The probes, oligonucleotides or PCR products, prior to the spotting process need to be dissolved in a print or spot buffer, according of the disposition method used. The purpose of the spot buffer is to perform spot trace that is circular, regular, with a homogeneous distribution of the samples (probes) and relative small. The spot trace should be small, first to reduce the spot area, consequently reducing the amount of hybridized material during the subsequent hybridization step, and second to concentrate the spotted sample to get a higher signal reducing the spotted material amount.

It is necessary to mention that the buffer has to deliver as little fluorescent signal as possible, to avoid false positive signals stemming from the buffer rather than from the probes. The spot buffer is also responsible for the viscosity of the spotting solution; it has to be remembered that high concentrated DNA solutions present a high viscosity. Normally the slides bought from a company are provided with the optimized spot buffer for the slides themselves and instructions for use according to the use of oligonucleotide or cDNA probes. In the case of customized slides the buffer consists of a solution of SSC (Shalon *et al.,* 1996; Khan *et al.,* 1999; Cheung *et al.,* 1999), SDS/NaOH (Call *et al.,* 2001), $NaPO_4$/SDS (Shearstone *et al.,* 2002), SSC/Betaine or SSC/NaCl (Huang *et al.,* 2002), DMSO/SSC (Hegde *et al.,* 2000;

Antani *et al.,* 2000), salmon sperm DNA, BSA, and obvious mixture of those components. The viscosity of the buffer in relation to the hydrophobic/hydrophilic nature of the slide surface against the buffer plays a central role for spot deposition. The DNA solution presents a remarkably high viscosity, which interferes with the disposition of the probe onto the slide. The increase of the viscosity of the buffer can cause problems at two stages: the loading of the probe in the pin and the disposition of probe on the slide surface. The viscosity of the spot buffer can influence the whole spotting process. A too fluid spot buffer does not allow for loading of sufficient sample on the pin to perform a considerable number of spots, in this way it obliges to refill the pin several time with enormous time expense. Moreover, a too fluid spot buffer produces a large spot during the deposition of the probe onto the slide, increasing the spot area and the dilution of the probe which in the spot trace delivers a weaker signal at the end of the process after the scan of the hybridized chip. In the case of a too viscous solution, the quantity of probe loaded by the pin will be too great and the first spots will not be regular, non-circular. Hence, a too viscous spotting buffer produces a non-regular spot trace.

The hydrophobic relationship between spotting buffer and slide has to be considered for the size of the spot trace. A hydrophilic surface in combination with an aqueous buffer delivers a large and irregular (non-circular) spot, a hydrophobic slide with a hydrophilic buffer concentrates the spot in the center delivering a high signal that sometimes may be too high.

Stable linkage of the probes to the solid matrix:

At the end of the spotting process the probes have to be linked (coupled) on the slide surface. This is a crucial event because, in the following steps, the slide will be in contact with other solutions and washing buffers several times. The coupling reaction is commonly performed by baking the spot slides in a warm and dry oven to allow the evaporation of the buffer and to supply sufficient energy to create a link between the probe and the polymer of the slide (Call *et al.,* 2001). Another method is exposure to Ultra Violet (U.V.) light to provide enough energy for the formation of covalent bond between the DNA of the probes and the functional group of the coated glass surface (Taylor *et al.,* 2003). After which the unlinked probes are usually washed and the slides are dried by centrifugation or N_2 stream. At that point, the slide can be stored in a dark dry cool place for several months, before use.

Hybridization

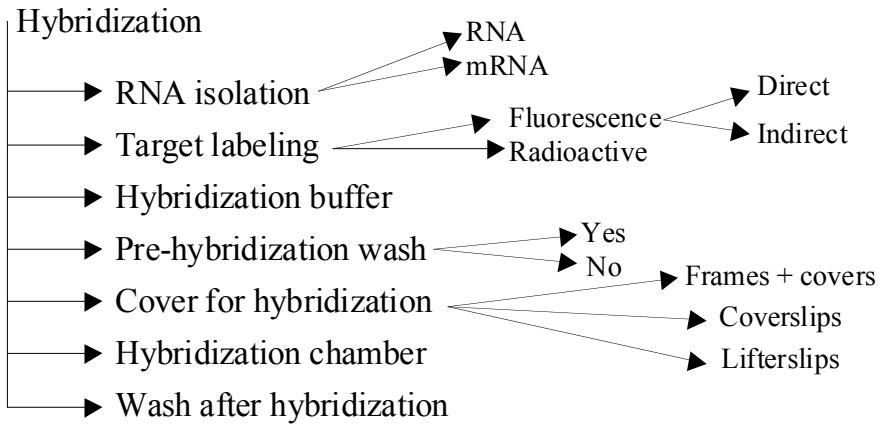

Fig. 10: Schematization of the preparation of the target samples for the hybridization, in the microarray technology.

RNA isolation:

The preparation of the probes starts with RNA isolation from the biological sample, for example, cell culture or tissues. The purity of the RNA is a critical factor for the hybridization performance, in the case of eukaryotes the isolation of mRNA can deliver a better result, but again is more expensive and time consuming. The sample RNA is converted into target cDNA by the use of the reverse transcriptase enzyme, for the reverse transcription of eukaryotic RNA oligo(dT) priming is recommended instead of a random priming to avoid the production of false positives in the form of immature mRNA or other types of short RNA (tRNA, rRNA).

Target labeling:

The sample can be labeled during the cDNA synthesis reaction using a pre-labeled primer for reverse transcription (Shi *et al.,* 2003) or by incorporating modified nucleotides into the cDNA (Zhao *et al.,* 2003) In both cases the label is usually a fluorophore: a Cyanine or an Alexa analogue (both molecules emit fluorescence when excited with a laser of a know energy and wave length). It is also possible to incorporate radioactive nucleotides like ^{33}P dCTP (Duggan *et al.,* 1999). The incorporation of modified nucleotides into the cDNA can be performed in two ways, and therefore it is possible to choose between a direct and indirect labeling. The handling of the first method is easier and less time consuming, it is also known as “one-step labeling”. It consists of the incorporation of modified nucleotides during the

reverse transcription of RNA (Yu *et al.,* 2002). The handicap of this method is its cost and the inability to stop the procedure, after the labeling, the target has to be hybridized to the probes as soon as possible. In the case of indirect labeling, also called "two-step labeling", instead of labeled nucleotides, modified nucleotides are incorporated during the cDNA synthesis, that present a group that allows for the formation of a covalent bond with the labeling molecule in a second step (Badiee *et al.,* 2003; Hui *et al.,* 2003). The advantages of this method are the lower costs and the ability to stop the reaction after cDNA synthesis and freeze the target samples performing the labeling of the cDNA in a second step. However "two-step labeling" is clearly more time consuming.

In the development of this technique some manufactures are attempting to improve the signal arising from the hybridization amplifying the cDNA, or the cRNA (Young *et al.,* 2004), to reach a larger copy number of genes with low expression levels. However there is no proof of linear duplication of the present genes, thus it may deliver false positive results.

In order to reduce the number of hybridization experiments of different targets to the probes spotted in the array, other researchers try to hybridize at the same time, on the same array, targets marked with three different labels, instead of the classical two, for example Cy3, Cy5 (Cyanine analogues commonly used as label) and Alexia 594 incorporated as dye-label, are able to reduce the expense and the time consumption to 50 % (Forster *et al.,* 2004), however the equipment required also include a scanner that can read all three fluorophores at the same time, this is uncommon for the available scanners.

Hybridization buffer:

For the hybridization reaction the hybridization buffer is key component, it is responsible for the efficiency of the interaction between the probes and target, consequently of the signal intensity, and it is also correlated to the background luminosity. A good hybridization buffer has to produce as little background signal as possible, to enhance hybridization of the samples, and to block unspecific binding of target to the probes, in order to avoid false positive signals.

Like for the spot buffer, the manufacture provides along with the slides a hybridization buffer. The basic composition of which is normally from SSC solution and other components in which SDS is often present. To block the interaction of unspecific samples with the probes an exogenous DNA can be used (like Salmon sperm DNA) or other registered substances.

Pre-hybridization wash:

Some slide producers have evolved a series of pre-hybridization washes to enhance the effectiveness of the hybridization, which also decrease the background signal. It can vary depending on the protocol, kind of slide or hybridization buffer.

Cover for hybridization:

The hybridization solution, consisting of the hybridization buffer and the dye-labeled samples, has to be applied to the spotted area of the slide in order to come into direct contact with the probes. To delimit and protect this area many covers are available, the choice depends on the kind of incubation procedure during the hybridization. Some covers close hermetic the spotted zone in contact with the hybridization solution, this allows to move the slide gently during the incubation and delivers a more equally distributed signal in the spotted area (like hybridization frames and cover from GeneScan, Germany). Other types of cover are in use, and many of them are simply slide covers (coverslips from Thermanox, USA) or slide covers with two parallel spacers positioned on the two edges to keep a free volume between the cover and the spotted area, this allows to use more hybridization solution along with better distribution (lifterslips from Eriescientific company, USA).

Hybridization chamber:

The hybridization must be carried out in a warm and moist chamber saturated by steam. There are many types available on the market. The temperature and the length of time of incubation are variable and have to be adjusted empirically. The temperature of hybridization should allow for annealing between the probes and the target sample, which depends on the length of the probes and its GC content. The oligonucleotides due to their single stranded need less time for incubation in comparison with double strand cDNA probes.

Wash after hybridization:

After hybridization the slides are washed by rinsing in different solutions, commonly SSC and SDS with decreasing stringency with to wash away the unlinked labeled samples from the slides. Washing the slides after hybridization with distilled water should be avoided, in this case the hybridization reaction would be disturbed resulting in a sensible decrease of performance of the signal.

At the end of the wash the slides have to be dried by centrifugation or N_2 stream. To the present time there are no indications concerning an influence of the way to dry the slides on the final result of the process, therefore it is only a matter of handling, costs and facilities.

End flow analysis

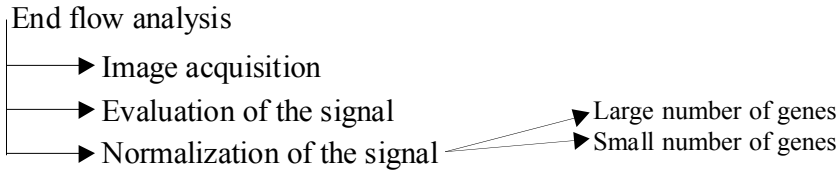

Fig. 11: Schematization of the steps involved in the end flow analysis of a hybridized microarray chip.

Scanning/image acquisition:

At the end of the process the hybridized array has to be evaluated by scanning with a specific laser which can read each dye individually. The setting of the parameters to scan a slide, the power of the laser and the PMT (photon multiplier tube) intensity, has to be adjusted for every slide. The ideal fit settings of parameters allow the acquisition of the slides with the higher signals, along with the possibility to detect the weaker signals. Concerning the settings for the intensity of the laser beam, reaching saturation of the higher signals must be avoided. When the saturation of the signal is reached the spot appears as a white mark and the instrument cannot distinguish any increase in the intensity of the signal. The intensity of the signal is directly correlated to the amount of labeled target linked to the probes affixed on the slide.

Evaluation of the signal:

After the scan of the biochip the signal stemming from the image has to be translated in an absolute value. Usually the scanner is provided with software for the evaluation of the signal. The software for the analysis of the signal presents many parameters which can be adjusted. The more parameters that are available, the more the software allows for a the fit of the evaluation method to an individual array. The most important parameter is the definition of the area from the signal which the samples stem from, and the area wherefrom the background signal derives. It is pretty simple to define as the sample's signal is the signal from the area of the spot, but the definition of the background area can vary, it can be the area surrounding the spot trace, or another non spotted area.

Normalization of the signal:

After subtraction of background signal, which eliminates the signal originating from the non spotted area, the signal has to be normalized. Normalization is necessary for three reasons. First, the dyes used for the labeling of the two samples may be incorporated with differing efficiencies. Second, one of the two dyes used for the labeling of the samples may deliver a higher signal intensity compared to the other dye, even when it is present in the same quantity, due to its characteristic. Third, in the gene expression analysis the isolated RNA quantity from the two samples may vary, this is due to normal experimental variability and

has to be normalized to avoid false results stemming from differing quantities of RNA isolated; as normally performed for the gene expression analysis by real time RT-PCR.

In the gene expression analysis, normalization of the signal is a central point to avoid having false positive results in the differential gene expression analysis. According to the size of the chip the tendency is to use three different methods, for normalization.

For large chips (more than 1000 spots/genes), normalization of the signal stemming from the two dyes can be performed by calculating the average signal of all spots; statistically working with a large number of genes, the differential expression of all genes is equal to zero, some genes will be up regulated and some other down regulated so resulting at the end in a general expression level that stays constant. In small chips (less than 1000 spots/genes) this procedure is impossible as the genes are too few to normalize according to previous method. In this case genes which are constitutively expressed have to be used. This is the case for housekeeping genes, their expression is not affected by the experimental conditions of the study. The choice of housekeeping genes have to be targeted, because under some experimental conditions, housekeeping genes can also vary in their expression levels. For example, in a study of food deprivation the gene for the glucose metabolism pathways should be avoided, because also when "normally" the genes of this pathways are constitutively expressed, the glucose metabolism is constant in standard condition, but when there is a paucity of the glucose itself this pathway is obviously repressed.

A "third" way for the normalization of the signal is the use of an external control. Some "spikes" are spotted together with the probes, spikes are oligonucleotides coding for foreign genes, for example genes of *Arabidopsis thaliana* used in experiment for gene expression in *Wistar rat*. During reverse labeling of the cDNA target samples, some mRNA coding for the genes present in the spikes is added to the reaction in a known quantity. Therefore, during hybridization the foreign labeled target can hybridize only with the "spikes" delivering a signal for the two dyes that differs only due to the natural fluorescence of the dyes, as the quantity of hybridized material is the same. This method presents the problem that is a completely external method of normalization of the signal. Using this method the mRNA quantity reverse transcribed and the efficiency of the reverse transcription for the two samples has to be exactly the same. Another example of "spikes" can be the polyT (Timidine) sequence present in every eukaryotic cDNA. In this case a polyA (Adenine) oligonucleotide is spotted in the array and it will find homology with all cDNA molecules reverse transcribed. This method also has the advantage of being an internal method of normalization of the

signal, because it is linked with the reverse transcription of the mRNA samples, and so it can, in addition, also be used to normalize the cDNA quantity of the two samples.

5.2 Real time RT-PCR

5.2.1 Real time RT-PCR origin and present application

The real time RT-PCR (reverse transcription polymerase chain reaction) technology has recently reached a level of sensitivity, accuracy and practical ease that supports its use as a routine bio instrument for gene expression analysis (Diel *et al.,* 2003, 2004), disease diagnosis (reviewed by Hui *et al.,* 2003; Aldape *et al.,* 2002), detection of pathogens (reviewed by Uhl *et al.,* 2002) or single nucleotide polymorphism (SNP) genotyping (Ulvik & Ueland, 2001).

5.2.2 Real time RT-PCR process description

The generalized method consists of a classical PCR reaction with online quantification of synthesized product after each amplification cycle. There are different products available on the market which differs in their detection mechanism for the PCR product. Our laboratory is equipped with the Icycler® real time RT-PCR (Biorad, USA). It uses SYBR green 1 as a fluorescent dye which when bounds to double stranded DNA, exhibits high fluorescence. The increase of fluorescence emission is measured after every cycle at the end of the elongation step resulting in a quantitative measure which represents the amount of double stranded DNA, the PCR product.

5.2.3 Role of the real time RT-PCR in my work

In my work, the real time RT-PCR is important as a reference method for gene expression analysis by microarray technology, a procedure already performed by other authors (Rajeevan *et al.,* 2001). At the beginning of the work the real time RT-PCR was also used for the identification of novel, along with the in depth characterization of known molecular markers for the estrogenic activity of different substances in *in vitro* and *in vivo* experiments.

6. The aim of my thesis

The major goal of my Ph.D. thesis was to establish the microarray technology and adapting it to the equipment present in the laboratory. This consisted of a spotter robot, epoxy-silane coated glass slides and a scanner equipped to detect fluorescence of Cy3 and Cy5 dyes. All necessary parts and steps to establish the microarray technology were optimized and the most efficient conditions chosen after a series of experiments.

Thereafter, a selection of 16 genes, reported at point 3.2 of this chapter, were used to test whether the molecular effects of the two synthetic estrogens EE and NP can be visualized in the cell line Fe33. Obtained results were verified by real time RT-PCR for 4 genes of the entire selection: IGFBP1, CaBP9k, PCNA and CAII and the housekeeping gene 1A as control gene for the normalization of the gene expression.

At the end of this work a DNA chip of 16 genes was tested and characterized.

In parallel, using real time RT-PCR, the gene expression of IGFBP1, CaBP9k and CAII, was investigated in the Fe33 cell line after exposure to Gen, Dai and 8PN. The gene expression profile of C3, PCNA and Clu was investigated in the RUCA-I cell line (rat adenocarcinoma cell line) after treatment with E2 and the pure antagonist ICI 182,780 (ICI) in differentially cultured cells, on a layer of an extra cellular matrix, collagen 1 or directly on the plate floor.

To relate the observed effects *in vitro* to the situation *in vivo*, effects of exposure to EE, Gen, Dai and 8PN were additionally analyzed by gene expression profiling of CA II and PCNA in both liver and uterus of ovariectomised rats. As positive control for the same experiment served expression profiling of C3 in uterus and IGFBP-1 in liver.

In this way the knowledge on the effect of xenoestrogens on cell proliferation is improved, which is important with the regard to safety issues. Regulation of CA II expression was investigated in depth in new target tissues, namely liver and uterus.

The study of the gene expression of C3 in uterus and IGFBP-1 in liver is crucial as a positive control for estrogenic activity of xenoestrogens in these two target tissues.

The estrogenic regulation of gene expression of a newly identified differential display fragment was investigated using real time RT-PCR. This fragment was investigated in the ovariectomised rat uterus and liver treated by EE, Gen, Dai, 8PN and 6DMAN. Since it was characterized by interesting response patterns to different estrogens and phytoestrogens, cloning of additional genomic information was pursued.

Materials and methods

1. Materials

1.1. Substances

Estradiol and Genistein: Sigma (Germany)

Ethinylestradiol: Schering AG (Germany)

Daidzein: Roth (Germany)

8-prenylnaringenin and 6-(1,1-dimethylallyl)naringenin: synthesized at the Institute of Organic Chemistry of Dresden University of Technology, as previously described (Gester *et al.,* 2001),

Nonylphenol: Hüls, technical mixture (Germany).

1.2. Media and media constituent

Culture medium: DMEM/F12 and 5 % w/v DCC (Biochrom, Germany).

1 % w/v Gelatin (Sigma, Germany).

1.3. Equipment

Cell culture plates (TPP, Switzerland).

Homogenizer Mikro-Dismembrator-s (B-Braun biotech international, Germany).

Agarose gel transilluminator Gel Printer 2000i (MWG-Biotech, Germany).

Real time RT-PCR tool: iCycler® Thermal Cycler with iQ real-time Detection System (BioRad, Germany).

96 wells thin-wall PCR plate (Biorad).

384 wells plate (Genomix, USA).

Robot spotter Microgrid II® (BioRobotics, United Kingdom).

Hybridization chambers: Flat block® (Biozym, Germany) incubated in the cycler PTC-200® (Byozim, Germany), ArrayIt Hybridization Cassette® (TeleChem international, USA).

Scanner: Scanarray 4000® (PerkinElmer, USA).

Scanner software: ScanArray®(PerkinElmer, USA).

Evaluation of signal software: Quantarray® (PerkinElmer, USA).

1.4. Reagents

TRIzol® RNA extraction kit (Invitrogen, USA).

DNase1® (Roche, Germany).

RNaseOUT® (Invitrogen, USA).

Superscript II RNase H^{-}® reverse transcripts Kit (consisting of: 5x first strand buffer, DTT 0,1 M and Superscript RNase H^{-}®) (Invitrogen, USA).

Platinum® *Taq* DNA polymerase (Invitrogen, USA).

Epoxy-silane coated slides ArrayLink hyphop® (GeneScan, Germany).

Spotting buffers: GeneScan: ArrayLink print buffer 2x (GeneScan, Germany),
PeqLab: Nexterion Spot buffer 2x (Schott Nexterion, Germany).

RNeasy® Mini Kit column (Qiagen, USA).

Cy-dye labeled nucleotides (Amersham, USA) (the supplier did not specify the concentration).

Cy-NHS esters (Amersham, USA)

RNase H (Invitrogen, USA).

Microcon YM-30® column (Millipore, USA).

Lifterslip (Eriescientific company, USA).

mRNA isolation kit: Nucleo Trap mRNA® mini kit (Macherey-Nagel, USA).

OneStep RT-PCR® (Qiagen, USA).

DNA ladder 100bp and 50bp (Invitrogen, USA).

Primers (MWG Biotech, Germany).

Oligonucleotides (Scenion AG, Germany).

2. Cell culture, animal experiments and RNA isolation

2.1 Fe33 cell line

Fe33 cell line (FTO-2B rat hepatoma cells, stable transfected with a human ERα expression plasmid by Kaling *et al.,* 1990, provided by Schering, Germany) was cultured in DMEM/F12 medium supplemented with 5 % w/v DCC at 37°C, 5 % v/v CO_2.

For treatment the Fe33 cells were cultured in tissue culture plates, coated with a layer of 1 % w/v gelatin. After one day in culture with DMEM/F12 medium supplemented by 5 % DCC, the cells were washed with 1 x PBS and cultured for 24 hours using DMEM/F12 medium supplemented with 1 % DCC. After washing the cells with 1x PBS, cultured in DMEM/F12 medium supplemented with 1 % DCC, the cells were treated with the substances in a concentration and time dependent manner. Because the substances were diluted in ethanol the ratio of the volume of substances to the total volume of the cell culture medium was 1:1000 (i.e. a volume of 10 µl of substance was used to treat the cells cultured in 10 ml of culture medium).

2.2 Animal experiments

The animal experiments were conducted by Dr. Patrick Diel in the department of Molecular and Cellular Sport Medicine, DSHS, in Köln (Germany), the materials and methods of the animal treatment were previously published (Diel *et al.,* 2004).

Briefly, female Wistar rats were ovariectomised (ovx) by ablation of the ovary, the natural source of female sex hormones. A first experiment consisted in the treatment of the ovx animals with subcutaneous (s.c.) injection of E2 and EE at the dose of 3 µg/kg/day, 10 mg/kg/day Gen for 7, 24 and 72 h and 20 mg/kg/day Dai for 72 h. A second experiment consisted in the treatment of the ovx animals with subcutaneous (s.c.) injection of 30 µg/kg/day E2 and 10 mg/kg/day 8PN. Investigation of the 8PN effect *in vivo* was performed only at the time point of 72 h due to the paucity of quantity of the substance at the time of the performed animal experiment. For both experiment the carrier of the substances was EtOH (negative control). A third experiment consisted in the treatment of the ovx animals with subcutaneous (s.c.) injection of E2 and EE at the dose of 4 µg/kg/day, 40 mg/kg/day Dai for 7 and 24 h and 10 mg/kg/day Gen for 24 h. The carrier of the substances was EtOH (negative control).

The animal experiment conducted in our laboratory under the supervision of Dr. Oliver Zierau consisted briefly, after ovariectomy of female Wistar rats, in the treatment with s.c. injection of E2 at the dose of 10 µg/kg/day, 15 mg/kg/day 6-(1,1-dimethylallyl)naringenin (6DMAN) or 8PN for 7 and 24 h. The carrier of the substances was EtOH (negative control).

2.3 RNA isolation

The RNA isolation was performed with the TRIzol® RNA extraction kit.

2.3.1 RNA isolation from tissues

Starting from material frozen in liquid nitrogen, the whole uteri or up to 200 mg of liver was homogenized using the homogenizer Mikro-Dismembrator-s. Each organ or part of them was homogenized with 1 ml of TRIzol®.

2.3.2 RNA isolation from cell culture

After treatment the cell culture plates were washed with 1x PBS followed by add of 1 ml of TRIzol® per plate. The cells were scrapped and the suspension was collected in 2 ml Eppendorf tube.

2.3.3 TRIzol® protocol for RNA isolation

Starting from a suspension of TRIzol® and cells or homogenized tissue, the material was incubated at room temperature for 5'. 0,2 ml of chloroform was added per ml of TRIzol, this mixture was shacked for 15" and incubated at room temperature for 2-3'. Centrifugation of the samples followed for 15' at 12.000 x g at 4°C. The mixture separated in two phases: the upper colorless aqueous and the lower red; the upper phase was transferred into a fresh tube and 0,5 ml of isopropyl alcohol per ml of starting TRIzol® was added, after incubation for 10' at 4°C a samples were centrifuged at 4°C for 10' at 12.000 x g, the supernatant was discarded. The RNA pellet at the bottom of the tube was washed with 1 ml of ice cold 75 % v/v EtOH per ml of starting TRIzol®, centrifugation for 5'at 7.500 x g at 4°C followed, the supernatant was again discarded. The pellet was dried, using a Speedvac centrifuge, the RNA pellet was dissolved in HPLC water. Samples were stored at –80°C.

3. PCR / real time RT-PCR

3.1 PCR primers

The PCR primers were designed using the freely available on-line software: "Primer3" (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the parameters listed in tab. 1.

Tab.1: Parameter for primer design.

PCR product size	150-250 bp
Primer size	20-24 bp
Max complementary	<4
Max 3' complementary	<2
Primer annealing temperature	59-61°C
GC%	<40%

The primers used for classical PCR analysis are listed in tab. 2a, those used for the real time RT-PCR are listed in tab. 2b. All primers were synthesized by MWG-Biotech (Germany).

Tab. 2a: PCR primers sequence

primer name	Forward primer	Reverse primer	PCR product size (bp)
r52	ccg gaa tag act acc ata cag ca	aga gag cca cct tca gat ttt tc	120
r52A	gac ata ctg cct cac tct act cca	cca aac cca c ttc tta tta atc	186
r52B	agt cct ggt ga tga aag tga gc	atg aag aaa gag cgg aaa gag ac	109
r52C	aac tac aga g gtg gca gat gat	cta ttc ata gtc caa gca gga tt	309
r52D	ggt gca tct tt tgc ttt tag ttg g	gca gtg aaa gt gta agc ctc agt	385
r52E	ttc aaa gtc tat cca ttt tcc tga	tgg aac aag tac aag tct caa cg	270
r52F	atg tgg gtt cta gga ttc agg tc	agt gtg cga ct gta tgt atg agg	385
r52G	tta ccc act aa cac aaa ctc ctc	aaa cca aac c aag caa aga aac	268
r52H	aga aat aag acc tca tta tat agc c	gga aaa gcg aga gag aga gag ag	310
r52I	ttc tgc att ag gac ttc att ggt	gct tcc cca gc tat ggt att att	299
r52L	aag acc tga agc cat ttt tgg	atttagaggct ggtatgatgtgc	205

Tab. 2b: Real time RT-PCR primers sequence

Gene	Forward primer	Reverse primer	PCR product size (bp)	Melting temperature of the PCR product	Blast ID gene
Cytochrome C oxidase subunit 1 (1A)	tga gca gga ata gta ggg aca gc	gag tag aaa tga tgg agg aag ca	261	86°C	343181
Complement C3 (C3)	aca gcc ttc ccg gga gca tca aca	agc gca cca cag gag gca cag agt c	276	92°C	NM 016994
Clusterin (Clu)	ccc tcc agt cca aga tgc tca aca c	cca tgc ggc ttt tcc tgc ggt att c	303	91°C	M64723
Proliferation cell nuclear antigen (PCNA)	gag caa ctt gga atg cca gaa cag g	cca agc tcc cca ctc gca gaa aac t	158	88,5/89°C	Y00047
Carbonic Anhydrase 2 (CAII)	gac tgg ctg ttt ggg tat ttt	taa tgg gtt cct tga gca cta tc	231	88,5/89°C	NM 019291
Calcium binding protein 9k dalton (CaBP9k)	tgt ctg act ctg gca gca ctc act g	cct tca gga ggc tgg gga act ctg	181	88,5°C	3287250
Insuline like growth factor binding protein 1 (IGFBP1)	caa cag aaa gca gga gat gag a	gaa gaa gga ggg agg aaa caa c	234	88,5°C	204736
r52	ccg gaa tag act acc ata cag ca	aga gag cca cct tca gat ttt tc	120	85°C	
r52E	ttc aaa gtc tat cca ttt tcc tga	tgg aac aag tac aag tct caa cg	270	84 °C	

3.2 DNase treatment

3 µg of total RNA was digested in 10 µl of total reaction volume containing 1 µl of 10x DNase1 buffer (Sambrook *et al.,* 1989) 1 µl of DNase1® 1 U/µl, 1 µl RNaseOUT® 40 U/µl, incubated at 37°C for 1 h. 1 µl of RNA after the DNA digestion was tested by real time RT-PCR using the housekeeping gene primer pair Cytochrome C oxidase subunit 1 (1A), to check the presence of remaining genomic DNA in the sample; as positive control a proofed cDNA solution was used, and HPLC water served as negative control. The DNA digestion was considered successful when the sample delivered a signal of the amplification similar to the negative control.

3.3 cDNA synthesis

After successful DNase digestion the samples were reverse transcribed using the kit: Superscript II RNase H^{-}® reverse transcriptase. The resulting RNA after DNA digestion was added to 1 µl of oligo (dT) primer (15T, 100 µg/µl), 1 µl of dNTP mix (10 mM), the volume was filled to 12 µl and heated for 5' at 65°C, and immediately cooled in ice. After a brief centrifugation 4 µl of 5x first-strand buffer, 2 µl DTT (0,1 M), 1 µl of RNaseOUT® was added, the tube was mixed gently by pipetting and incubated at 42°C for 2', the addition of 1 µl of Superscript II RNase H^{-}® (200 U/µl) followed with gently mixing by pipetting. The sample was incubated at 42°C for 50' followed by heating to 70°C for 15'. The obtained cDNA was stored at –20°C.

3.4 PCR reaction

The PCR was performed using Platinum® *Taq* DNA polymerase. PCR reactions consisted of a first denaturing cycle at 95°C 3', followed by 50 cycles of 10" at 95°C, 10" at 60°C and 1' at 72°C per 1000 bp of amplified region. The PCR conditions are summarized in tab. 3.

The resulting PCR product was run on agarose gel (1 % w/v) stained by ethidium bromide and compared to the DNA 1kb ladder. The gel was visualized by the gel transilluminator Gel Printer 2000i.

Tab. 3: PCR condition.

PCR reagents	
	final concentration
MgCl2 50mM	4mM
primer mix (forward and reverse) 10µM	0,1µM
10x PCR buffer	1x
dNTP 10mM	0,1mM
Platinum® Taq DNA polymerase 5U/µl	0,5U
HPLC H20	to 49µl

3.5 Real time RT-PCR analysis

The real time RT-PCR was performed with Platinum® *Taq* DNA polymerase using the iCycler® Thermal Cycler with iQ real-time Detection System. All reactions were run in triplicates. After vortexing 50 µl aliquots of the mix were pipetted in each well of the 96 wells thin-wall PCR plate. PCR reactions consisted of a first denaturing cycle at 95°C 3', followed by 50 cycles of 10" at 95°C, 10" at 60°C and 20" at 72°C. Fluorescence was quantified during the 60°C annealing step and product formation was confirmed by melting curve analysis (55°C–95°C). The conditions for PCR are summarized in tab. 4.

Tab. 4: PCR condition for each real time RT-PCR primer.

real time RT-PCR reagents									
reagents	final concentration of reagents								
primer pair	1A	C3	Clu	PCNA	CalI	IGFBP1	CaBP9k	r52	r52E
MgCl2 50mM	4mM	4mM	4mM	4mM	3mM	2mM	4mM	5mM	3mM
primer mix (forward and reverse) 10µM	0,1µM	0,2µM	0,2µM	0,2µM	0,1µM	0,4µM	0,2µM	0,1µM	0,1µM
2x SYBR green in 10x PCR buffer	1x								
dNTP 10mM	0,1mM								
Platinum® Taq DNA polymerase 5U/µl	0,5 U								
Fluorescein calibration dye 400nM	10nM								
HPLC H20	to 49µl								

3.6 Calculation of data

The relative mRNA amounts of the target genes were calculated after normalization against an endogenous reference gene, the house keeping gene 1A, and relative to the negative control, with the arithmetic formula $2^{-\Delta\Delta CT}$ (Winer *et al.,* 1999) and schematized below.

C_T: automatically calculated by the iCycler software. It indicates the PCR cycle thereafter the PCR product reach a definitive value detectable from the instrument.

R: relative expression of the probe compared to the standard (sample treated by the carrier substance). The R value is at the exponent because there is a doubling of DNA quantity at every cycle.

Target: target gene studied.

Standard: reference gene (housekeeping gene) that is independent of the treatment.

$R= 2^{-\Delta\Delta CT} = 2^{\Delta CT(target)-\Delta CT(standard)}$

where

$\Delta C_{T\ target}$: $C_{T\ control\ (target)} - C_{T\ probe\ (target)}$

$\Delta C_{T\ standard}$: $C_{T\ control\ (standard)} - C_{T\ probe\ (standard)}$

(Arithmetic formula kindly provided by Georg Kretzschmar, master thesis).

4. Microarray analysis

4.1 Spotting

The spotting of the probes (oligonucleotides) on the slide was performed using the robot spotter Microcrid II®. Each sample loaded by the pins was pre-spotted on 3 pre-spotting slides, performing a single spot for each pre-spotting slide, thereafter it was spotted on the target slide by a triple strike (three times spot at the same position). Before to load the pin with a new sample it was washed twice in HPLC water bath for 2" and once in a HPLC water flow for 0,5" and dried for 2,5" by air flow.

4.1.1 Oligonucleotides

The oligonucleotides, listed in tab. 5a and 5b, were designed and synthesized by Scenion AG (Germany). The oligonucleotides were provided in two different forms: with and without an amino (-NH2) modification at the 5´ end of the oligonucleotide. They were stored at –20°C at the concentration of 100 pmol/µl (µM).

Tab. 5a: Oligonucleotide sequences. Oligo ID: identification number of the oligonucleotide by the company Scenion, gene: gene name, short: abbreviation of the gene name, blast ID gene: identification number of the sequence in the NCBI databank, length: length in nucleotides of the oligonucleotides, hyb zone: zone of sequence homology between the oligonucleotide and the sequence reported in the databank.

Oligo ID	gene	short	blast ID gene	CDS	Length	hyb zone
13592132-0-0	Beta-actin	ß-act	gi:13592132	1-1128	50	941-990
1373280-0-0	Estrgen receptor ß	ERß	gi:1373280	417-1874	51	2426-2476
14010866-0-0	Heat schock protein 70 kDa	HSP70	gi:14010866	178-2103	52	2308-2359
207523-0-0	Clusterin	Clu	gi:207523	63-1406	51	1384-1434
27718982-0-0	Ki-67 (Proliferation marker)	Ki-67	gi:27718982	316-792	50	650-699
3207507-0-0	Hexokinase 1	HK1	gi:3207507	92-2848	52	3590-3641
460557-0-0	Cyclooxigenase-2	COX-2	gi:460557	125-1939	51	3821-3871
56434-0-0	Insulin Growth Factor 1	IGF-1	gi:56434	1-327	53	241-293
56861-0-0	Prolifering cell nuclear antigen	PCNA	gi:56861	63-848	60	897-956
6978814-0-0	Estrogen receptor alpha	ER @	gi:6978814	210-2012	52	2030-2081
8394008-0-0	Cyclophilin	Ppia	gi:8394008	43-537	50	659-708
9506444-0-0	Carbonic anhydrase 2	CA2	gi:9506444	9-791	52	1150-1201
207644-1-0	Calcium binding protein 9k dalton	CaBP9k	gi:207644	63-302	58	325-382
34317	Cytochrom-c Oxygenase subunit 1A	1A	gi:343182	1418-2962	52	1944-2044
NM_016994	Complement C3	C3	gi:8393023	42-5033	56	1019-1074
204736	Insulin Growth Factor Binding Protein 1	IGFBP1	gi:204736	121-939	53	793-845
PoliA	Poli A (54 A)	PoliA			54	

Tab. 5b: Oligonucleotides sequences. Hybridization zone: description of the region of homology between the oligonucleotide and the sequence reported in the databank, sequence: sequence of the oligonucleotide.

short	note of the hybridization zone	Sequence
ß-act	in the CDS region	AGAAGGAGATTACTGCCCTGGCTCCTAGCACCATGAAGATCAAGATCATT
ERß	in the not translated zone	GACCCTTGGGTTACATCCTTAGAGCCTGCTTATTTGGTCTGTCTGAATGTG
HSP70	in the not translated zone	ATCTAAGTAGCTGTGTAAAAATGGTGTTTCCTTCCTGCGAACACCTCAGCAC
Clu	50% in the CDS, 50% in the not translated region	GCAGGAAAAGCCGCATGGAATGAGACAGAAGCATCAGTTTTCTATATGTAG
Ki-67	in the CDS region	CTAGAGTCACTTTGGACAATGAACCTAAGCCAAGAGTAACTCGAGGGGCC
HK1	in the not translated zone	ATTTCCATCGCCCGAATTCTTTAGTGAGCCATTGTTGTACGTCTAGTAAACT
COX-2	in the not translated zone	AAACTGCGTAGAGCCAATATTGACTCACCTACACACGTTATACAGATTGAC
IGF-1	at the 3' extremity region of translated region	CACACTGACATGCCCAAGACTCAGAAGGAAGTACACTTGAAGAACACAAGTAG
PCNA	in the not translated zone	TGCCAGCGTGTTCTGAGGTCTTTTCTGTCACCAAGTTTGTACCTGAGTATTCTTAAATAT
ER a	in the not translated zone, not similarity with hER@	ACAAGGTTCTGCGAATCCCTGAAACGTTTTACCCATGTCCTGTATGACTTTA
Ppia	in the not translated zone	CCATATTTTCCTCATTCCCCTTCAAGTCTAGCAGGATTGCAAAGTTAAGT
CA2	after a short poliA, not in CDS	TCCACATCATGAGACAAACTGAGGCAATTTAGGCAAATCAGGTAAAACAGTC
CaBP9k	in the not translated zone	ACCACCTACTGATTGAATCCTATCCAATCCCAAAGATCTAGCTGTGAGAGCAAGATAC
1A	in the CDS region	CGT CCT ACT ACT TCT CTC ACT GCC AGT ATT AGC AGC AGG TAT CAC TAT ACT C
C3	in theCDS in zone	CTC TGT CAC TGT TAT CCT GCA CTC AGG TAG CGA CAT GGT AGA GGC AGA GCG CAG TG
IGFBP1	in the CDS region	TAT CAC AGC AAA CAG TGC GAG ACA TCT CTG GAT GGA GAA GCT GGG CTC TGC TG
PoliA		AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA

4.1.2 Slides

The oligonucleotides were spotted on epoxy-silane coated slides ArrayLink hyphop®, the slides were not pre-treated.

4.1.3 Spotting buffer

The oligonucleotides, before spotting, were mixed with equal volume of the different spotting buffers and pipetted into a 384 well plate.

Spotting buffer list (the final concentration is showed):

GeneScan printing buffer® 1x

3x SSC/1,5 M Betain

GeneScan printing buffer® 1 x + 1,5 M Betain

GeneScan printing buffer® 1 x + 10 % v/v DMSO

GeneScan printing buffer® 1 x + 100 µg/ml BSA

GeneScan printing buffer® 1 x + 2 µg/ml Salmon sperm DNA

4.1.4 Coupling reaction

The coupling reaction (linkage of oligonucleotides on the slides surface) could be performed using a warm steam saturated bath or by irradiation with 600-1.000 mJoule of UV light.

For the warm bath treatment the slides were placed on a support in a plastic box with the bottom covered by wet paper towels. The box was closed and incubated in a water bath at 50°C for 2 h, this resulted in a saturation of steam in the box.

The UV irradiation was conducted by exposing the slides at 254 nm UV radiation for various lengths of time. We used a 4 W 254 nm UV lamp, at a distance of 10 cm from the slides.

4.1.5 Wash of unlinked oligonucleotides from the slides

The slides, after the spotting and the coupling reaction, were washed twice in a freshly prepared solution of 2 x SSC/0,1 % SDS and twice in double distilled water for 5', shaking at room temperature. The slides were then dried by centrifugation and stored in a dark dry box.

4.2 Hybridization

4.2.1 cDNA synthesis and labeling

After RNA isolation from cell culture, the RNA was treated with DNase1. 100 µg of RNA was digested for 3 h at 37°C in a volume of 100 µl, in the presence of 10x DNase1 buffer, 20 units of DNase1®(1 U/µl), 800 units RNaseOUT® (40 U/µl).

1 µl of the resulting RNA after DNA digestion was tested by real time RT-PCR using the housekeeping gene primer pair 1A, to check for the presence of remaining genomic DNA in the sample; as positive control a reference cDNA solution was used, and HPLC water as negative control. The DNA digestion was considered successful when the sample delivered a

signal of the amplification similar to the negative control. The samples were stored at –20°C. Before the labeling of the samples the RNA contained in the DNase reaction was purified through the RNeasy® Mini Kit column.

Direct labeling

To reverse transcribe and direct label the samples the Superscript II RNase H- reverse transcriptase kit modified by GeneScan (Germany) was used. Starting with 50 µg of total RNA, 5 µl of oligo(dT) (15T, 100 µg/µl) primer solution was added for a resulting total volume of 23,5 µl. The sample was heated to 65°C for 10' and cooled on ice for 5'.

5 µl of dNTP mix without dTTP (2 mM per nucleotide), 2,5 µl dTTP (2 mM), 3 µl of Cy3-dUTP or Cy5-dUTP (1 mM), 10 µl 5x first-strand buffer, 5 µl DTT 0,1 M, 1 µl Superscript II RNase H- (200 U/µl) was added. After incubation at 42°C for one hour 1 µl Superscript II RNase H- was added, mixed by pipetting and incubated for one hour at 42°C. 1 µl of RNase H (5 U/µl) was then added, mixed by pipetting and incubated at 37°C for 10'. Then stored in dark and ice.

Indirect labeling

To perform reverse transcription and indirect labeling the Superscript II RNase H- reverse transcriptase kit was used. The protocol was obtained from Yu et al. (2002).

1st step: 25 µg of purified RNA in 16 µl HPLC water was added to 2 µl oligo(dT) (15T, 100 µg/µl) primer, incubated at 70°C for 10' and immediately cooled in ice for 5', then quickly spinned down. To the RNA sample: 6 µl of 5x first strand buffer, 3µl of DTT 0,1M, 20U of RNase OUT® (40 U/µl), 1,5 µl of Superscript II RNase H- reverse transcriptase (200 U/µl), 0,6 µl of 50x dNTP stock solution (dATP 25 mM, dCTP 25 mM, dGTP 25 mM, dTTP 5 mM, aa-dUTP 20 mM from Sigma, USA) was added. The samples were incubated at 42°C for 1 h, then 1 µl of Superscript II RNase H- was added and mixed gently by pipetting. The incubation mixture was incubated one more hour at 42°C. Followed by addition of 1 µl of RNase H (5 U/µl) which was mixed by pipetting and incubated at 37°C for 10'. The resulting cDNA solution was adjusted to a volume of 500 µl with HPLC water and pipetted in a microcon column YM-30®, centrifuged at 14.000 x g for 12', the filtrate was discarded, the column was washed with 400 µl HPLC water and centrifuged at 14.000 x g for 12'. To recover the cDNA 23 µl of HPLC water was added to the microcon column and it was transfered upside down in a new tube, centrifugation at 1.000 x g for 2' followed. The resulting volume of cDNA was 20 µl. The cDNA was stored at –20°C.

2nd step: 1 µl of $Na(CO_3)_2$ (pH: 9, 1 M) was added to the cDNA solution up to a final concentration of 50 mM. The resulting 21 µl solution of cDNA and sodium carbonate were

transferred into the brown Eppendorf tube containing the Cy-NHS ester (the ester of the Cy3 or Cy5 ready to react with the aminoallyl group of aa-dUTP to form the covalent bound between the aa-dUTP incorporated in the cDNA and the Cy-NHS ester) and mixed by pipetting, incubated in darkness and at room temperature for 2 h. The quenching reaction (block of the spare dyes, after the link of Cy-NHS esters to the aa-dUTP) was performed by pipetting 4,5 µl of hydroxylamine (4 M) into the sample, and the reaction mixture was left in darkness for 15' at room temperature.

Independently from the labeling before hybridization the cDNA was cleaned using a microcon column YM-30®. The resulting labeled cDNA was adjusted to a volume of 500 µl with HPLC water and pipetted into a microcon column YM-30®, centrifuged at 14,000 x g for 12', the filtrate was discarded, the column was washed with 400 µl HPLC water and centrifuged at 14,000 x g for 12'. 23 µl of HPLC water was added to the microcon column to recover the cDNA and it was place upside down in a new tube, centrifugation at 1,000 x g for 2' followed, the final volume of cDNA was 20 µl. The cDNA was stored in ice and dark until the start of hybridization process.

4.2.2 Hybridization protocol

We tested four different hybridization protocols from different companies: GeneScan (Germany), Genetix (UK), Amersham (USA) and Peqlab (Germany).

The hybridization protocols are summarized in tab. 6.

Tab. 6: Hybridization protocol.

protocol	pre-treatment	hybridization solution	pre-heat sample	slide wash	dry
GeneScan	no	cDNA 20µl H20 95,9µl 20xSSC 30,8µl 2% SDS 7,8 µl Arrayblock solution 15,5µl	100°C 1' ice 5'	1x 2xSSC/0,5%SDS 65°C 5' 1x 0,5xSSC RT 5' 1x 0,1xSSC RT 5'	stream of N2
Genetix	no	genHYB 2x cDNA ratio 1:1	45°C 2'	2x 2xSSC/0,1%SDS RT 5' 2x 2xSSC RT 5'	stream of N2
Amersham	slide: 5xSSC/0,2%SDS/1%BSA 60°C 20' sample: cDNA 94°C 3' ice 5' +1µg PoliA (54A) per slide final volume 7,5µl 70°C 45'+ RT 30'	cDNA-PoliA 7,5µl Hybridization buffer 4x 7,5µl formamide 100% 15µl	no	1x 1xSSC/0,1%SDS 55°C 5' 2x 0,1xSSC/0,1%SDS 55°C 10' 1x 0,01xSSC/0,1%SDS 55°C 10'	stream of N2
PeqLab	slide:no wash of unlinked oligonucleotides after the spot 1x 0,1% Triton-x100 RT 5' 2x HCl 0,0037% RT 2' 1x 100mM KCl RT 10' 1x H20 RT 1' 1x Nexterion QMT Blocking solution 50°C 15' 1x H20 RT 1'	Nexterion hyb buffer cDNA ratio 2:1	no	1x 2xSSC/0,2%SDS RT 10' 1x 2xSSC RT 10' 1x 0,2xSSC RT 10'	stream of N2

4.2.3 Hybridization of the samples

The hybridization had to be conducted in a humid hybridization chamber, three different chambers were tested.

Using the Flat block® hybridization chamber the slides were incubated in the cycler PTC-200®, the Flat block was filled with 8 ml of water.

Following the user protocol of the ArrayIt Hybridization Cassette® the slides were placed in the cassette with 400 µl H_2O and incubated in a water bath at 50°C.

A third type of hybridization chamber that reaches the saturation of steam was built using a plastic box with the bottom covered by wet paper towels, the slides were placed inside on a support to avoid contact between the slides and the wet towels. The box was closed and incubated in a bath at 50°C to reach a saturation of steam in the box.

The slides were placed in the hybridization chamber, the area with the spotted oligonucleotides on the slides was covered by a lifterslip 18x18 mm, 15 µl of hybridization solution was pipetted directly under the lifterslip carefully avoiding the formation of air bubbles between the slide and the cover (lifterslip).

4.3 Scan and analysis of the hybridized slides

The scan of the hybridized slides was performed using the scanner Scanarray_4000® in combination with the software ScanArray® 3.1. The resulting images from the scan of the slide acquired by the Scanarray_4000® were evaluated using the software Quantarray® 3.0.

4.4 Grubb's statistical outlier test

Each sample was spotted in replicates (3-7) and subjected to the test. The significance was set to 95 %. The "P" values of significance are reported in tab. 7. A "P" value greater than the limiting value reported in the tab. 7, according to the number of values composing the analyzed group, did not pass the test. The equation of Grubb's outlier test (P: limiting value, $V_{(max/min)}$: the maximum or minimum value of the group, MW: average value, SD: standard deviation).

$$P=\frac{|V_{(max/min)}-MW|}{SD}$$

Tab. 7: Limiting values of P setting the significance of test to 95% (n: number of values analyzed).

n	95%	n	95%	n	95%
3	1,153	6	1,822	9	2,109
4	1,436	7	1,938	10	2,177
5	1,671	8	2,031	12	2,287

4.5 Normalization and gene expression analysis

To normalize the signal stemming from Cy3 and Cy5 which is affected by variable incorporation rates and fluorescent signal intensities of the Cy-dyes (Cy5 delivers a higher fluorescent signal intensity than Cy3). The value of Cy5 was divided by a factor stemming from the average values of the ratio Cy5/Cy3 of the housekeeping genes (ß-Act, 1A, Ppia and HK1). This factor was calculated in the following way: each housekeeping gene group of values was subjected to the Grubbs test, and then the average for each housekeeping gene and the ratio Cy5/Cy3 was calculated. The average value of the Cy5/Cy3 ratio calculated from all housekeeping genes was taken as normalization factor.

After the normalization of all Cy3 values, the relative gene expression of each sample was calculated from the ratio between treated and untreated samples labeled by the two different Cy-dyes.

5. Sequencing

5.1 mRNA isolation

Starting with total RNA extracted from uterus, a DNA digestion was performed (for reagents see at 3.2). 100 µg of RNA was treated in a total volume of 300 µl containing: 10x DNase1 buffer, 15 U of DNase1 (1 U/µl), 600 U RNase OUT (40 U/µl), and incubated at 37°C for 2 h. 1 µl of the resulting RNA after DNA digestion was tested by real time RT-PCR using the housekeeping gene primer pair 1A. As positive control a reference cDNA solution was used, and HPLC water served as negative control. The DNA digestion was considered successful when the sample delivered an amplification signal similar to the negative control.

The RNA contained in the DNase reaction was purified through the RNeasy® Mini Kit column and eluted in 100 µl. Thereafter the mRNA was isolated using the Nucleo Trap mRNA® mini kit, the RNA was eluted in 90 µl, quantified, and stored at –20°C.

5.2 cDNA library screening

Liver cDNA library of adult Wistar rat created by Bento Soares, with insert of size of 1kb was screened (RZPD, Germany). The library consists in 6 primary pool and secondary pool. The bank was screened by real time RT-PCR using gene specific primers (r52, 1A, C3) as probe.

5.3 Gene specific reverse transcription

The gene specific reverse transcription was performed using the kit OneStep RT-PCR®. The resulting mRNA after isolation by Nucleo Trap mRNA® was used as template for the reverse

transcription. 150 ng mRNA was added to 10 µl of 5x Qiagen OneStep RT-PCR Buffer®, 2 µl dNTP (10 mM), 5 µl specific primer for reverse transcriptase (6 µM) reported in tab. 8, 2 µl Qiagen OneStep enzyme®, 40U RNase OUT®(40 U/µl), in a total volume of 50 µl, incubated at 50°C for 30'. Sample was stored at –20°C.

Tab. 8: Sequence of the primer for the gene specific reverse transcription

Primer name	Sequence	Annealing temperature
cDNA1	aga gcc acc ttc aga tt	50°C

5.4 Sanger method with labeled primer

For the sequencing reaction the Sanger method with labeled primer was used (Sanger *et al.,* 1977). The primer should have a length of 15-20 bp, an annealing temperature of 55-60°C and a label of IRD800, fluorescent marker, at the 5' end. The primers are listed in tab. 9.

Tab. 9: Sequence primer, modified at the 5'end by IRD800 used for the sequence reaction.

Primer name	Sequence
r52seq1	ggg gca aag cag tca tc
r52seq2	gcc aac ata gaa tcc tgt cc
r52seq3	gtg ccc ttt cgc tta gg

The sequencing reaction was conducted by Andreas Blecha in the institute for Genetics of Prof. Dr. Gerhard Rödel at the Technische Universitaet Dresden.

5.5 *In silico* studies of the fragment r52

The resulting sequences of r52 were compared and analyzed using the software "Blast" (basic local alignment and search tool) (Altschul *et al.,* 1992), "blast2sequences" and "Rat genome project" present at the web site of National Center of Biotechnology Information (http://ncbi.nlm.nih.gov). To search for ORFs (open reading frames) in the sequenced region of r52 I used the software "ORF finder" present at the web site of National Center of Biotechnology Information (http://ncbi.nlm.nih.gov). To study the promoter region of the r52 sequence the following software were used: "promoter finder", "promoter 2.0 prediction server", "GC Promoter Finder" and "Eukaryotic Promoter Database" from the web site http://www.bioinformatics.vg/biolinks/bioinformatics/Promoter%2520Scan.shtml, "match Public" from http://www.gene-regulation.com. The software "Mfold" (Zuker M., 2003) was used for the prediction of RNA secondary structure.

Results

This chapter is subdivided into three main categories: results of the gene expression analysis using real time RT-PCR, the establishment of the microarray technology in the laboratory and its first application to verify the reliability of the method, and the study of a newly identified sequence that presents an interesting gene expression profile in the uterus.

The effects of estrogens and xenoestrogens on gene expression were always correlated with the control treatment (carrier of the substance) set to one. A change larger than twice the gene expression of the control was considered to be stimulation, a reduction to less than 50% of control level was repression.

Significance of all data obtained using the real time RT-PCR was determined by the Student's t-test (* $p < 0,05$). Expression analysis performed on the micro array was performed just once, which does not allow for statistical analysis.

1. Gene expression analysis using the real time RT-PCR

1.1 Gene expression analysis in uterus models

1.1.1 Results of the vitro experiments in the RUCA-I cell line

The cell culture, cell treatment and RNA extraction was performed in the Institute for Molecular Medicine at the University of Lübeck (Germany) in the group of Prof. Dr. Günter Vollmer. The method of cell culture was already published (Hopert *et al.,* 1998; Tüshaus *et al.,* 2003). Reverse transcription and analysis of gene expression was performed in our laboratory; every result comes from the average of at least two independent cDNA synthesis and two independent real time RT-PCR analyses per cDNA synthesis, delivering a mean value deriving from at least four independent values.

The cells were cultured on a layer of reconstituted basement membrane (ECM), Collagen I (COL1) and without any coating of the plate floor (plastic). The genes C3, CLU, PCNA and CAII were studied.

The effects of E2 treatment on the regulation of C3 are reported in fig. 1. There was a detectable effect only in culture on ECM (fig. 1a), the presence of some substances with estrogenic activity in the cell medium was detected by down regulation in response to the pure antagonist ICI 182,780 (ICI) that resulted in a reduction of C3 expression level. No effect was detectable by any substances culturing the cell on the plastic floor of the cultural plate (fig. 1b) or on the floor coated by a layer of COL1 (fig. 1c).

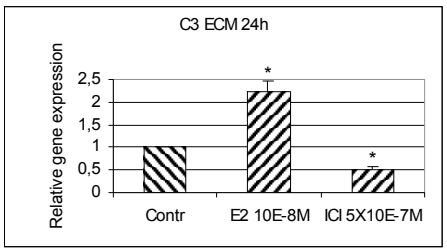

Fig. 1a

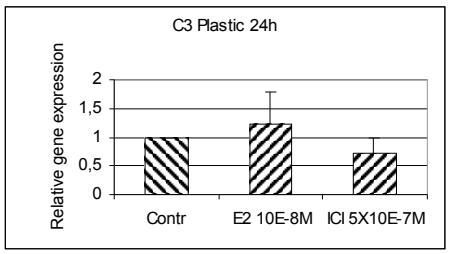

Fig. 1b

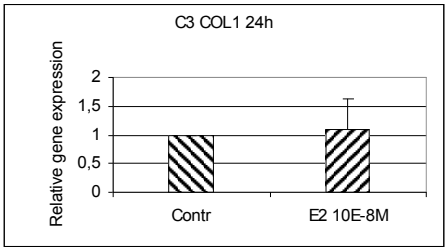

Fig. 1c

Fig. 1: Gene expression analysis by real time RT-PCR, correlated to control (set to one) of C3 expression after 24 h of treatment, in RUCA-I cell (rat uterus cancer cell line). ECM: cell culture plate coated by reconstituted basement membrane, Plastic: cell culture plate not coated, COL1: cell culture plate coated by Collagen 1. Cells were treated by EtOH (Contr), E2 and ICI. Significance: Student's t-test *: $p<0,05$.

The effects of E2 treatment on the regulation of CLU are reported in fig. 2. A stimulation of expression was visible only in culture on a layer of ECM (fig. 2a).

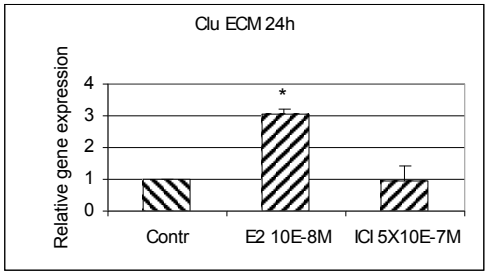

Fig. 2a

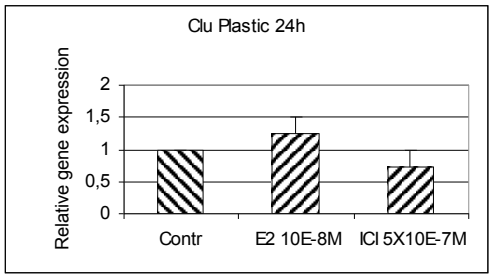

Fig. 2b

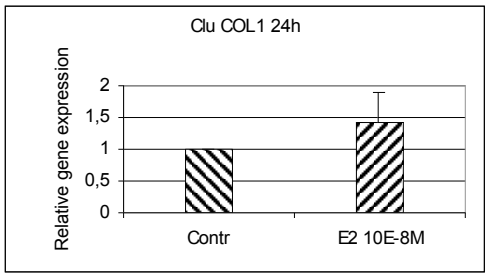

Fig. 2c

Fig. 2: Gene expression analysis by real time RT-PCR, correlated to control (set to one) of Clu after 24h treatment, in RUCA-I (rat uterus cancer cell line). ECM: cell culture plate coated by extra cellular matrix, Plastic: cell culture plate no coated, COL1: cell culture plate coated by Collagen 1. Cells were treated by EtOH (Contr), E2 and ICI. Significance: Student's t-test *: $p<0,05$.

The expression of PCNA was also studied, but it was not affected by any of the treatments and appeared to be independent of cell culture substrate (data not shown).

The gene expression of CAII in RUCA-I using the real time RT-PCR could not be verified. This might be due to a low level of CAII expression in RUCA-I that could not be detected using this method.

1.1.2 Results of the in vivo experiments in rat uterus

After the *in vitro* tests, the focus was moved to a more complex level, an entire organism. The animal experiments from which the material analyzed and reported in fig. 3, 4, 5, 6, 7, 8, 9 and 10 were performed by our collaborator Dr. Patrick Diel in the department of Molecular and Cellular Sport Medicine, DSHS, in Köln (Germany), as reported in Materials and methods 2.2.

In our laboratory RNA was extracted from tissue (Materials and methods 2.3.1), pooled according to the treatment, with at least 6 animals per treatment, and reverse transcribed to cDNA (Materials and method, 3.2 and 3.3).

Each result derives from the mean value of at least three independent cDNA syntheses and two independent real time RT-PCR analyses per cDNA synthesis, delivering a mean value calculated from at least from six independent experiments.

The animal experiments, from which the material for the analyses reported in fig. 11 were derived, were performed in our laboratory under the supervision of Dr. Oliver Zierau.

In fig. 3 the effects of E2 and EE on C3 gene expression in ovariectomised rat for 7, 24 and 72 h are shown, both substances induced a time dependent up regulation of C3 gene expression.

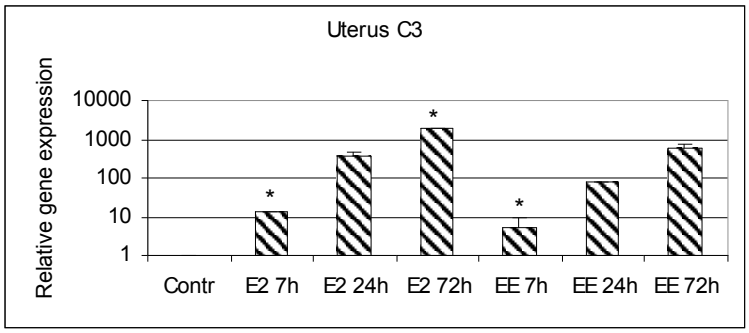

Fig. 3: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of C3 in a time dependent manner (7-24-72h) by a treatment (s.c. injection) of EtOH (Contr), E2 (3µg/kg/day) and EE (3µg/kg/day). Significance: Student's t-test *: $p<0,05$.

In fig. 4 the effects of isoflavones on C3 expression are shown whereas Gen was measured in a time dependent manner (7, 24, 72 h) with Dai at the time point of 72 h. Only the treatment by Gen for 24 h triggered a significant increase of the relative gene expression to 5 times that of the control, no significant effect was detectable by Gen at all other time points of treatment and following administration of Dai.

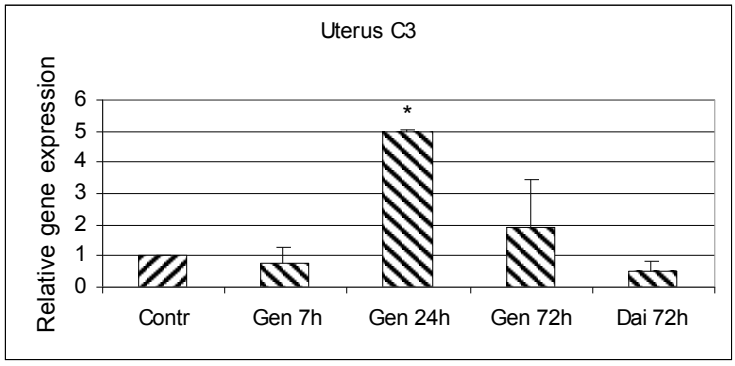

Fig. 4: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of C3 in a time dependent manner (7-24-72h) by a treatment (s.c. injection) of EtOH (Contr), Gen (10mg/kg/day) and Dai 20mg/kg/day. Significance: Student's t-test *: $p<0,05$.

In fig. 5 the effect of 8PN administration for 72 h on C3 expression is shown. The 8PN mimicked the activity of the positive control E2 (at a 1000 higher concentration dose) with roughly half the increase of C3 gene expression reached by E2.

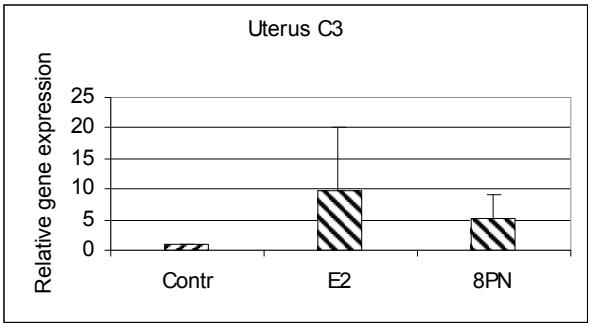

Fig. 5: Gene expression analysis, through real time RT-PCR, correlated to control (set to one), in rat uterus of C3 after 72h treatment (s.c. injection) by EtOH (Contr), E2 (30µg/kg/day) and 8PN (10mg/kg/day). Significance: Student's t-test *: $p<0,05$.

The same material was used to analyze the gene expression of PCNA and CAII. The effects of E2, EE, and Gen for 7, 24 and 72 h and Dai for 72 h, on PCNA gene expression are shown in fig. 6. The treatment with E2 and EE induced an up regulation of the relative gene expression of PCNA to more than 200 % compared to the control level after 7 and 24 h but this was shown to not be statistically significant. The administration by Gen and Dai did not have any remarkable effect.

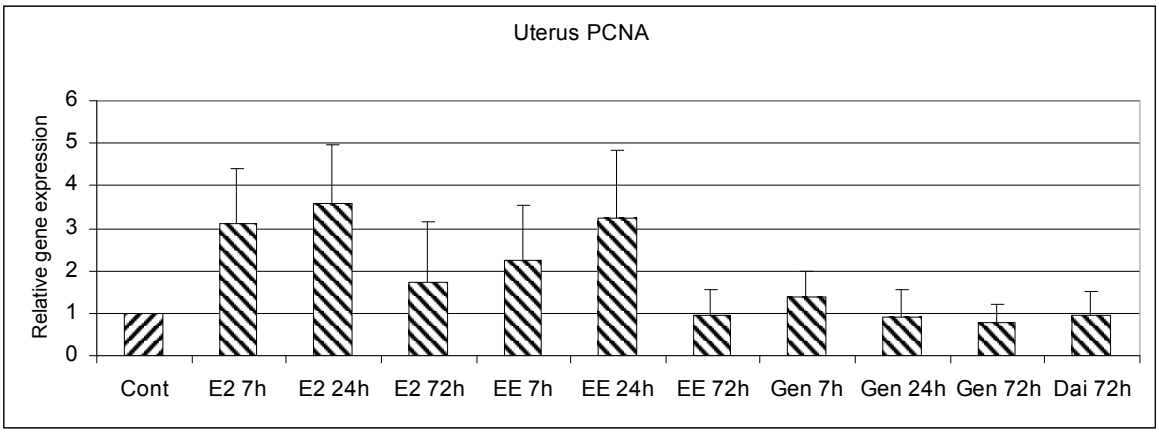

Fig. 6: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of PCNA in a time dependent manner (7-24-72 h) by treatment (s.c. injection) of EtOH (Contr), E2 (3µg/kg/day), EE (3µg/kg/day), Gen (10mg/kg/day), Dai (20mg/kg/day). Significance: Student's t-test *: $p<0,05$.

In fig. 7 the effects of 8PN and E2 following a 72 h treatment on PCNA expression were compared. E2 triggered an increase of the relative gene expression of PCNA to more than double that of control which was mimicked in part by 8PN. For both samples no significance was found.

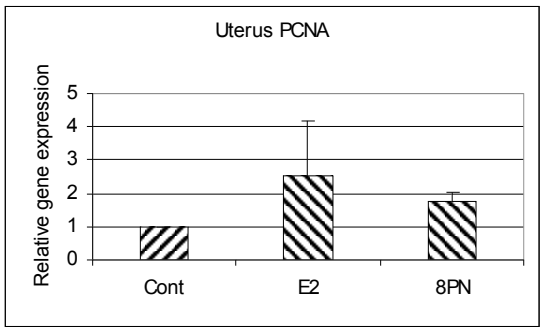

Fig. 7: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of PCNA after 72 h of treatment (s.c. injection) by EtOH (Contr), E2 (30µg/kg/day) and 8PN (10mg/kg/day). Significance: Student's t-test *: p<0,05.

The effects of E2, EE and isoflavones on CAII expression are shown in fig. 8. Estrogen and EE induced a down regulation of CAII gene expression which became more pronounced according to the progression of time (7, 24 and 72 h). Gen triggered a down regulation reaching roughly 50 % of control expression, with a significant effect after 7 and 24 h of treatment. Dai after 72 h did not show any particular activity.

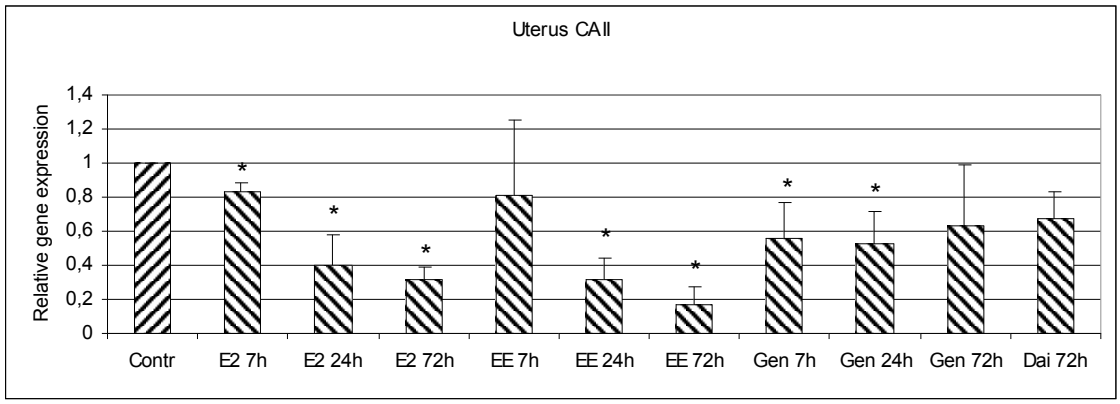

Fig. 8: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of CAII in a time dependent manner (7-24-72h) by treatment (s.c. injection) of EtOH (Contr), E2 (3µg/kg/day), EE (3µg/kg/day), Gen (10mg/kg/day), Dai (20mg/kg/day). Significance: Student's t-test *: p<0,05.

Treatment by 8PN for 72 h did not show any influence on CAII expression (fig. 9).

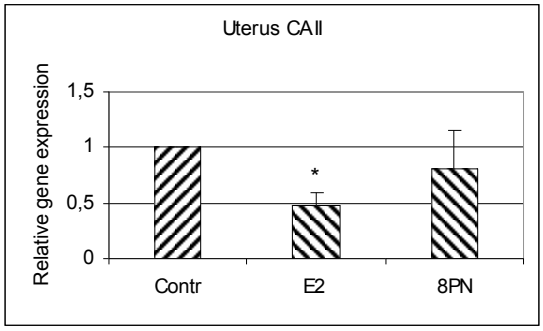

Fig. 9: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of CAII after 72h of treatment (s.c. injection) by EtOH (Contr), E2 (30µg/kg/day) and 8PN (10mg/kg/day). Significance: Student's t-test *: p<0,05.

In another animal experiment the effects of E2 and Dai for 7 and 24 h, and Gen for 24 h, in rat uterus were studied only for C3 expression and are shown in fig. 10. E2 induced a strong up regulation of C3 gene expression with an increase from 7 h to 24 h, to seven and thirty times respectively in comparison to the control level. After 24 h only Gen induced a significant up regulation of C3 at the level reached by E2 after 7 h of treatment. Treatment with Dai did not lead to any remarkable change.

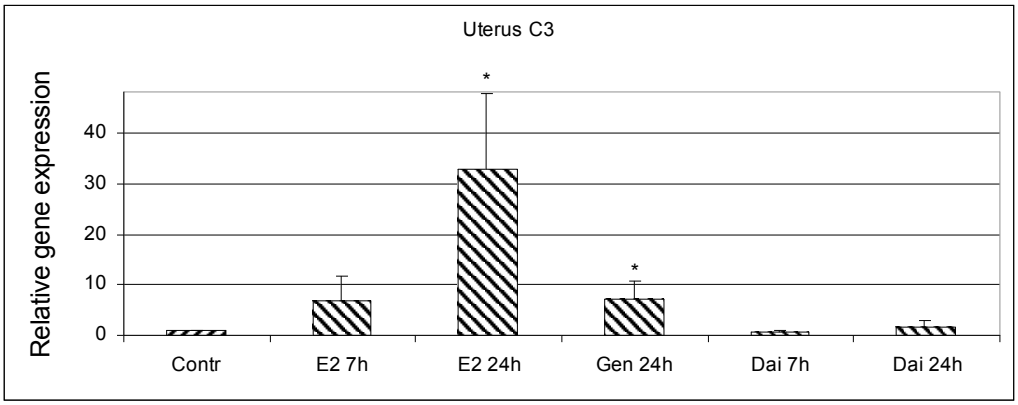

Fig. 10: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat uterus of C3 in a time dependent manner (7-24h) by treatment (s.c. injection) of EtOH (Contr), E2 (4µg/kg/day), Gen (10mg/kg/day) and Dai (40mg/kg/day). Significance: Student's t-test *: p<0,05.

The animal experiments performed in our laboratory are reported in fig. 11. C3 gene expression was analyzed. The effects of E2 and the two flavones 8PN and 6DMAN are presented in fig. 11a and b, for 7 and 24 h of treatment respectively. After the first 7 h E2 and the two flavones induced a similar up regulation, reaching to the doubling of the relative gene expression of C3. After 24 h E2 showed a stronger effect in comparison to 6DMAN and 8PN, which reached a significant up regulation to 43 and 16 times of relative gene expression respectively at a dose that is 1000 higher the positive control E2.

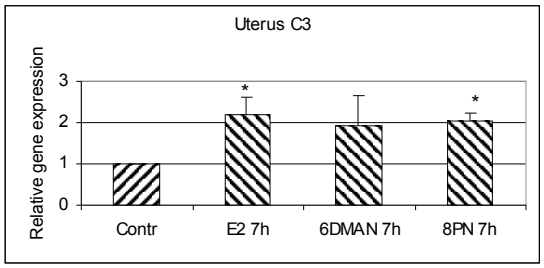

Fig.11a

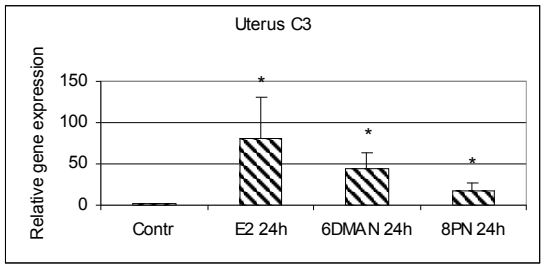

Fig.11b

Fig. 11: Gene expression analysis, through real time RT-PCR, correlated to control (set to one), in rat uterus of C3 in a time dependent manner (7-24h) by treatment (s.c. injection) of EtOH (Contr), E2 (10µg/kg/day), 6DMAN and 8PN (both 15mg/kg/day). Significance: Student's t-test *: $p<0,05$.

1.2 Gene expression analysis in liver models

1.2.1 Results of in vitro experiments in the Fe33 cell line

The genes IGFBP1, CaBP9k, PCNA and CAII were studied in the Fe33 cell culture model, they were cultured and treated as reported in the "Material and methods 2.1".

A single cell culture experiment was performed per treatment. Each result comes from the average of at least three independent cDNA syntheses and two independent real time RT-PCR analyses per cDNA synthesis, delivering a mean value deriving from at least six independent values.

The effect of E2 and the phytoestrogens Gen, Dai and 8PN for 7 and 24 h treatment on IGFBP1, CABP9k and CAII gene expression are shown in fig. 12a, 12b and 12c respectively. In each of them, a clear up regulation of their expression is seen in the case of E2 treatment, which is the positive control.

The phytoestrogens Gen and 8PN mimics the effects of the positive control after 7 h of treatment. The other genes show no significant effects after treatment with the three phytoestrogens.

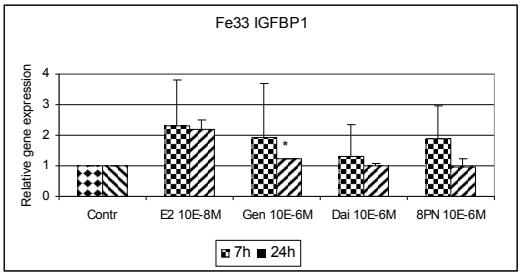

Fig. 12a

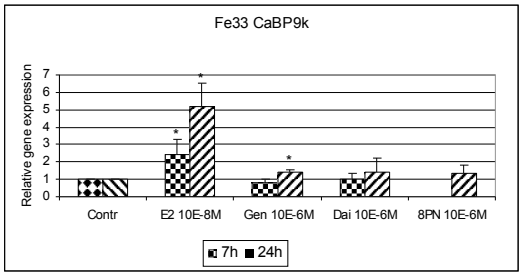

Fig. 12b

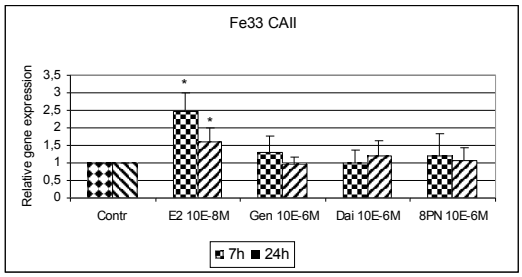

Fig.12c

Fig. 12: Gene expression analysis of IGFBP1 (12a) CaBP9k (12b) and CAII (12c) using real time RT-PCR, in rat hepatoma cell line Fe33. Cells were treated with EtOH (Contr), 10^{-8}M E2, 10^{-6}M Gen, Dai and 8PN for 7 and 24 h. Significance was determined by Student's t-test (* $p < 0,05$).

The effect of the treatment by EE in Fe33 in a dilution series from 10^{-9}M to 10^{-6}M and in a time course of 1, 3, 7 and 24 h on the expression of IGFBP1, CaBP9k, PCNA and CAII, is shown in fig. 13, 14, 15 and 16 respectively.

Fig.13 shows a clear up regulation of IGFBP1 gene expression after the treatment with E2 and EE. The potency of EE was always comparable or even stronger than the positive control.

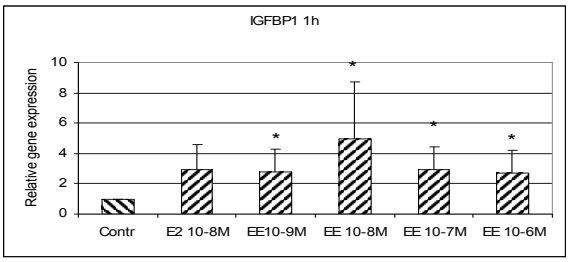

Fig. 13a

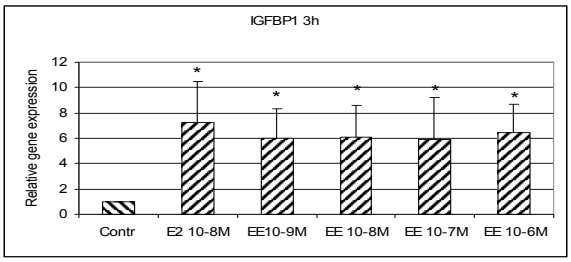

Fig. 13b

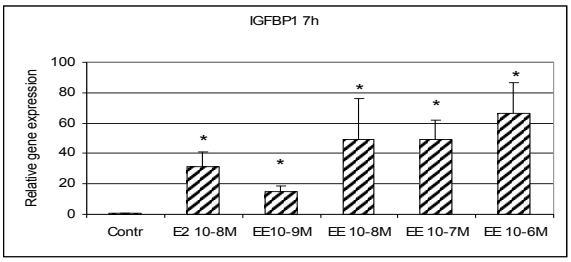

Fig. 13c

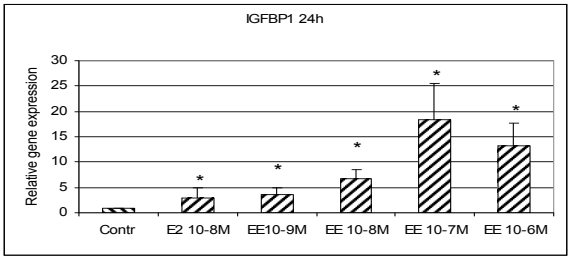

Fig. 13d

Fig. 13: Gene expression analysis of IGFBP1 using real time RT-PCR, in the rat hepatoma cell line Fe33. Cells were treated with EtOH (Contr), 10^{-8}M E2 and EE in a dose dependent manner for 1 (a), 3 (b), 7 (c), and 24 h (d). Significance was determined by Student's t-test (* $p < 0,05$).

In fig. 14 the effect of treatment by EE on the expression of CaBP9k is shown. In all figures a clear up regulation of CaBP9k gene expression is detectable. The effect of EE was always comparable to that of E2, and was sometime greater. The substances triggered a time and dose dependant up regulation of CaBP9k expression.

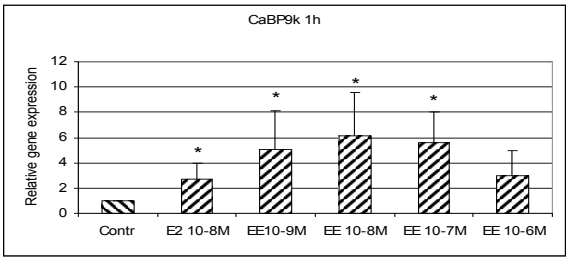

Fig. 14a

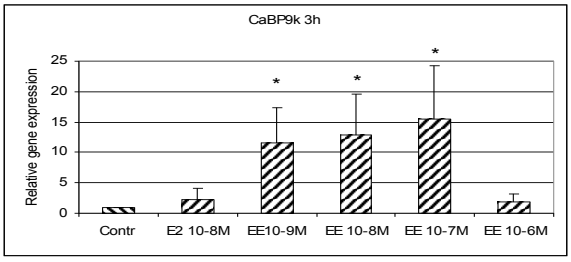

Fig. 14b

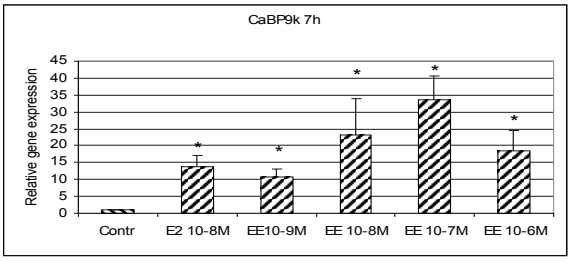

Fig. 14c

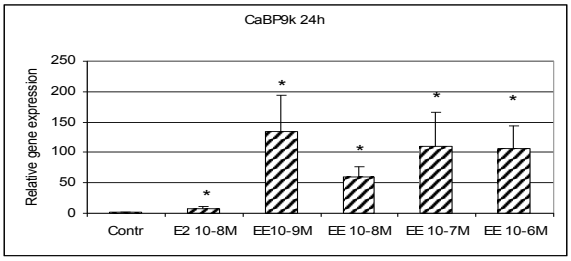

Fig. 14d

Fig. 14: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CaBP9k. Cells were treated with EtOH (Contr), E2 and EE in a dose dependent manner for 1 (a), 3 (b), 7 (c), and 24h (d).Significance Student's t-test *: $p<0,05$.

Fig. 15 shows the effects of EE treatment on PCNA gene expression in the cell line Fe33. The stronger effect of PCNA stimulation was detectable after 1 h (Fig. 15a) by EE 10^{-8}M. The effect on stimulation of PCNA declined according to later time points, until to a tendency of down regulation after 24 h of treatment for the doses of 10^{-8}, 10^{-7} and 10^{-6}M (fig. 15d). E2 and EE present always comparable results.

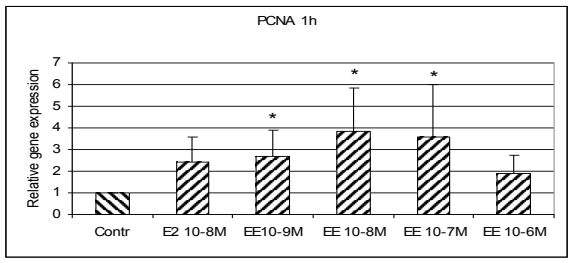

Fig. 15a

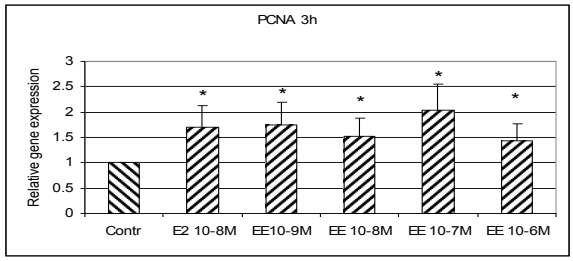

Fig. 15b

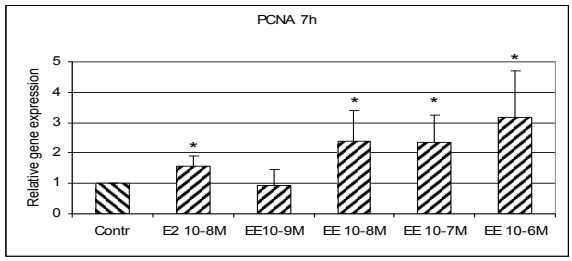

Fig. 15c

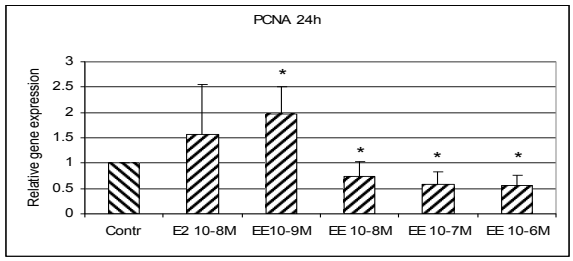

Fig. 15d

Fig. 15: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in Fe33 (rat hepatic cell line) of PCNA. Cells were treated with EtOH (Contr), E2 and EE in a dose dependent manner for 1(a), 3(b), 7(c), and 24 h(d).Significance: Student's t-test *: $p<0,05$.

Fig. 16 shows the effects of administration of EE for 1, 3, 7 and 24 h, at different doses, to the Fe33 cell line on the expression of CAII. A clear time and dose dependent effect was detected. The most prominent effect on the up regulation of gene expression was seen after 7 h of treatment by a dose EE 10^{-6}M (fig. 16c). The effects of EE were always comparable to those of the positive control, E2 10^{-8}M.

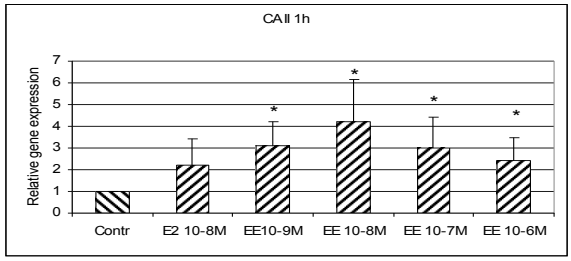

Fig. 16a

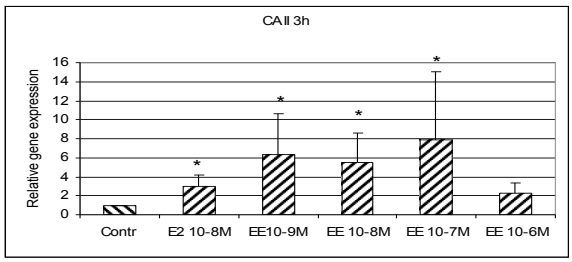

Fig. 16b

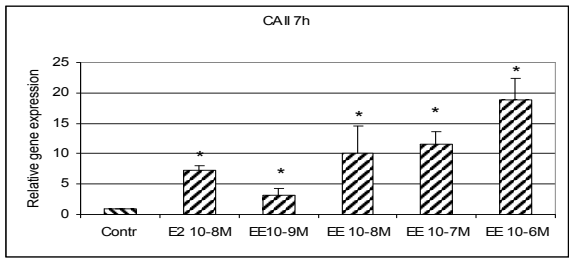

Fig. 16c

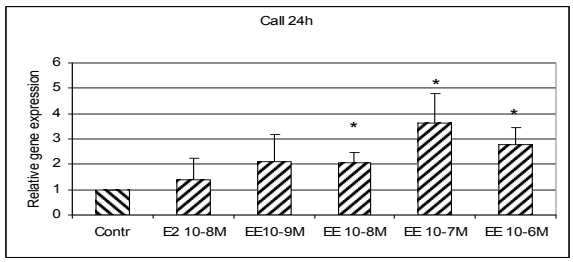

Fig. 16d

Fig. 16: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CAII. Cells were treated with EtOH (Contr), E2 and EE in a dose dependent manner for 1(a), 3(b), 7(c), and 24 h(d).Significance: Student's t-test *: $p<0,05$.

The effects of treatment with NP in Fe33 in a dilution series from 10^{-8}M to 10^{-5}M and in a time course of 1, 3, 7 and 24 h on the expression of IGFBP1, CaBP9k, PCNA and CAII, is shown in fig. 17, 18, 19 and 20 respectively.

Fig. 17a shows that treatment with NP for 1 h induces an up regulation of IGFBP1 gene expression only at a dose of 10^{-6}M. After 3 and 7 h (fig. 17b and 17c) E2 and NP 10^{-5}M the relative gene expression level of IGFBP1 doubled.

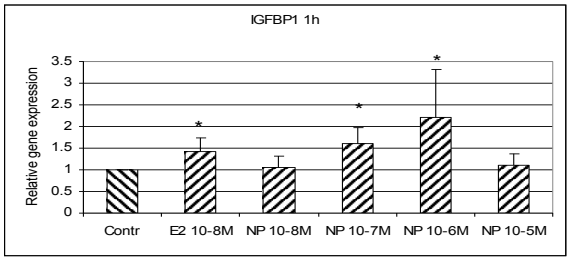

Fig. 17a

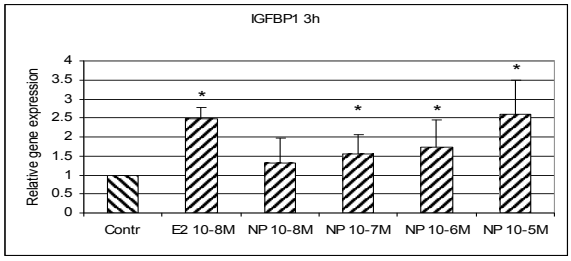

Fig. 17b

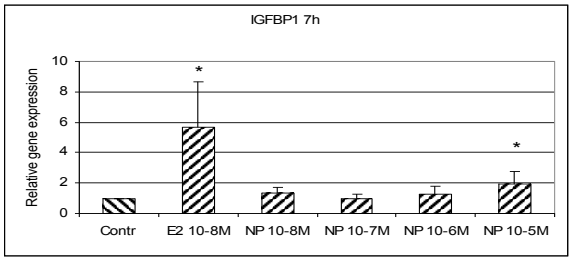

Fig. 17c

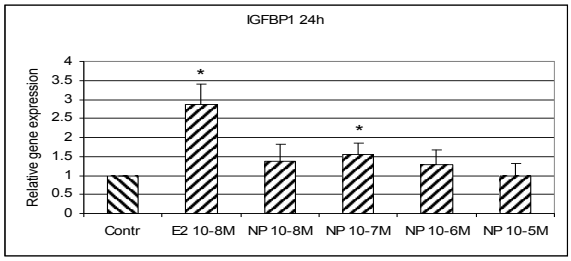

Fig. 17d

Fig. 17: Gene expression analysis of IGFBP1 using real time RT-PCR, in the rat hepatoma cell line Fe33. Cells were treated with EtOH (Contr), 10^{-8}M E2 and NP in a dose dependent manner for 1 (a), 3 (b), 7 (c), and 24 h (d). Significance was determined by Student's t-test (* $p < 0,05$).

Gene expression of CaBP9k was only slightly influenced by treatment with NP (fig. 18). It was shown to only have stimulation statistically significant by NP treatment with a dose of 10^{-7}M after 24h of exposure (fig. 18d).

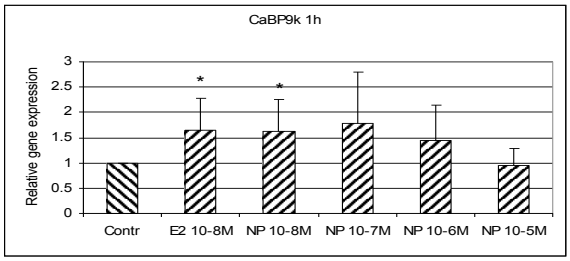

Fig. 18a

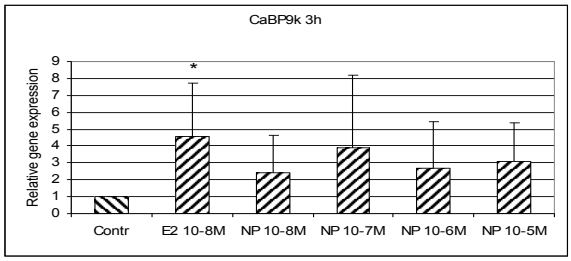

Fig. 18b

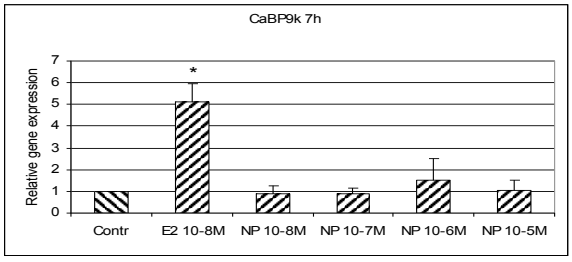

Fig. 18c

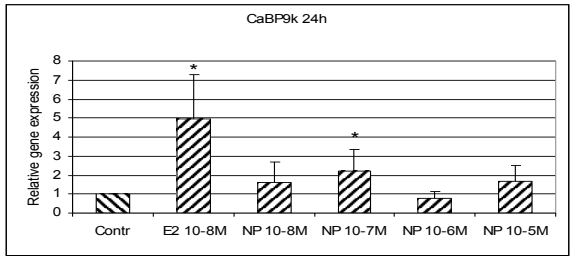

Fig. 18d

Fig. 18: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CaBP9k. Cells were treated with EtOH (Contr), E2 and NP in a dose dependent manner for 1(a), 3(b), 7(c), and 24 h (d). Significance: Student's t-test *: $p<0,05$.

Fig. 19 shows the effects of NP administration on the expression of PCNA in the cell line Fe33. The only effect on gene regulation induced by NP appeared after 3 and 24 h (fig. 19b and 19d). For the earlier time point treatment with a dose of 10^{-5} and 10^{-7}M induced a doubling of gene expression that was significant according to the Student's t-test, and comparable to the positive control E2. After 24 h only the doses of 10^{-7} and 10^{-8}M lead to an increase in gene expression that was also statistically significant, as with treatment with E2.

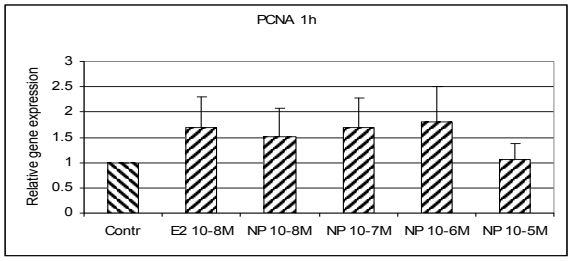

Fig. 19a

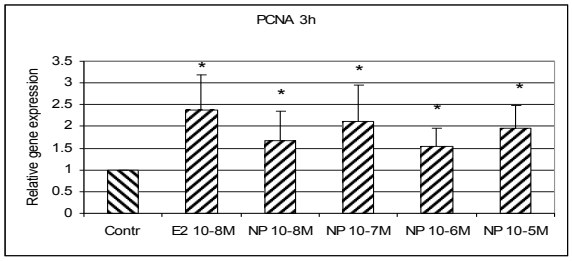

Fig. 19b

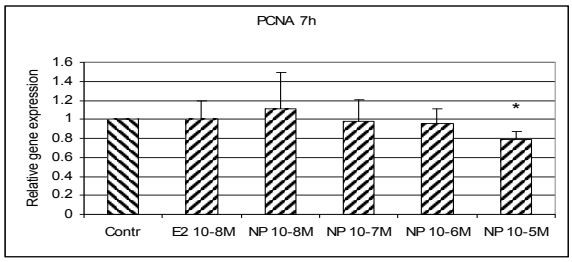

Fig. 19c

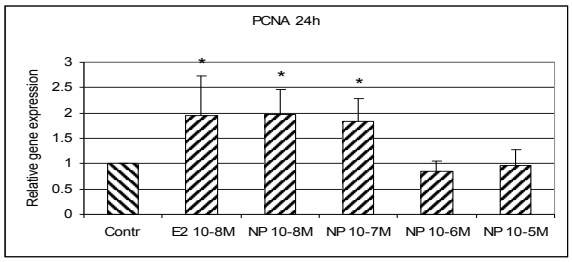

Fig. 19d

Fig. 19: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in Fe33 (rat hepatic cell line) of PCNA. Cells were treated was EtOH (Contr), E2 and NP in a dose dependent manner for 1(a), 3(b), 7(c), and 24 h (d). Significance: Student's t-test *: $p<0,05$.

Treatment with NP in Fe33 resulted in a slight general stimulation of CAII gene expression (fig. 20). The only clear stimulation of CAII expression was obtained after 3 h of treatment at the highest concentration (fig. 20b), which is statistical and biological significant, with the doubling of relative gene expression and is perfectly comparable to the positive control, E2. For the other time points no remarkable changes were detected.

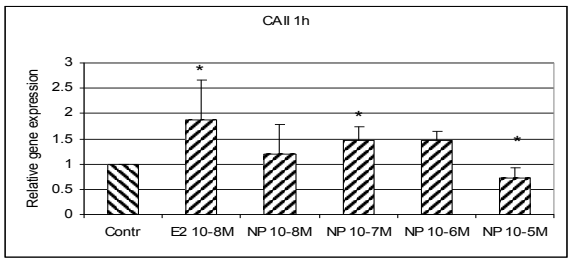

Fig. 20a

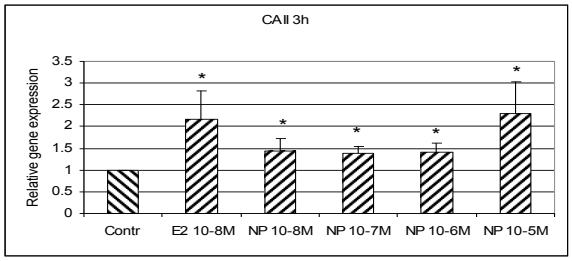

Fig. 20b

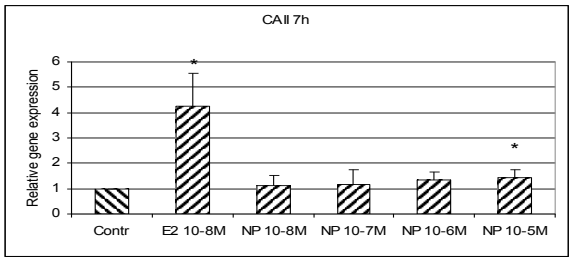

Fig. 20c

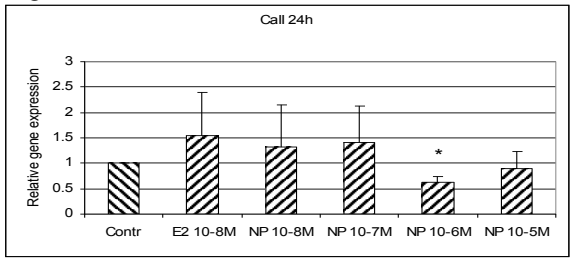

Fig. 20d

Fig. 20: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CAII. Cells were treated with EtOH (Contr), E2 and NP in a dose dependent manner for 1(a), 3(b), 7(c), and 24 h (d). Significance: Student's t-test *: $p<0,05$

1.1.2 Results of in vivo experiments in rat liver

After these preliminary *in vitro* tests on the substances, attention was moved to a more complex level, the situation in an intact organism. The logical evolution of the level complexity starting from a rat hepatic cell line was the *in vivo* analysis with the investigation of effects on the rat liver.

The RNA material analyzed in fig. 21, 22, 23, 24 and 25 is the same as already described for the uterus analysis in point 1.1.2 of this chapter (fig. 3-9).

The gene expression of IGFBP1, PCNA and CAII was studied.

The conditions for the analysis of IGFBP1 gene expression were established in our laboratory by Renate Geis (Geis *et al.,* 2005).

The effects of E2 and EE, and the isoflavones, Gen and Dai, on IGFBP1 gene expression are shown in fig. 21. E2, EE and Dai showed stimulation of IGFBP1 expression after 72 h of treatment. EE and Gen stimulated the expression of IGFBP1 after 7 h of treatment. Only Gen produced a change in the gene expression level which showed a statistical significance after 72 h of treatment with a reduction of the IGFBP1 expression to approximately 40 % of the control level.

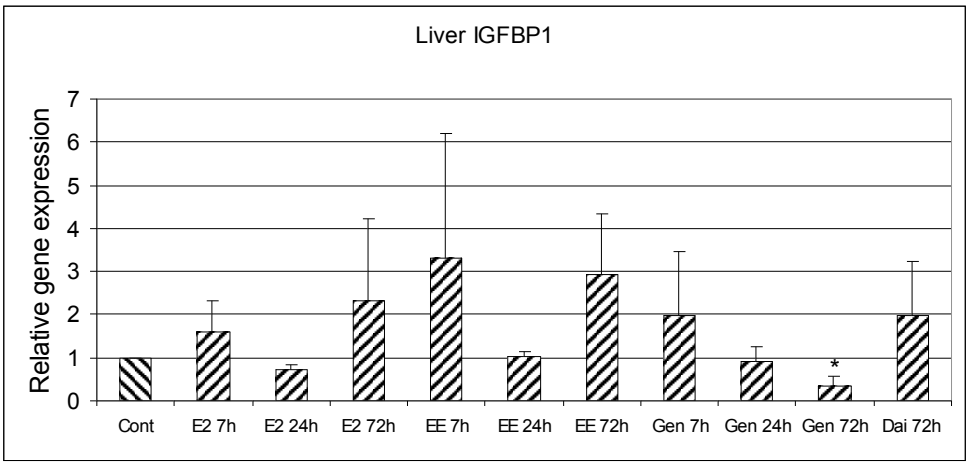

Fig. 21: Gene expression analysis of IGFBP1 using real time RT-PCR, in liver of ovx rats. Animals were treated in a time dependent manner (7, 24 and 72 h) by s.c. injection of EtOH (Contr), E2 and EE (3µg/kg/day), Gen (10mg/kg/day) and Dai (20mg/kg/day). Significance was determined by Student's t-test (* $p < 0,05$).

E2 and 8PN in a 72 h treatment protocol induced a stimulation of the IGFBP1 expression to roughly 200 % (fig. 22), but only treatment by E2 was statistical significant.

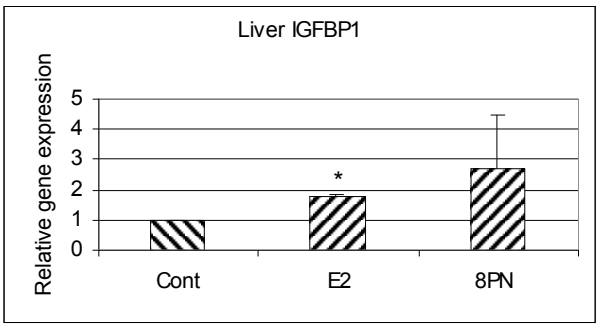

Fig. 22: Gene expression analysis of IGFBP1 using real time RT-PCR, in liver of ovx rats. Animals were treated for 72 h by s.c. injection of EtOH (Contr), E2 (30µg/kg/day) and 8PN (10mg/kg/day). Significance was determined by Student's t-test (* $p < 0,05$).

Fig. 23 shows the effects of E2, the xenoestrogen, EE, and the isoflavones Gen and Dai on the gene expression of PCNA. After 72 h E2 induced a slight repression of the gene expression to 60 % of the control level, delivering a significant value according the Student's t-test. For the other treatment protocols no regulation could be shown.

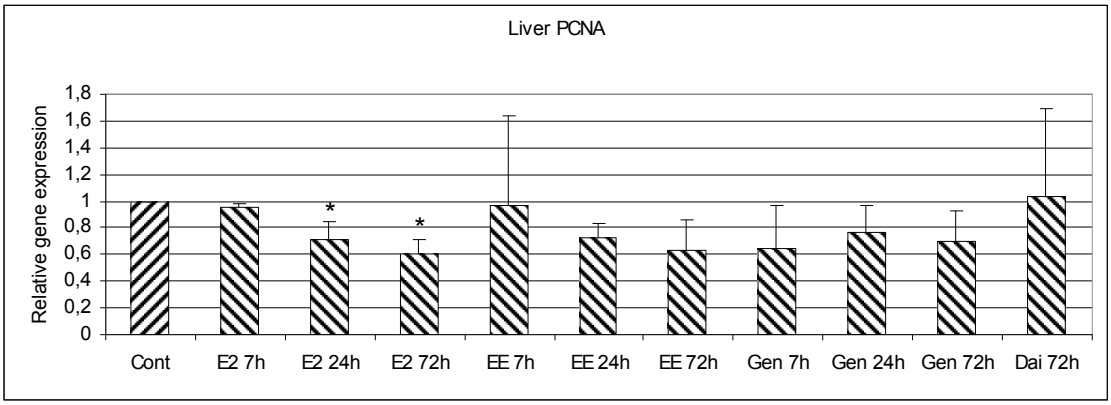

Fig. 23: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat liver of PCNA in a time dependent manner (7-24-72h) by treatment (s.c. injection) of EtOH (Contr), E2 (3µg/kg/day), EE (3µg/kg/day), Gen (10mg/kg/day), Dai (20mg/kg/day). Significance: Student's t-test *: $p<0,05$.

The expression of PCNA in liver was not affected by neither E2 or 8PN after 72 h (data not reported).

In fig. 24 the gene expression analysis of CAII is shown. E2 and EE triggered, after 72 h, a significant change in CAII expression decreasing it to 50 % of the control level. Gen showed a similar effect after 7 h of treatment, with the same statistical significance. No alteration to gene expression was seen with Dai or the other treatment conditions.

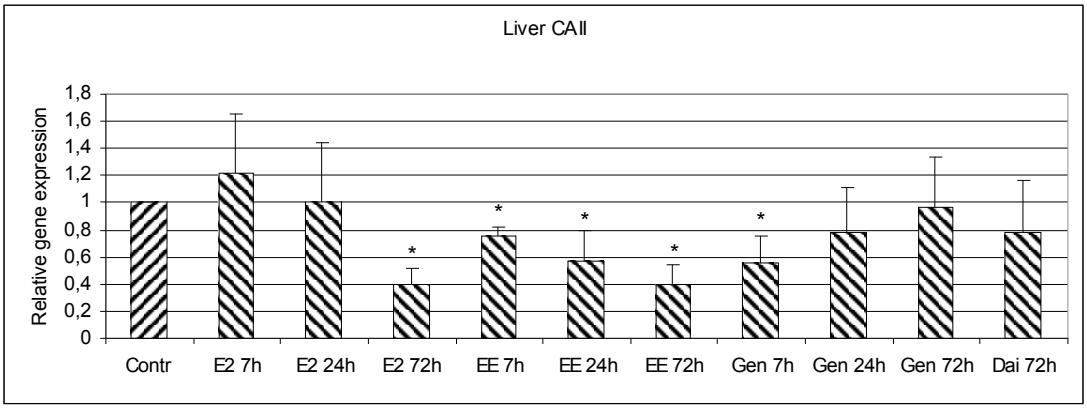

Fig. 24: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat liver of CAII in a time dependent manner (7-24-72 h) by treatment (s.c. injection) of EtOH (Contr), E2 (3µg/kg/day), EE (3µg/kg/day), Gen (10mg/kg/day), Dai (20mg/kg/day). Significance: Student's t-test *: $p<0,05$

In fig. 25 the effects of E2 and 8PN on CAII expression after 72 h of treatment are shown. For both treatments a slight reduction of gene expression became apparent, E2 reached 60 % and 8PN reached about 80 % of the control level.

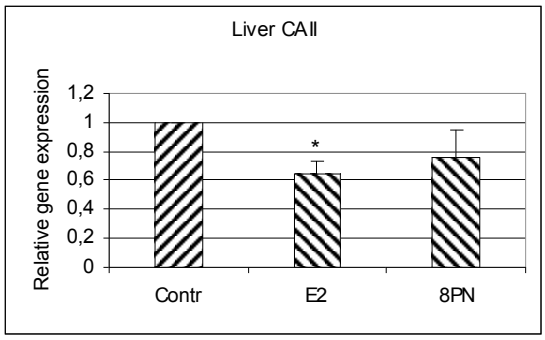

Fig. 25: Gene expression analysis by real time RT-PCR, correlated to control (set to one), in rat liver of CAII after 72h of treatment (s.c. injection) with EtOH (Contr), E2 (30µg/kg/day) and 8PN (10mg/kg/day). Significance: Student's t-test *: $p<0,05$.

2. Establishment of the microarray technology

One task of my thesis was the establishment of the microarray technology in our laboratory. The microarray is a complex process which presents many opportunities of choice for every step, as already reported in the "Introduction" at the point 5.1.1.

All the steps that present different options and that were performed in this thesis are reported in table 1.

The results of the most important experiments are reported and briefly illustrated.

Tab.1: Summary of the optimization of the microarray technology.

Step	Different possibility	Result	Reason	Illustrated at point
Process: chip production				2.1
probe disposition	spot/print	spot	presence of the spotter	
kinds of probes	oligonucleotides/PCR product	oligonucleotide	stability, time saving, practical hadling	
kind of matrix	glass slide/membrane	glass slide	best support, currently the only one used	
slide coat	epoxy silane Poly-L-lysisn amino silane	epoxy silane	best fit with oligonucleotides	Call et al., 2003; Warsen et al., 2004
oligonucleotide modification	no modification/ NH2 group at 5' end	modification	more stability of oligonucleotides throught the washes	2.1.1
spot buffer	home made/ GeneScan/ PeqLab	GeneScan	best fit with the slides best signal	2.1.2 Fig. 26-27
spot concentration of oligonucleotide	same/different amount	different sample specific	every gene presents a different expression level	2.1.3 Fig.28
link of oligo on slide	warm wet chamber/ U.V. cross link	warm wet chamber	best reproducibility across all samples Cy5/Cy3 ratio close to one	2.1.4 Fig.29
Process: hybridization				2.2
RNA isolation	total RNA/mRNA	total RNA	cheaper/ no difference	
hybridization buffer	suppliers: GeneScan (bankrupt) Genetix/ Amersham/ PeqLab	PeqLab	PeqLab producer best result	2.2.1 fig.30-31
prehybridization wash	protocol of the supplier of hybridization buffer /no prewash	prewash	best result, to test because of different source of slide and hybridization buffer	2.2.2 fig.32
amount of hybridization sample	different amount reverse transcribed RNA	12,5ng starting RNA	best compromise between background/sample signal	2.2.3 fig.33
target labeling	fluorescence direct/indirect radioactivity	fluorescence direct	no possibility to use radioactivity _ direct, better result	2.2.4 fig.34
Cy3/Cy5 ratio in labeling of sample	balanced/unbalanced amount of Cy dye	3:1 Cy3:Cy5	best result of signal intensity of samples cheaper	2.2.5 fig.35
hybridizatio chamber	3 mosted chambers: Flat Block/ Array IT/ self made	Flat Block	best handling, reproducibility and quality of results	2.2.6 fig.36
hybridization time and temperature	time courses: 2-24h tempeatures: 37-60°C	20h 45°C	best result signal intensity of sample compared to background	
Process: end flow analysis				2.3
normalization of the signal	average of all spot/ external control/ house keeping genes	house keeping genes	best suitable solution for a little array (less than 1000 spot)	
quantitation of the signal through the software	Fixed circle/ Histogram/ Adaptative	Fixed circle	best fit with a self spotted chip array	2.3 fig.38

2.1 Spotting

2.1.1 Oligonucleotide modification

The oligonucleotide modification was suggested using epoxy silane coated slides, to improve the binding of the oligonucleotides to the surface of the slides improving their stability during the many washes to which the slides are exposed.

In our case the amino modified oligonucleotides always gave a higher signal compared to the not amino modified oligonucleotides, not influencing the background signal.

2.1.2 Spotting buffer

The optimization of the spot buffer was a key issue for the establishment of the microarray technology, the quality the shape of the spot and the distribution of the oligonucleotides within the spot mark is dependent on this optimization. The viscosity of the DNA solution plays a central role during the spotting of the probes, and, for this reason different spot buffers were tested.

The effects of two different spotting buffers on the spot signal distribution are presented in fig. 26. The buffers were provided by the companies GeneScan and PeqLab. The first difference that was apparent from the comparison of these buffers was the magnitude of the signal, the GeneScan spotting buffer, on the left, gave a lower signal (the scale of the signal intensity was: blue, green, yellow and red, the saturation of the signal is indicated by the white) and the PeqLab spotting buffer was better distributed in all surfaces of the spot trace, on the right, gave a higher signal which were more concentrated in the middle of the spot. A preferential spot mark was considered the better distributed signal from GeneScan.

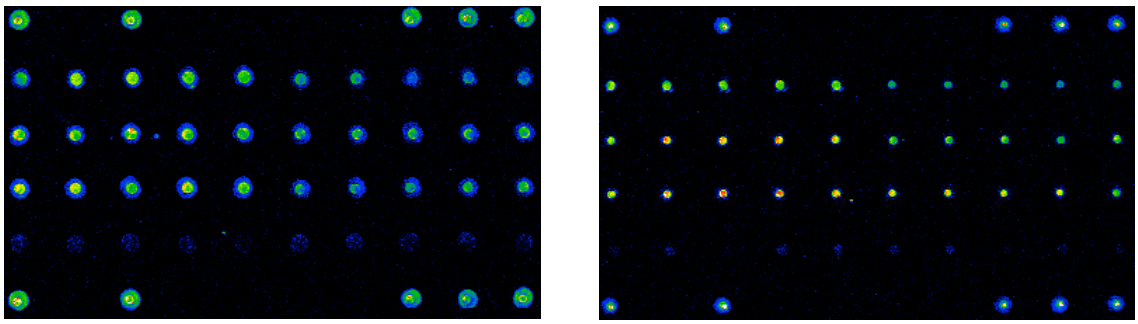

Fig. 26: The results of a scan of a slide of microarray spotted using two different spotting buffers are presented. The spotting was performed using two different spotting buffers, GeneScan on the left and PeQLab on the right, with the same pattern of oligonucleotides.

To optimize the distribution of the signal in the spot trace, the oligonucleotides corresponding to the gene 1A were spotted with different spotting buffers and different additions of substances to the GeneScan spotting buffer.

The results of this test are shown in fig. 27. The signal from the spot with GeneScan spotting buffer provided the second higher signal, which was also well distributed in the spot trace. The spotting buffer from GeneScan with the addition of betaine 1,5 M or DMSO 10 % (v/v) or 3X SSC/1.5 M betaine, gave undesirable results in all three cases, with a spot signal that was too concentrated in the center of the spot mark. The GeneScan spotting buffer containing additionally BSA (100 µg/ml) or salmon sperm DNA (2 µg/ml) provided superior results. Following BSA addition the spot was well distributed within the spot mark, unfortunately it

was possible to identify a signal also from the relative negative control the same buffer spotted without nucleotide. The signal that came from this negative control could be produced by linking of the labeled samples to the BSA protein or by the fluorescence of the protein itself. For gene expression analysis the negative control of GeneScan spotting buffer used with the addition of BSA (100µg/ml) was subtracted from the signal of oligonucleotides spotted with the same spotting buffer.

The results using salmon sperm DNA added to GeneScan spotting buffer delivered a signal that was higher than from the GeneScan spotting buffer alone, however in this case the negative control also exhibited a strong signal (data not shown) and for this reason, it was abandoned.

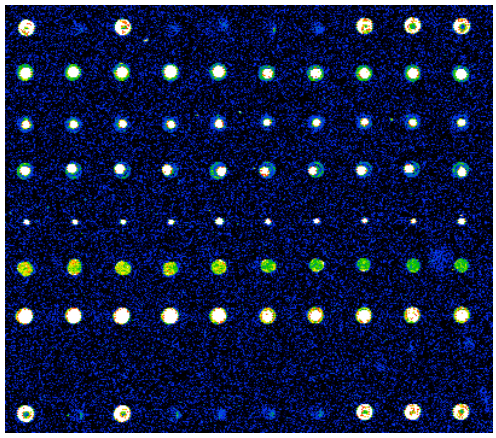

Fig. 27: Effects of different spotting buffers and compounds added to the GeneScan spotting buffer. Laser Power and PMT gain set to 100%. The distribution of the spotted samples was reported in table 2.

Tab. 2: Distribution of the spotted samples on the slide: 10 columns and 9 rows. The oligonucleotides are reported with the spot concentration and the spotting buffer used. Negative controls: GeneScan spotting buffer + one volume of HPLC water (GS), GeneScan spotting buffer supplemented with 100µg/ml of BSA + one volume of HPLC water (GS BSA), PoliA 0,1µM was spotted with GeneScan spotting buffer. The oligonucleotide 1A was spotted 5 times on one row.

	1	2	3	4	5	6	7	8	9	10
1	PoliA 0,1µM	GS BSA	PoliA 0,1µM	GS BSA	GS BSA	GS BSA	GS BSA	PoliA 0,1µM	PoliA 0,1µM	PoliA 0,1µM
2	1A 2µM in GeneScan spotting buffer					1A 1µM GeneScan spotting buffer				
3	1A 2µM in 3XSSC/1,5M Betaine					1A 1µM 3XSSC/1,5M Betaine				
4	1A 2µM in GS spotting buffer+Betaine 1,5M					1A 1µM GS spotting buffer+Betaine 1,5M				
5	1A 2µM in GS spotting buffer+DMSO 10%					1A 1µM GS spotting buffer+DMSO 10%				
6	1A 2µM in GS spotting buffe+ BSA 100µg/ml					1A 1µM GS spotting buffe+ BSA 100µg/ml				
7	1A 2µM in GS spotting buffer+ Salmon sperm DNA 2µg/ml					1A 1µM GS spotting buffer+ Salmon sperm DNA 2µg/ml				
8	GS	GS	GS	GS	GS	GS	GS	GS	GS	GS
9	PoliA 0,1µM	GS BSA	PoliA 0,1µM	GS BSA	GS BSA	GS BSA	GS BSA	PoliA 0,1µM	PoliA 0,1µM	PoliA 0,1µM

2.1.3 Oligonucleotides spot concentration

The oligonucleotide spot concentration was optimized for every gene. This allows for the acquisition of the slides with the higher intensity of Laser Power and PMT (photon multiplier tube) gain, allowing for detection of signals from low expressed targets, without reaching saturation in the case of highly expressed genes.

In fig. 28 the optimization of the 16 target genes was shown. In tab. 3 the optimized spotting concentration of the target oligonucleotides are summarized.

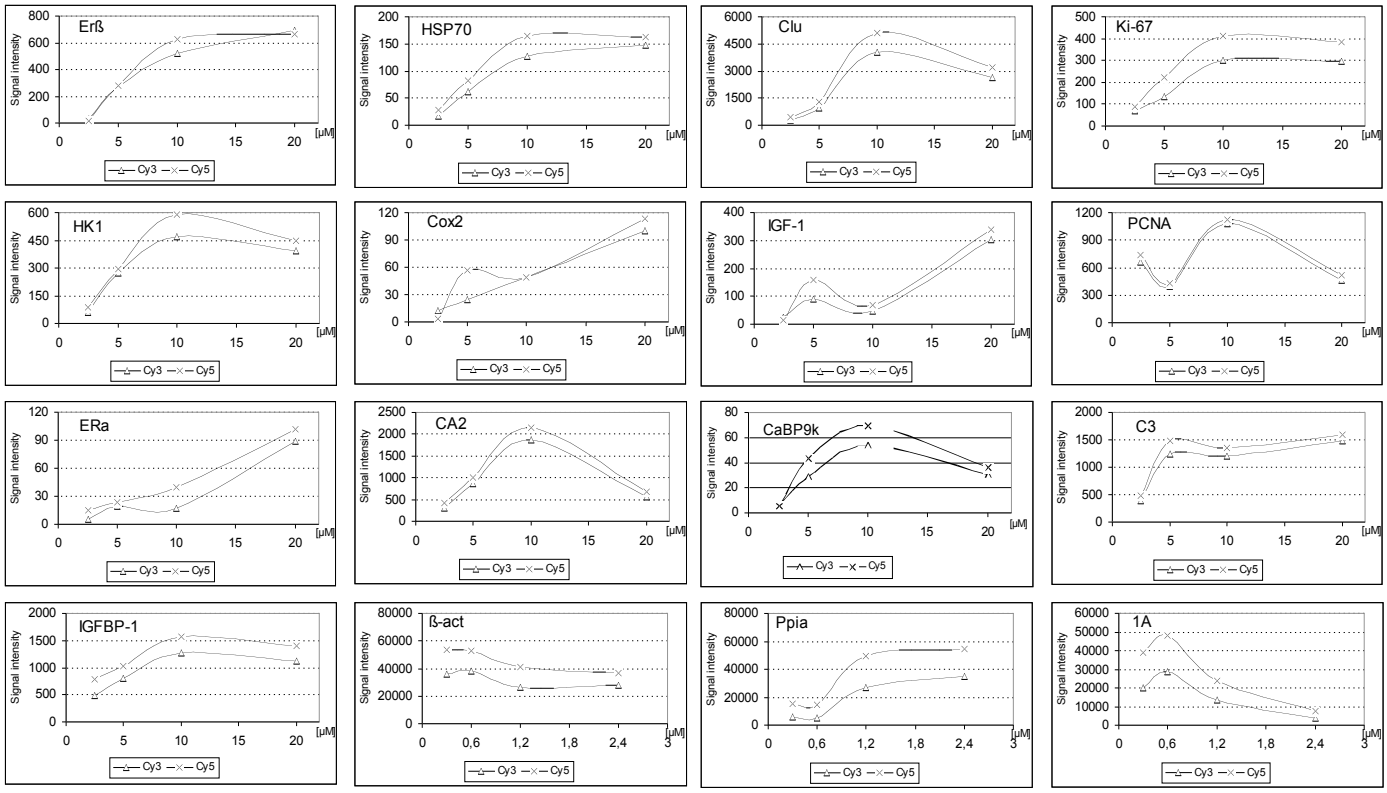

Fig. 28: Influence of quantity of spotted oligonucleotides on the signal intensity. Oligonucleotides reported: ERß, HSP70, Clu, Ki-67, HK1, Cox2, IGF1, PCNA, Era, CA2, IGFBP1, C3, CaBP9k, ß-act, Ppia, 1A. Signal intensity after signal background subtraction is shown on the Y axis, concentration of spot oligonucleotides (µM) is reported on the X axis. Labeling of the cDNA samples: dUTPCy3 and dUTPCy5.

Tab. 3: Determined spot concentrations of the target oligonucleotides.

Target oligonucleotide	Spot concentration	Target oligonucleotide	Spot concentration
ERß	15µM	ER$^{\alpha}$	15µM
HSP70	15µM	CA2	10µM
Clu	10µM	CaBP9k	15µM
Ki-67	10µM	C3	5µM
HK1	15µM	IGFBP-1	10µM
Cox2	20µM	ß-act	1µM
IGF-1	20µM	Ppia	1µM
PCNA	10µM	1A	1µM

2.1.4 Coupling (linkage) of the oligonucleotides on slide surfaces after the spotting

The effectiveness of the coupling reaction was crucial for the establishment of the microarray technology because of the many washing steps to which the slides are exposed. Two different methods were tested: coupling by UV irradiation for different length of time, and coupling in a humid, steam saturated warm box.

Across the slides coupled by different protocols the warm steam saturated box produced the superior result (Fig. 29). The lower right image, coupled by warm bath, presents the higher signal from the spots indicating a higher quantity of oligonucleotides present on the slide.

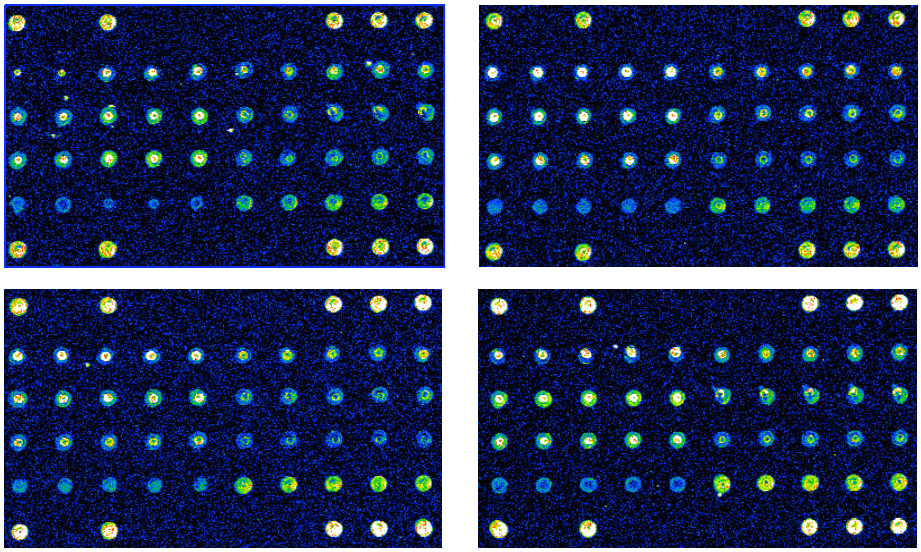

Fig. 29: Comparison of different coupling treatment. The oligonucleotides were coupled to the slides after the spot by irradiation of UV light with 254nm of wave length, for 1' (left upper panel), 10' (right upper panel) and 30' (left lower panel), or incubated in a saturated of steam chamber for 2 h at 50°C (right lower panel).

2.2 Hybridization

2.2.1 Hybridization buffer

The hybridization buffer is one of the key part of the microarray procedure, many factors are dependent on its quality: the quality of the hybridization between the target oligonucleotides spotted on the slides and the cDNA samples labeled by Cy-dye, the signal from the spots that was correlated to the quality of the hybridization and the signal background, which should be as weak as possible. Usually the producers supplied the slides together with the relative hybridization buffer, but after the bankruptcy of our slide supplier (GeneScan) and having a large amount of slides ready to use, I was required to substitute the hybridization buffer. For this reason the following experiments were necessary.

In fig. 30 are shown the results of three different hybridization buffers, from three different companies: GeneScan, Genetix and Amersham. The slides were evaluated with fitted setting of Laser Power and PMT gain to avoid saturation of the signal, and to have standard

experimental conditions. The image of the array hybridized using GeneScan hybridization buffer (fig. 30a) indicated the high quality of this buffer as there was a low background signal and the oligonucleotides gave a clear signal that could be easily evaluated. The image of the array hybridized using the Genetix hybridization buffer (fig. 30b), showed a strong background that in some cases was stronger than the signal from the oligonucleotides. For this reason they were not usable anymore. The last image (fig. 30c), produced using the Amersham hybridization buffer was the least satisfactory.

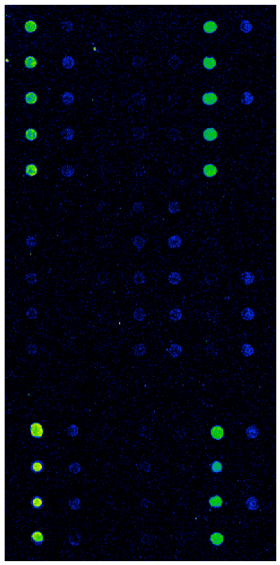

Fig. 30a

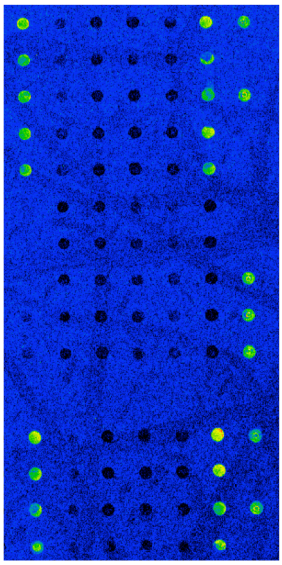

Fig. 30b

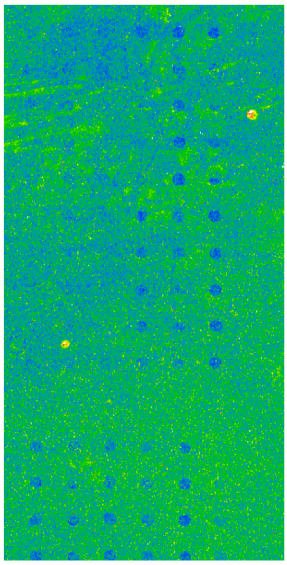

Fig. 30c

Fig. 30: Presentation of the results using three different hybridization buffers: fig. a shows the result of a slide hybridized using GeneScan hybridization buffer, fig. b shows the result of a slide hybridized using Genetix hybridization buffer and in fig. c shows the result of a slide hybridized using Amersham hybridization buffer. Cy3 channel is shown.

After the unsatisfactory results obtained from the Genetix and Amersham buffers, the hybridization buffer from PeqLab was tested.

Fig. 31 shows two arrays, spotted on the same slide to reduce any variation, hybridized with the two different hybridization buffers. The slide was scanned only once with fitted setting of Laser Power and PMT gain to avoid any saturation of the signal. The slide was treated according the GeneScan protocol (no pre-wash, and wash after hybridization as described for the Genescan hybridization buffer), unfortunately the choice to use the same slide had the disadvantage of restricting the hybridization procedure to just one. The choice was made to follow the GeneScan protocol to be sure to have a signal at the end of the hybridization. In this case the GeneScan hybridization buffer, on the left side, resulted as the better buffer. It produced less background and higher level of signal intensity from the oligonucleotides. The better result from the GeneScan hybridization buffer was understandable because the parallel

use of the GeneScan epoxy silane coated slides and the GeneScan hybridization protocol was followed. This is a good example that in the microarray technology the use of components derived from the same supplier is crucial. However, the results obtained from the PeqLab hybridization buffer were satisfying.

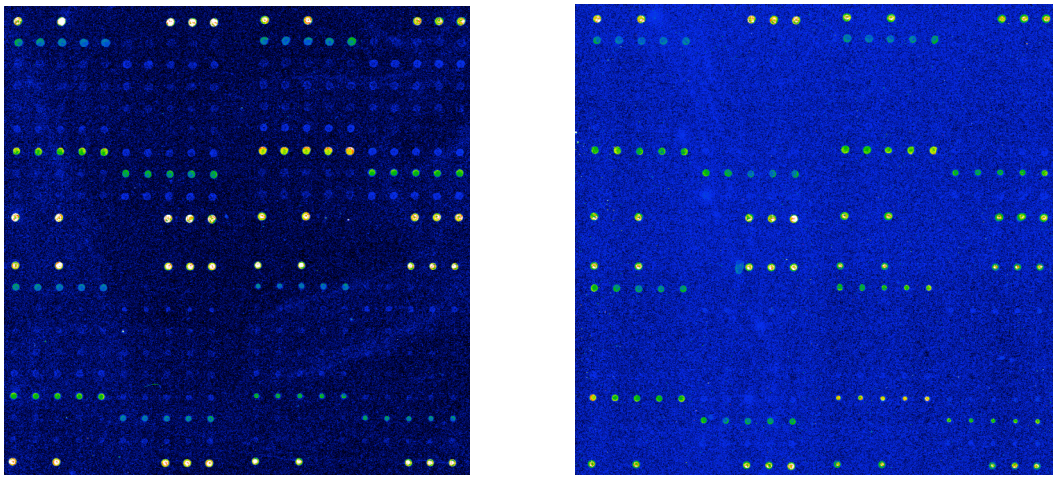

Fig. 31: Representation of the results using two different hybridization buffers. GeneScan on the left, and PeqLab on the right. Cy3 channel is shown.

2.2.2 Pre-hybridization treatment of the slides

The PeqLab hybridization protocol suggested, performing a pre-hybridization treatment of the slides (Material and methods 4.2.2). To control the effectiveness of this pre-treatment with our slides, produced by another company (GeneScan), the pre-hybridization procedure starting from three different points of the protocol was tested: the complete pre-hybridization treatment, a pre-hybridization treatment starting from the step of incubation in the Nexterion QMT blocking solution for 15' at 50°C and no pre-hybridization treatment.

The results are shown in fig. 32, note that not all nucleotides could be quantitatively evaluated. In tab. 4 an overview of the oligonucleotides according to the ability to analyze their signal is shown. After background subtraction the signal of some nucleotide resulted as negative values.

It was possible to determine that the complete pre-hybridization protocol produced always a more stable result. It delivered a small standard deviation, also when in some cases where the signal intensity was smaller than the one produced by a pre-hybridization starting at the blocking step.

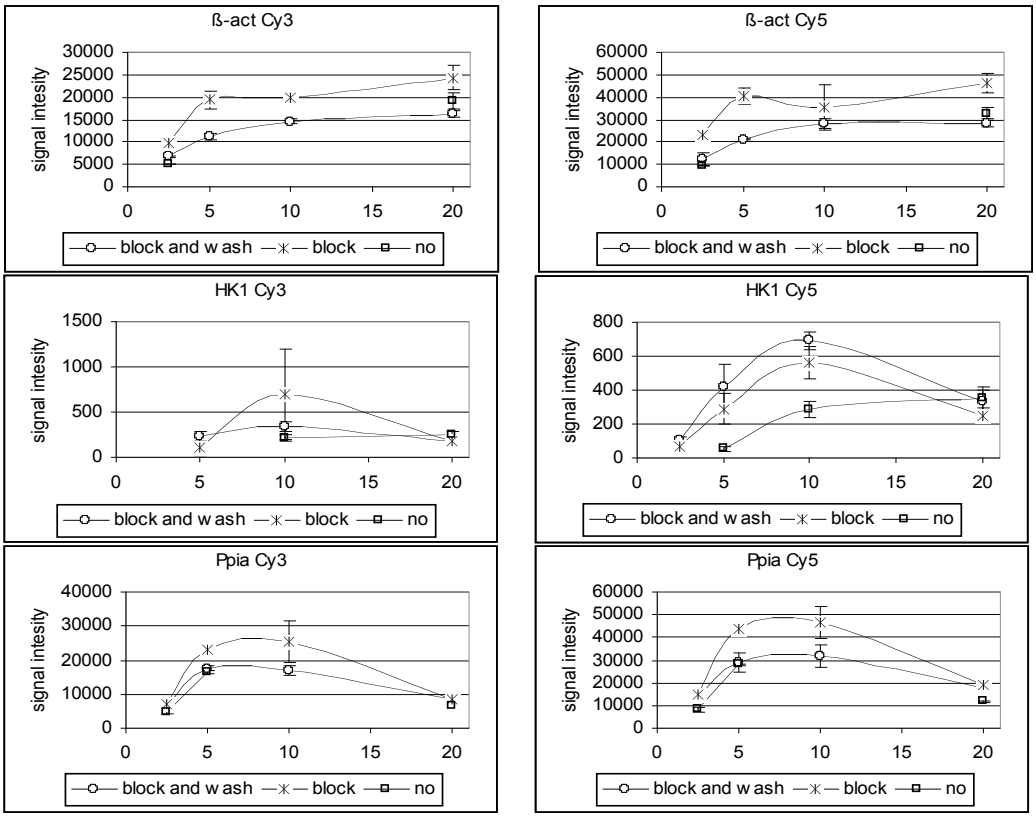

Fig. 32: Comparison of the effects of pre-hybridization treatment of the slides performed according the PeqLab protocol and started at different points: complete pre-hybridization protocol (block and wash), a pre-hybridization treatment starting from the Nexterion QMT blocking solution for 15' at 50°C (block) and no pre-hybridization (no). The housekeeping genes ß-act, HK1 and Ppia were tested. The signal intensity (after background subtraction) is reported on the Y axis, the spot concentration (µM) is reported on the X axis.

Tab. 4: Overview of signals which could be evaluated, after background subtraction, according to pre-hybridization treatment, oligonucleotide spot concentration and gene.

Oligonulceotide/	Block and wash				Block				no treatment			
Gene	20	10	5	2,5	20	10	5	2,5	20	10	5	2,5
ß-act	I	I	I	I	I	I	I	I	I	0	0	I
HK1	I	I	I	0	I	I	I	0	I	I	0	0
Ppia	I	I	I	I	I	I	I	I	I	0	I	I
	I quantitation				0 no quantitation possible							

2.2.3 RNA quantity hybridized to the array

Another point to optimize in the establishment procedure of the microarray technology was the quantity of RNA used in the reverse transcription and labeling of the samples, which reflectes the quantity of sample hybridized to each array. In fig. 33 the influence of the quantity of RNA hybridized per array is shown. Two quantities were tested, 6,25 µg RNA per array (reported as low) and 12,5 µg RNA per array (high).

For this test the housekeeping genes 1A, Ppia, HK1 and ß-act were used. It was possible to show that the intensity of the signal, after background subtraction, using more RNA was always higher for each oligonucleotide than using less RNA. It has to be noted that the background increased when using more RNA, however it was compensated by an increase in the absolute signal. It is remarkable that the oligonucleotides spotted at the concentration of 20 µM delivered a intensity of signal generally lower than the signal intensity delivered by the probes spotted at the concentration of 10µM.

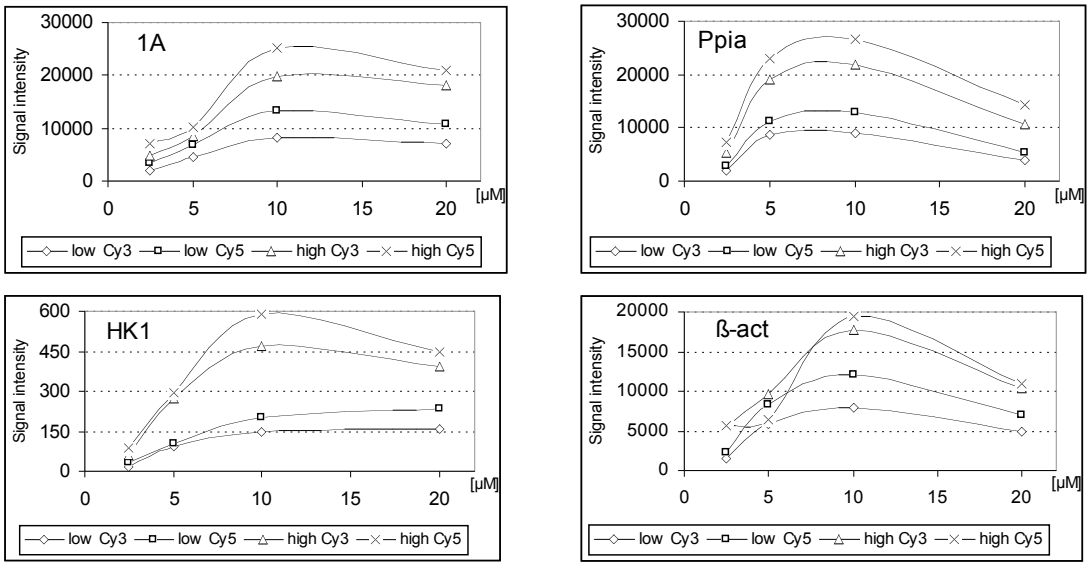

Fig. 33: Influence of quantity of RNA on the signal intensity of the oligonucleotides. The housekeeping genes 1A, Ppia, HK1 and ß-act were used. Low: 6,25µg RNA/array, high: 12,5µg RNA/array. Signal intensity (background subtracted) reported on the Y axis, concentration of spot oligonucleotides (µM) on the X axis. Both channel are shown (Cy3 and Cy5)

2.2.4 Direct/indirect labeling of the samples

The labeling of the cDNA samples during the reverse transcription of RNA presented the possibility to choose between two protocols: direct labeling (one-step labeling) and indirect labeling (two-steps labeling) as described in the Introduction 5.1.2 and the Materials and methods 4.2.1. Both options were tested.

In fig. 34 images produced by two slides hybridized using the different protocols are shown. The slides were scanned with Laser Power and PMT gain set to 100 % to better visualize the effects of the two different protocols. Comparing the left image (direct labeling) with the right (indirect labeling), it is clear that the general fluorescence (background) image was lower in the left image. The negative signal stemming from some spots shown by the indirect labeling, those were darker than background (the overall signal resulted negative because the background was brighter than the spot). Moreover, in the right image, it is clearly possible to

identify the zone where the lifterslip (the cover set on the spotted array area where the hybridization solution was pipetted) was posed. In the lower part of the image the is a clear border between a green region (lifterslip) and a blue on (not hybridized zone). The left image presents good background intensity although the scan set was so high that some oligonucleotides reached the signal saturation (white spots), due to the scan performed with setting of Laser Power and PMT gain to 100%.

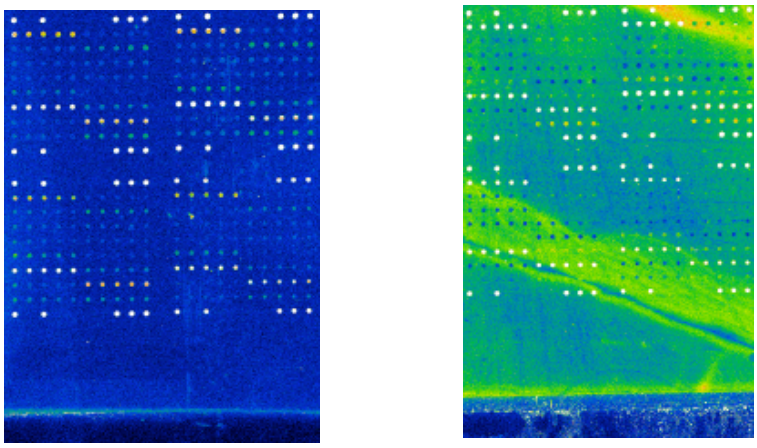

Fig. 34: Comparison of direct to indirect labeling of cDNA samples. Slide hybridized by cDNA labeled by direct protocol (left image) and slide hybridized by cDNA labeled by indirect protocol (right image). Slides were scanned with Laser Power and PMT gain set to 100%. The Cy3 channel is shown

2.2.5 Cy-dye ratio in the labeling reaction

For gene expression analysis an extra step is the normalization of the values. As Cy5 dye gave always higher signal intensity in comparison to Cy3 dye. The Cy-dye labels were also one of the most expensive reagents. To try to reduce as far as possible the different of signal intensities using the two Cy-dyes, and their consumption, three different quantities and ratios of the Cy-dyes were tested: 3µl (from the stock solution reported in Material and Methods) of both dUTP-Cy3 and dUTP-Cy5 (pro RNA reverse transcription and labeling), 3 µl of dUTP-Cy3 and 1 µl of dUTP-Cy5, and 1µl of both dUTP-Cy3 and dUTP-Cy5. The resulting ratio of Cy5/Cy3 was calculated after the subtraction of background signal.

The results of three different experiments are shown in fig. 35. The images of the slides were obtained with a fitted setting of Laser Power and PMT gain. A good signal ratio of Cy5/Cy3 was considered to be close to one. The housekeeping genes 1A and Ppia were investigated spotted at a concentration of 1 or 2µM.

The ratio of the signal intensity Cy5/Cy3 was generally close to one, independent of the quantity of Cy-dye used and for all three housekeeping genes. The least expensive but valid solution appeared to be the use of Cy3:Cy5 at the ratio of 3:1.

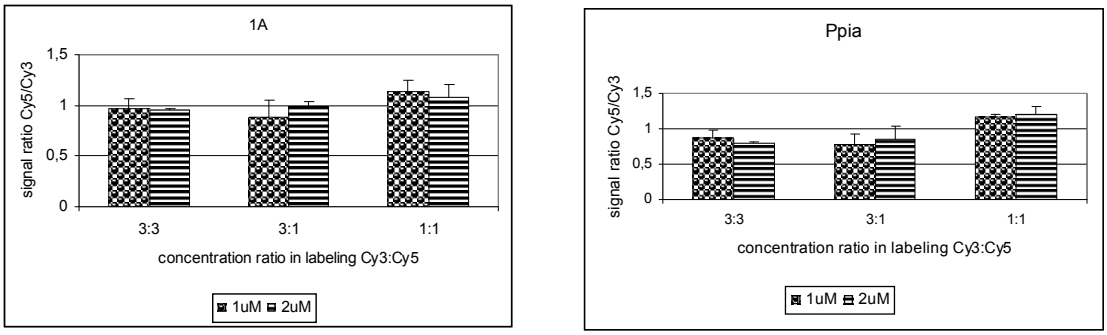

Fig. 35: Signal ratio Cy5/Cy3, according to different ratio of dUTPCy3 and dUTPCy5 dye (Cy3 and Cy5) used in the cDNA synthesis and labeling. The signal ratio Cy5/Cy3 (after background subtraction) of different oligonucleotides (1A and Ppia) is shown on the Y axis, according to different concentration of spotted oligonucleotides (1-2 µM). The slides were scanned with fitted setting of Laser Power and PMT gain. An average over three experiments is shown.

2.2.6 Hybridization chamber

To hybridize the slide a warm humid chamber was needed. During the establishment procedure of the microarray technology three different hybridization chambers were tested: Flat block hybridization chamber, ArrayIt Hybridization Cassette hybridization chamber and a self made steam saturated box (described in the Materials and methods, 4.2.3), the results are shown in fig. 36.

For the Flat block (on the left), the image shown was really good, the signal from the oligonucleotides was sharp and no shadows around the spot field were presents along with a low background signal. The low background signal was also visible comparing to the lower side of the image. There it is possible to recognize the mark of the lifterslip the green line. There is no difference in fluorescence outside or inside the lifterslip zone.

Moving attention to the other images, a clear reduction of quality is seen. The middle image (ArrayIt Hybridization Cassette hybridization chamber) presents a lower quality compared to the left image, the right image (self made hybridization chamber) is of the lower quality

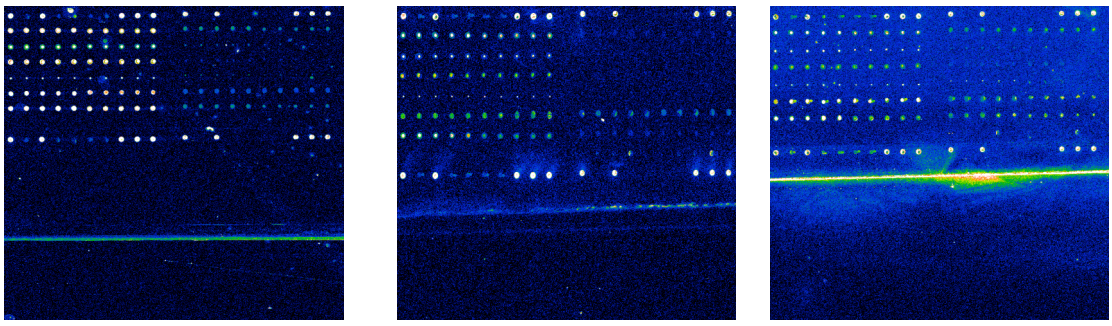

Fig. 36: Influence of different hybridization chambers. Flat block hybridization chamber (left image), ArrayIt Hybridization Cassette hybridization chamber (middle image) and a self made steam saturated box (right image). The hybridization was conducted for 16h at 45°C for all hybridization chambers. The slides were scanned with Laser Power and PMT gain set to 100%. Channel Cy3 is shown.

2.3 End flow analysis

After the scan of the slides, performed using a scanner that can measure fluorescence emitted by the labeling compound, in this case Cy3 and Cy5 dyes, the images had to be evaluated using software that recognizes the signal stemming from the samples against the general signal (background), and is able to translate it into a numeric value.

The software provided with the scanner, Quantarray presented the possibility to choose from three different options for the evaluation of the signal coming from the spot field, usually a round circle, and from the background, the not spotted area, exemplified in fig. 37.

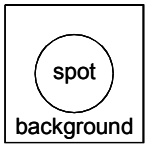

Fig. 37: exemplification of spot/background zone.

The histogram option decomposed the spotted area in pixels and measures the signal of each, without distinction between spot and background signal. It considered as signals value all pixels with a value higher than a threshold, and as background all signal below, independent of the position of the pixel, inside or outside the spot mark. The fixed circle method uses a mask that was produced according to two variable parameters: the diameter of the spot zone and the diameter of the background zone. It considered all pixels inside in the spot zone to be signal, and all pixels in the background zone to be background. The adaptive method uses the same mask of the fixed circle but adapted the diameter of the spot field to each spot. In fig. 38 the results derived from the three methods for the calculation of the signal are compared.

It was possible to note that the adaptive and the fixed circle methods gave similar values of intensity, in contrast to the histogram method which reported lower signal intensity. Between the adaptive and the fixed circle methods, the fixed circle was considered more suitable to this study for its stability independently from the spot quality.

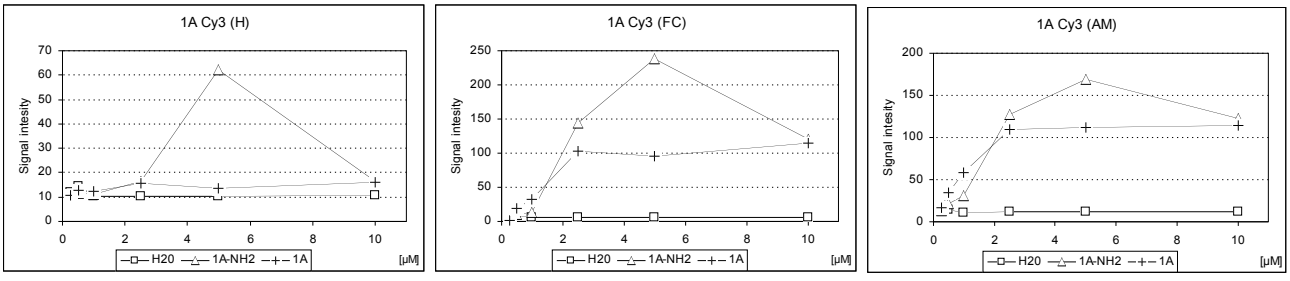

Fig. 38: The influence of different analytic options of the software Quantarray on the resulting signal intensity is reported ion the Y axis. Concentration of the spotted oligonucleotides (0,25-0,5-1-2,5-5-10 µM) are shown in the X axis. Samples: negative control (H2O), 1A amino modified at the 5'end (1A-NH2) and unmodified 1A (1A). Analytical methods: adaptive method (AM) (upper left diagram), fixed circle (FC) (upper right diagram) and histogram (H) (lower diagram).

2.4 Final conditions for the microarray experiment

After the microarray technology was optimized, the decision on materials, methods and tools employed for the gene expression analysis are reported in tab. 5

Tab. 5: List of the materials, methods and tools used in the microarray gene expression analysis.

step	tool
oligonucleotide modifiaction	aminomodification at the 5'end
oligonucleotide spot concentration	see table 1
spotting buffer	GeneScan spotting buffer for all nucleotides, GeneScan spotting buffer + BSA 100µg/ml for ß-act, 1A, Ppia
coupling reaction	steam saturated box at 50°C for 2h
hybridization buffer	PeqLab hybridization buffer
pre-hybridization of the slides	PeqLab pre-hybridization of the slides
RNA quantity pro array/hybridization	12,5µg RNA
labeling	direct labeling
dye quantity/ratio	3µl dUTPCy3/1µldUTPCy5
hybridization chamber	Flat block hybridization chamber
hybridization duration	20h
hybridization temperature	45°C
evaluation method	fixed circle

The image shown in fig. 39 represents one example of the final result after the establishment of the microarray method. The background was low and the signal from the oligonucleotides spots is sharp.

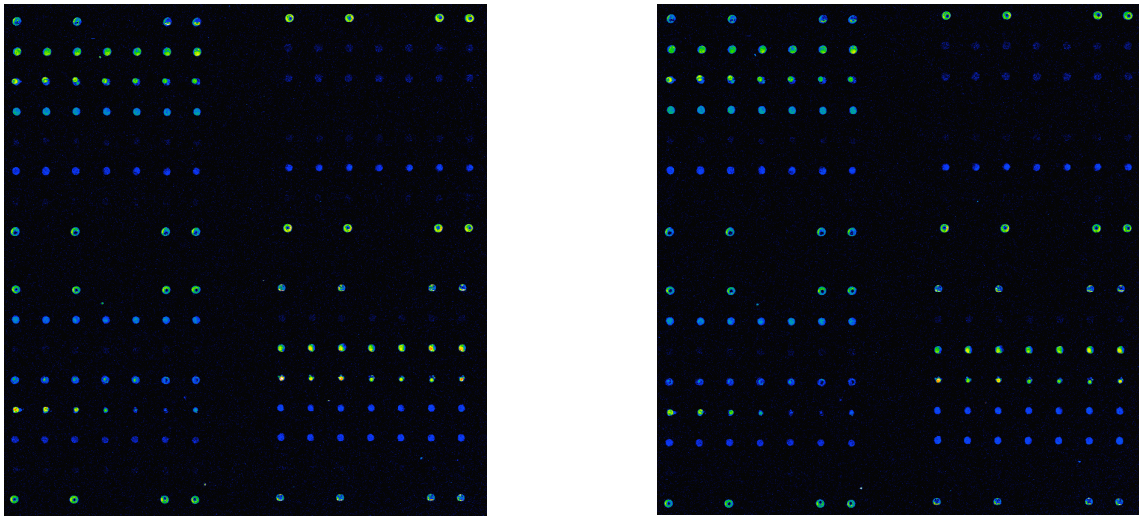

Fig. 39: Resulting images after the optimization process of the microarray (particular, central array), the parameters used for spot, hybridization and calculation of the slides are reported in table 5, in the left image the Cy3 fluorescence was acquired with Laser Power set to 75 and PMT gain to 80, in the right image the Cy5 fluorescence was acquired with Laser Power set to 57 and PMT gain to 80.

3. Gene expression analysis using the Microarray

At the end of the process for the establishment of the microarray method in our laboratory, this method was tested for its capacity in the analysis of the gene expression, compared to a more conventional procedure. To achieve this task the same RNAs extracted from the Fe33 cell line treated by EE and NP were analyzed by the real time RT-PCR and by microarray (the gene expression analysis using the real time RT-PCR was conducted only for four genes reported above in this chapter: IGFBP1, CaBP9k, PCNA and CAII, and already shown in point 1.2 of this chapter). In this case the real time RT-PCR served as reference method, to verify the results of the gene expression derived from analysis made using the microarray.

The RNAs extracted from Fe33 cells were analyzed just once. Each slide was spotted with three array areas, and scanned twice, once avoiding any saturation of signal (standard setting) and a second time at a higher intensity of scanning, to get a signal from every spotted oligonucleotide. In this second run some oligonucleotides reached saturation of the signal and they were not suitable for the gene expression analysis and were discarded. The final result were six value groups per gene: three from the three arrays acquired according the first set of parameters, and three from the three arrays acquired with the second setting of parameters. Some arrays on the slides could not be evaluated.

The hybridization and scanning procedure described is exemplified in fig. 40. The control sample was labeled by dUTP-Cy3 and the treated sample by dUTP-Cy5, no array swap was performed.

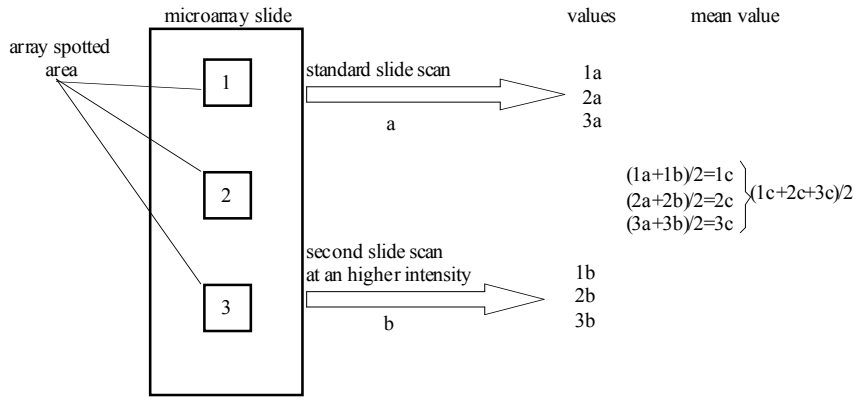

Fig. 40: Description of the procedure for the calculation of the mean value using the microarray for the gene expression analysis. Array spotted area: area where the oligonucleotides probes are affixed; “1”, “2” and “3”: the three array spotted areas and the relative deriving value; “a” and “b”: the value after the scan of the relative spotted area; “1c” “2c”, “3c”: the mean value calculated using the two values which stem from the same array area.

The first microarray DNA chip containing 16 genes (Introduction 3.3) was designed for the investigation of the estrogenic effect of different substances in different tissues of the organism. To establish the method of the microarray in our laboratory the liver cell line Fe33 was chosen. The gene expression reported in this part of the work concern only the gene that are responsible to exposure to estrogens only in this cell line, and they are reported in tab. 6. Some of those genes were shown by the analysis performed with the microarray to not be regulated in the cell line Fe33, even when they had already been shown to be estrogen responsive in this cell line. This result can be explained by the lower sensitivity of this method of gene expression analysis compared to other already proved tools for gene expression investigation present in our laboratory, eg. real time RT-PCR. In the case where the expression of some genes was not affected by estrogen exposure no diagram was reported.

Tab. 6: Estrogen responsiveness of different genes representing 3 main gene groups after treatment with estrogenic substances (EE and NP) in the gene expression analysis by microarray. E2 served as positive control.

Gene class	Gene	Regulated by treatment		
		EE	NP	E2
Estrogen Receptors	Estrogen Receptor α	No	No	No
	Estrogen Receptor ß	No	Yes	No
Cell cycle progression	Proliferating Cell Nuclear Antigen	No	No	No
	Ki-67	No	No	No
Liver	Insulin-like Growth Factor 1	No	No	No
	IGF binding protein 1	Yes	No	Yes
	Calcium Binding Protein 9 k Dalton	Yes	No	Yes
	Carbonic Anhydrase II	Yes	No	Yes

3.1 Effects of 17α-Ethinylestradiol on gene expression in Fe33cell line in a time and dose dependent manner

The effects of administration of EE on the gene expression of IGFBP1 are reported in fig. 41. After 7 h of treatment the relative gene expression of IGFBP1 was stimulated to levels of more then 250 % by E2, EE 10^{-6} and 10^{-7}M. After 24 h only EE 10^{-6}M reached an up regulation to more then three times compared to the control level.

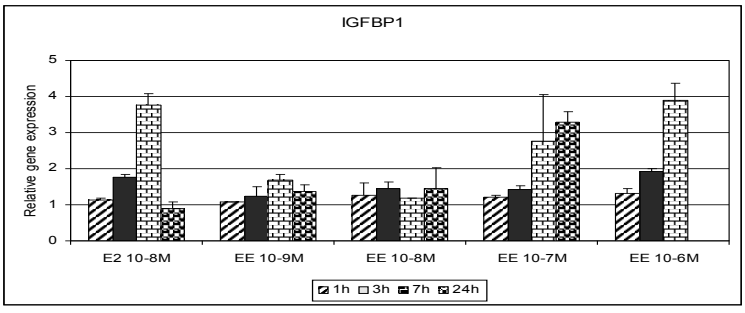

Fig. 41: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of IGFBP1. Cells were treated with EtOH (control), E2 and EE in a dose dependent manner for 1, 3, 7 and 24 h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

The effects of the administration of EE on the gene expression of CaBP9k are reported in fig. 42. EE at the dose of 10^{-6}M induced a clear up regulation after 7 h of treatment. EE at the concentration of 10^{-7}M stimulated CaBP9k expression after 3h with a higher effect after 24 h. EE 10^{-8} and 10^{-9}M induced a doubling of gene expression level of CaBP9k after 3 and 24 h of treatment.

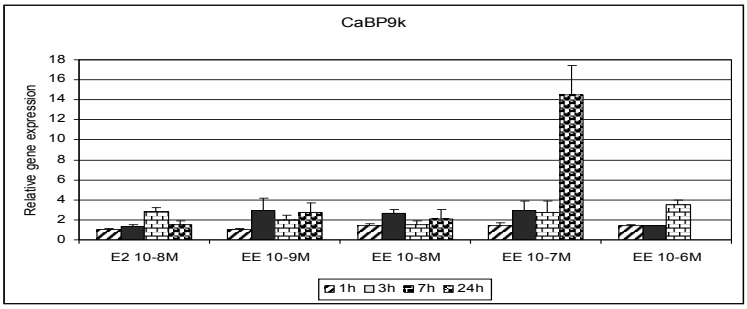

Fig. 42: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CaBP9k. Cells were treated with EtOH (control), E2 and EE in a dose dependent manner for 1, 3, 7 and 24 h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

The effects of the administration of EE on gene expression of CAII are shown in fig. 43. The treatment with EE for 1 h induced a doubling of the relative gene expression only at the concentration of 10^{-6}M. After 3 h EE at every dose, except for the concentration of 10^{-6}M, triggered a stimulation of the gene expression reaching at least 250 % of the control level. After 7 h of treatment, only EE at the concentration of 10^{-6} and 10^{-7}M stimulated clearly CAII expression. At 24 h of treatment only EE 10^{-6}M reached more than double of relative gene expression.

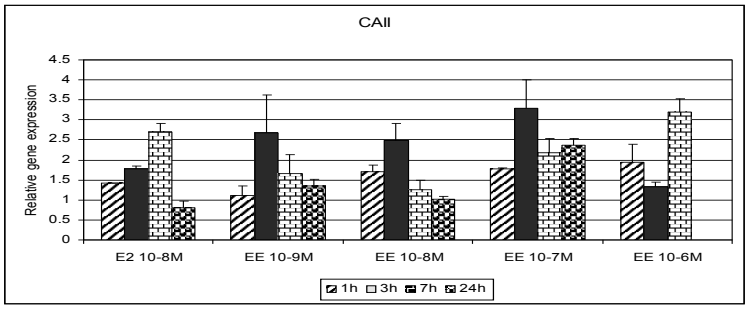

Fig. 43: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CAII. Cells were treated with EtOH (control), E2 and EE in a dose dependent manner for 1, 3, 7 and 24 h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

3.2 Effects of Nonylphenol on gene expression in Fe33cell line in a time and dose dependent manner

The effects of the administration of NP on the gene expression of ERß are reported in fig. 44. NP induced after 3 h of treatment a clear stimulation of the relative gene expression of ERß, at a concentration of 10^{-5} and 10^{-6}M reaching a doubling of relative gene expression. For the other doses and time points of treatment with NP no effects were detectable.

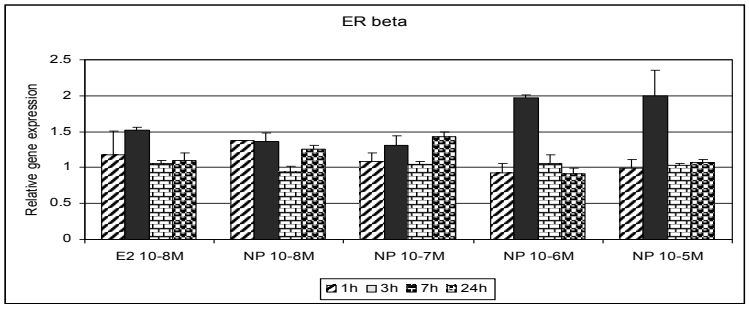

Fig. 44: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of ER beta. Cells were treated with EtOH (control), E2 and NP in a dose dependent manner for 1, 3, 7 and 24h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

The effects of administration of NP on the gene expression of IGFBP1 are reported in fig. 45. The administration of NP to Fe33 cells did not trigger any remarkable effect. E2 stimulated only after 7 h of treatment IGFBP1 gene expression (similar to what shown in fig. 41).

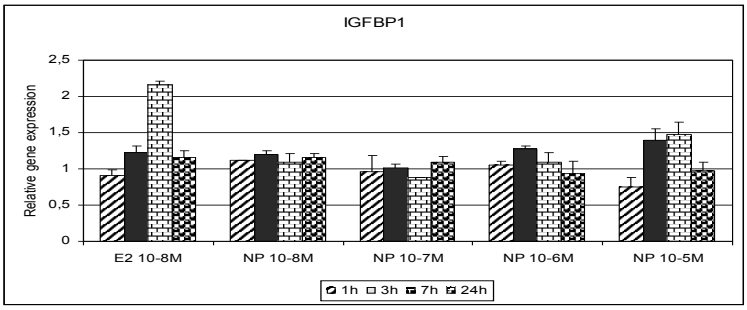

Fig. 45: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of IGFBP1. Cells were treated with EtOH (control), E2 and NP in a dose dependent manner for 1, 3, 7 and 24h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

The effects of administration of NP on gene expression of CaBP9k are reported in fig. 46. The administration of NP to Fe33 cells did not trigger any remarkable effect on the CaBP9k gene expression. E2 induced an up regulation of the relative gene expression to more than 350 % after 7 h of treatment (the same was already shown in fig. 42).

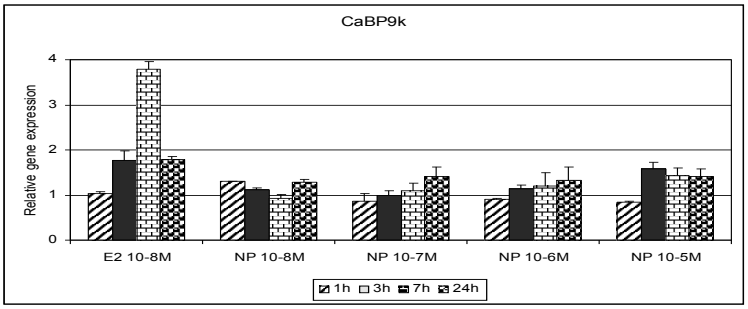

Fig. 46: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CaBP9k. Cells were treated with EtOH (control), E2 and NP in a dose dependent manner for 1, 3, 7 and 24 h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

The effects of administration of NP on gene expression of CAII are reported in fig. 47. The treatment with NP did not affect gene expression of CAII. Only E2 after 7h of treatment stimulated the expression of CAII (as reported in fig. 43).

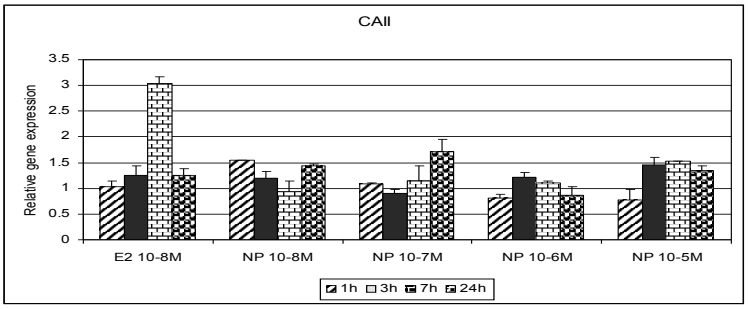

Fig. 47: Gene expression analysis by oligonucleotides microarray, correlated to control (set to one), in Fe33 (rat hepatic cell line) of CAII. Cells were treated with EtOH (control), E2 and NP in a dose dependent manner for 1, 3, 7 and 24 h. Dye labeling: control sample by dUTPCy3, treated sample by dUTPCy5. This experiment was conducted just once, the significance could not be calculated.

3.3 Validation of the gene expression measured by microarray

To verify the results produced by gene expression analysis using the microarray, real time RT-PCR was used as reference method. To have direct comparison identical RNA materials were analyzed by the two methods. Both results were already shown in this chapter, here a direct comparison was performed.

3.3.1 Validation of the gene expression analysis performed by microarray with real time RT-PCR in Fe33 cell line treated with 17α-Ethinylestradiol in a time and dose dependent manner

IGFBP1(fig. 48): IGFBP1 was clearly up regulated by estrogens in the Fe33 cell line and it was possible to demonstrate this using both methods: real time RT-PCR and microarray. Through this it is possible to recognize a trend in the gene regulation. The magnitude of the relative gene expression level differed considerably, the real time RT-PCR showed changes in the gene expression levels that were ten times higher than the changes detected by the microarray. It was hard to find a stable correlation factor for gene expression between the two methods.

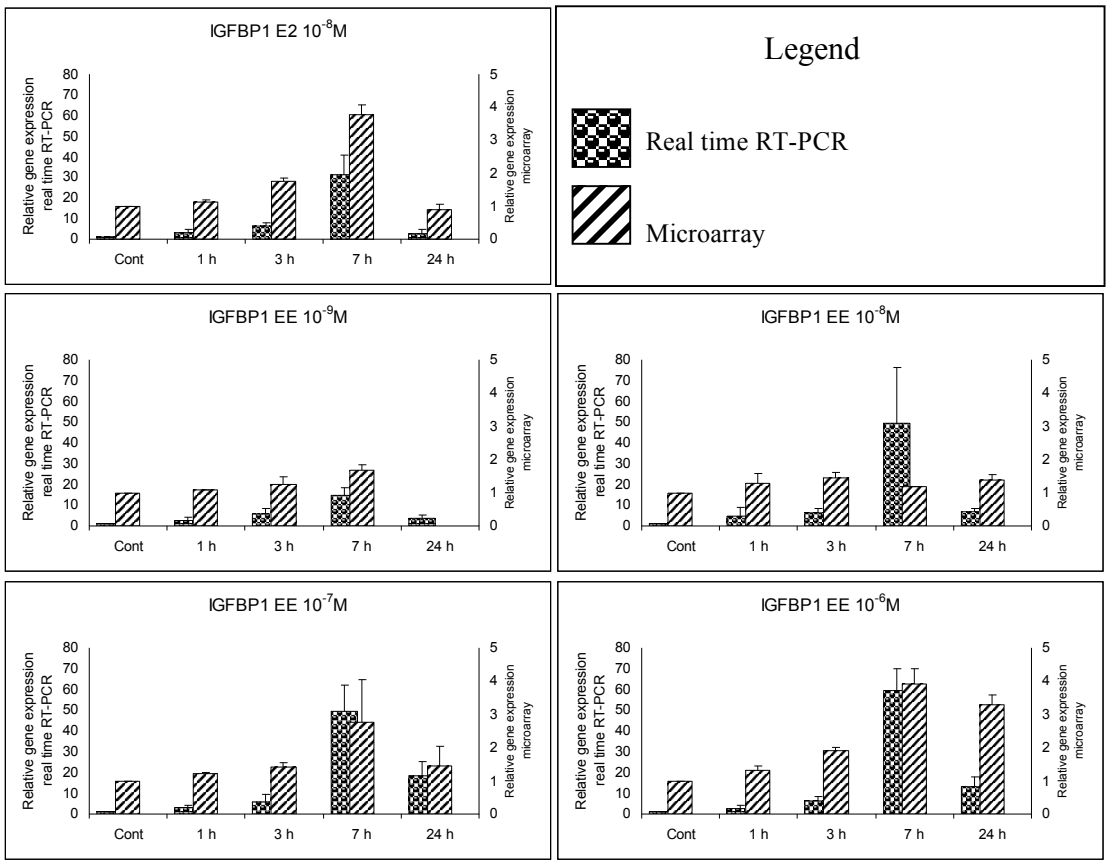

Fig. 48: Comparison of the gene expression pattern of IGFBP-1 using microarray technology and real time RT-PCR. Real time RT-PCR in the left side Y axis (black points), and microarray in the right side Y axis (obliques lines). IGFBP1 gene expression was analyzed in Fe33 cells (rat hepatic cell line). Cells were treated with EtOH (Contr), E2 and EE in a time and doses dependent manner. Dye labeling for the microarray method: control sample dUTPCy3, treated sample dUTPCy5. Calculation of significance was not possible, this experiment was conducted just once.

CaBP9k (fig. 49): CaBP9k gave a strong response to estrogens in Fe33 cells. It showed as in the case of IGFBP1, a clear stimulation of gene expression using both methods. The graph stemming from treatment with EE 10^{-6}M is a good example, the two columns indicating the two different methods overlap almost perfectly. Also treatment with E2 and EE 10^{-8}M are good example of the similarity of the trend of gene regulation detectable by both methods.

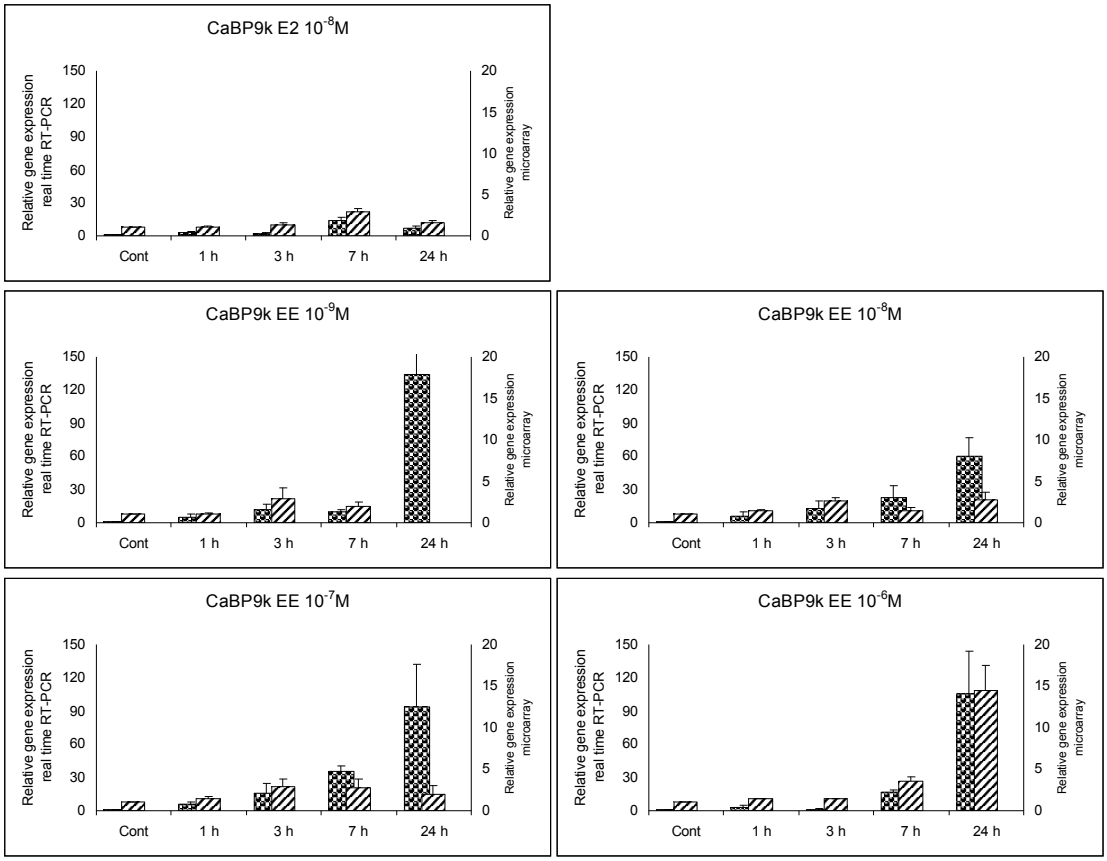

Fig. 49: Comparison of the gene expression pattern of CaBP9k using microarray technology and real time RT-PCR. Real time RT-PCR in the left side Y axis (black points), and microarray in the right side Y axis (oblique lines). CaBP9k gene expression was analyzed in Fe33 (rat hepatic cell line). Cells were treated with EtOH (Contr), E2 and EE in a time and doses dependent manner. Dye labeling for the microarray method: control sample dUTPCy3, treated sample dUTPCy5. Calculation of significance was not possible, this experiment was conducted just once.

PCNA: PCNA did not show a strong sensitivity to the estrogens in Fe33 cells. The microarray did not report any regulation of PCNA to estrogens exposure (data not shown). No comparison between the methods was possible.

CAII (fig. 50): The intensity of the response of CAII to the estrogens treatment was quite strong. A good example of conserved trend was the treatment with E2 10^{-8}M or EE 10^{-9}M. For the other cases only the direction of the gene regulation showed similarity.

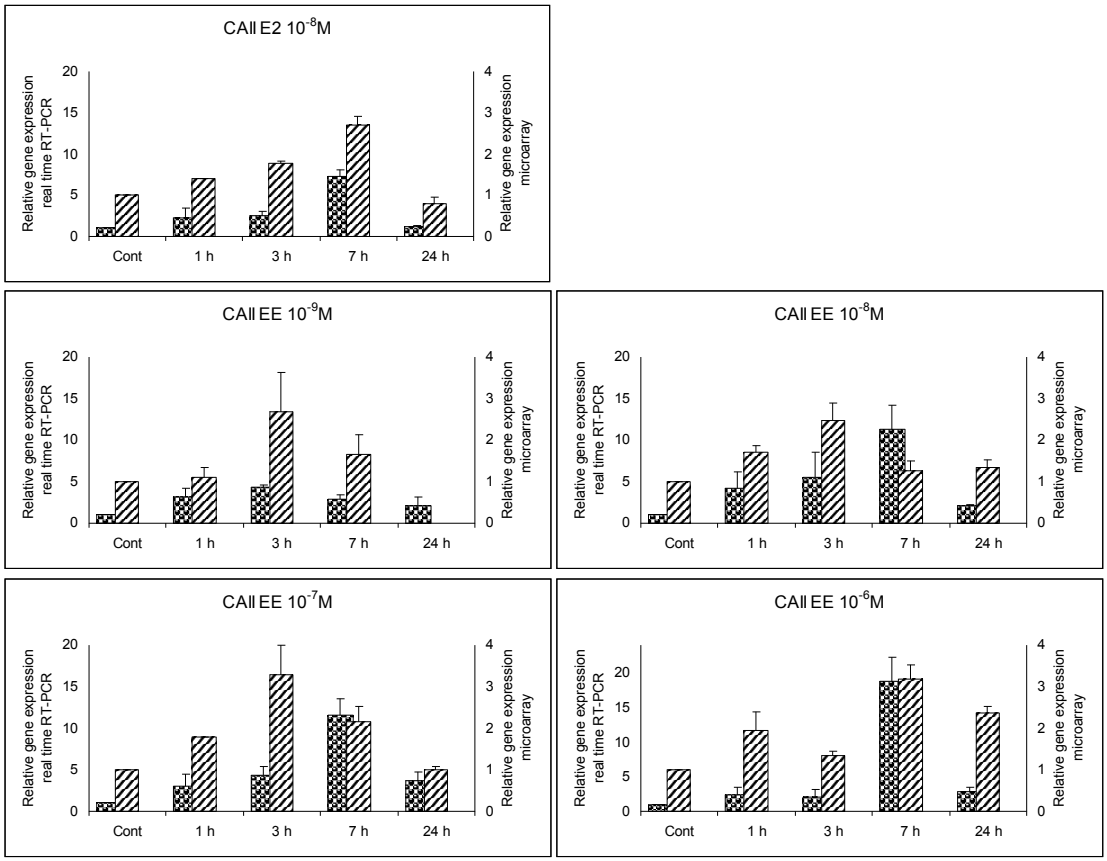

Fig. 50: Comparison of the gene expression pattern of CAII using microarray technology and real time RT-PCR. Real time RT-PCR in the left side Y axis (black points), and microarray in the right side Y axis (oblique lines). CAII gene expression was analyzed in Fe33 cells (rat hepatic cell line). Cells were treated with EtOH (Contr), E2 and EE in a time and doses dependent manner. Dye labeling for the microarray method: control sample dUTPCy3, treated sample dUTPCy5. Calculation of significance was not possible, this experiment was conducted just once.

3.3.2 Validation of the gene expression analysis performed by microarray with real time RT-PCR, in Fe33 cell line treated with Nonylphenol in a time and dose dependent manner

The microarray showed a regulation of the genes IGFBP1, CaBP9k and CAII only in treatment with E2 10^{-8}M for 7 h (data not shown) reporting a stimulation that is roughly the half on that reported by real time RT-PCR. No regulation was shown for other time point and the NP administration, for this reason no comparison was possible between the results on gene expression of real time RT-PCR and microarray.

3.3.3 Final consideration on the gene expression correlation factor

According to these results it was not possible to find a stable correlation factor for gene expression detected by the two methods used. The largest difference between the two tools for

the gene expression analysis appeared to be sensitivity, both methods delivered comparable results however, with different orders of magnitude, the trends were similar.

3.4 Dye-swap

The dye swap is an experiment that was intended to change, to swap, the label dye of the sample. It consists of two experiments. In the first labeling, during the reverse transcription of RNA and the labeling reaction, the control sample was labeled with a dye, in this case Cy3, and the treated sample with the other dye, Cy5. The experiment was repeated a second time with the same kind of RNA but labeling the samples in the opposite way, control sample labeled with Cy5 and the treated sample with Cy3. The aim of this experiment was to test if the result of the analysis of gene expression is independent of the labeling reaction of the samples. The experiment can be considered successful when both experiments produce the same result in gene expression. This experiment was conducted only once, and therefore it was not possible to calculate any significance.

The experiment reported here was only part of the full series of experiments performed during the thesis. In this case it was used the RNA extracted from Fe33 treated by EE 10^{-7}M for 1, 3, 7 and 24 h. In the first experiment the RNA was labeled with "standard": the control sample treated by EtOH was labeled by Cy3 and the treated sample, EE 10^{-7}M, by Cy5. In the swap experiment the labeling was inverted, indicated by "swap". They are reported only for two examples. An identity of the result independent of the choice of labeling ("standard" or "swap") was found.

In fig. 51 the gene expression analysis of the gene CAII and IGFBP1 treated with EE 10^{-7}M for 1, 3, 7 and 24 h is shown. In both cases the result produced in the "standard" and the "swap" labeling were similar. Therefore the control experiment could be considered successful.

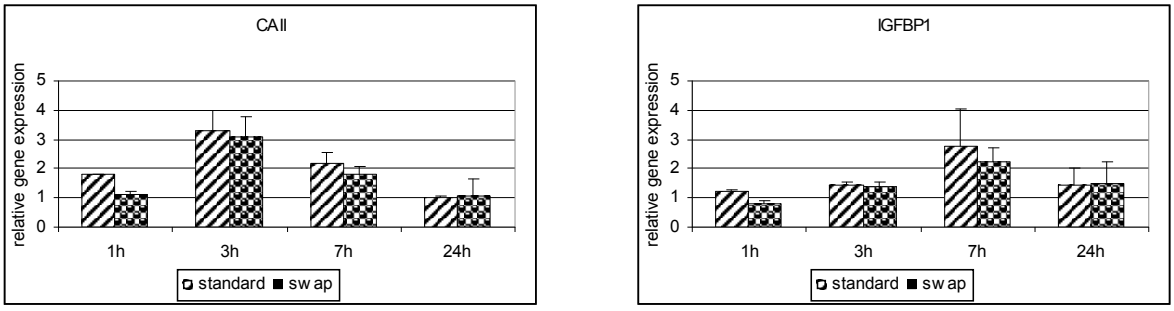

Fig. 51: Comparison of gene expression analysis, correlated to control (set to one), in Fe33 by oligonucleotides microarray, with the change of label on the sample. Labeling: control-Cy3, treated-Cy5 (standard) and control-Cy5 treated-Cy5 (swap). The cells were treated with EtOH (control) and EE 10^{-7}M in a time dependent manner (1-3-7-24 h). Genes investigated: CAII and IGFBP1. No statistical significance is calculated, because each experiment was conducted just once.

4. Investigation of the fragment r52

The fragment r52 was identified using the differential display RT-PCR (ddRT-PCR) (Liang & Pardee, 2002) by Dr. Patrick Diel in the uterine tissue of differentially treated ovariectomised rats, E2 against the control. This sequence was selected for further characterization due to its sensitivity to E2 treatment, resulting in a strong repression following treatment. This technique consists, roughly, in a RT-PCR using a anchored oligo dT primer (8-10 T with 2 specific bases at one extremity), to reverse transcribe mRNA starting directly at the point of the poli A tail, the other primer is a random decamer. To avoid any DNA contamination the RNA extracted from the samples is treated with DNAse. The results of the RT-PCR are separated through a poly acryl amide gel. Due to the low stringency condition this method delivers many false positive, for this reason every PCR product obtained has to be sequenced. The sequence of r52 fragment is reported in sequence A (286 bp)

Seq. A

(5´)- GTCAGTGAC NGAACCGGT ATGATTTGA AAACAAACA AGATGTGTG AGAAATTGG
AAAGATCAA GATGACTGC TTTGCCCCC TTCTTTTTA AATCTAAGC AACCGGAAT
AGACTACCA TACAGCATC TGTGAATTT TCAGGCAGT GCAGTCACT TTTACATAC
ATGACTGAG ACAGCTACT AAACACCAA GAGTTCTGG GAAAAATCT GAAGGTGGC
TCTCTGTAC CAAGCAGTT CTAAGAGAA GTTTGGCTA AAATTAGTA AGACCATCA
TCACCAGAA AAAAAAA - (3´)

4.1 Study of the sequence of the fragment r52

The sequence identified by a differential display RT-PCR corresponds to the 3' end of a putative mRNA that should be associated with a gene. The aim of this part of the work was to characterize the gene by discover the section of the gene upstream the already known sequence, searching for the 5' end, and the promoter region along with identifying possible binding sites which confer estrogen responsiveness and could explain the strong estrogenic sensitivity of this gene.

4.1.1 Primer walking sequence strategy

The sequence A was "blasted" against the NCBI nucleotide data bank (http://ncbi.nlm.nih.gov). The standard blast result of the sequence A against the NCBI data bank did not deliver any suitable results, with only short matches of maximum 22 nucleotides. The sequence was also blasted against the gene bank of the "Rat Genome Resources" presents on the same web site. This time it found homology in the 4th chromosome, with an identity of 95% (275/287bp), in the reverse strand of the sequenced chromosome (strand plus/minus) between 15.605.504 and 15.605.788 bp.

The sequence was contained in a region indicated as “similar to cyclin D-interacting myb-like protein 1” that in this position was the result of a computational analysis using the gene prediction method: GNOMON (a computational gene prediction method).

A schematization of the homology region of the sequence A in chromosome 4 (purple) is shown in fig. 52. The sequence A (blue) found homology in the intron (yellow) region between the 4th and 5th exons (red) of the sequence indicated in the Rat Genome Project as “similar to cyclin D-interacting myb-like protein”.

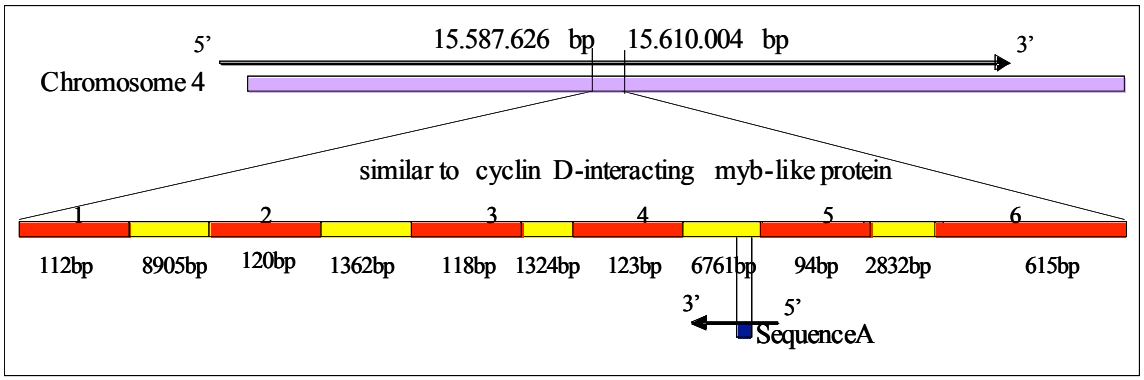

Fig. 52: Scheme showing the homology between sequence A (blue) and chromosome 4 of rat (purple), in the region of the gene “similar to cyclin D-interacting myb-like protein”. In red is shown the exon regions with the respective number, in yellow the intron regions are represented. The length of the sequences is reported in base pairs. The length of the fragments is not to scale.

Using the chromosome sequence, 10 primer pairs were designed, r52A-r52L, to amplify the boundary regions of the sequence A (reported in Materials and methods, 3.1). The positions of the primer pairs in the sequence and the length of the amplified fragments are summarized in tab. 7. The start position was considered to be the first base of sequence A at the 3’ end. The template for the PCR reaction total RNA isolated according the point 5.1 of Materials and methods was used but without final mRNA isolation. The total RNA was reverse transcribed into cDNA as described in the Materials and methods 3.3. The resulting PCR products were analyzed with a run through an agarose gel.

Tab. 7: Primer pairs listed with their positions in the sequence, considering as start position the first nucleotide of sequence A at the 3’ end, and length.

primer pair	position in the sequence	length
r52	103-222	120
r52A	668-854	186
r52B	403-512	109
r52C	988-1296	308
r52D	1475-1859	384
r52E	2129-2398	269
r52F	2519-2903	385
r52G	3075-3342	267
r52H	3531-3840	309
r52I	4187-4485	298
r52L	(-280)-(-75)	206

The scheme of the homology region of the primer pairs in the "similar to cyclin D-interacting myb-like protein" is shown in fig. 53. The numbers represent the distance in base pairs from the first base of sequence A at the 3' end. The PCR products resulting from the use of the primer pairs reported in tab. 7 are indicated in green.

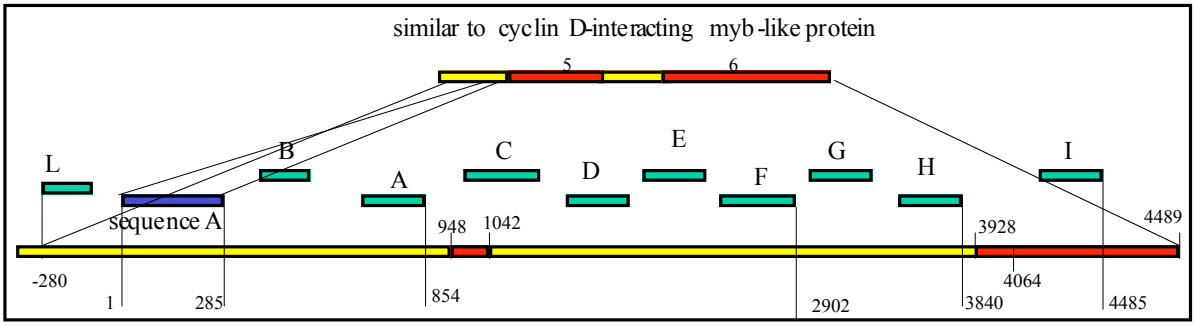

Fig. 53: Scheme of the homology of the different amplification primer pairs (green) A-L in the terminal part of the gene "similar to cyclin D-interacting myb-like protein". Exons 5 and 6 are represented in red, the introns are represented in yellow, and sequence A is represented in blue. The numbers in the image represent the distance in base pairs from the first nucleotide of sequence A. The length of the segments is not to scale.

The primer pairs were also combined together, as reported in tab. 8, to amplify different parts of sequence and to determine if interruptions in the continuity of the cDNA sequence were present.

Tab. 8: List of the primer pair combinations and lengths of the resulting amplified sequences

primer mix	amplify length
r52rev+r52Brev	448bp
r52rev+r52Arev	790bp
r52Bfwd+r52Arev	431bp
r52Cfwd+r52Drev	871bp
r52Dfwd+r52Erev	923bp
r52Efwd+r52Frev	774bp
r52Afwd+r52Crev	629bp
r52Afwd+r52Drev	1192bp
r52Afwd+r52Erev	1731bp
r52Dfwd+r52Frev	1426bp
r52Ffwd+r52Grev	823bp
r52Gfwd+r52Hrev	765bp
r52Hfwd+r52Irev	/
r52fwd+r52Lfwd	500bp

After analysis of the different amplified fragments, it was possible to conclude that there was no interruption in the cDNA used as template starting from the primer r52L forward until the primer of r52H reverse, it was not possible to get a signal of amplification using the primer r52Hfwd in combination of the primer r52Irev. It has to be mentioned that the two primers found themselves at the boundary of an intronic/exonic region of the gene "similar to cyclin D-interacting myb-like protein"; after the 4064 bp of its 3'UTR region, as reported in the

databank. The results are schematized in fig. 54 indicating with the azure segment the proved continuous fragment of cDNA.

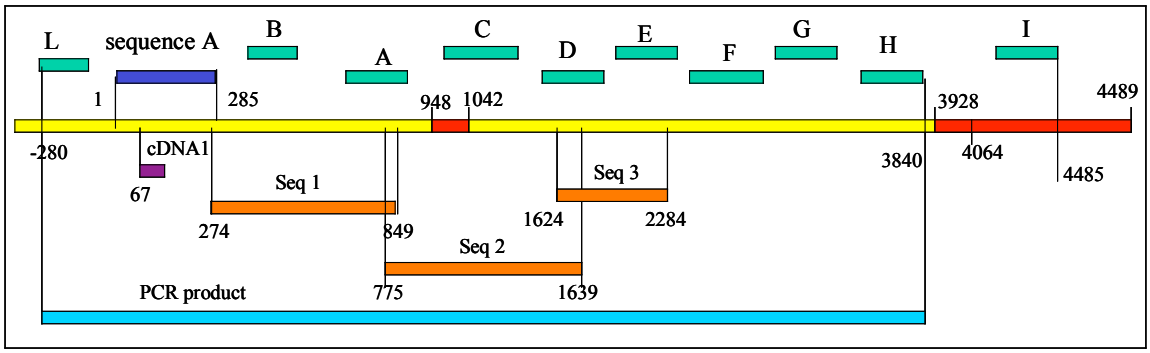

Fig. 54: Scheme of the homology of the different amplification primer pairs (green) A-L, the PCR product (azure bar) is the result of the amplification of the cDNA using different mixture of the primer pairs A-L. In orange is the homology region of the sequences 1, 2 and 3 in the terminal part of the gene "similar to cyclin D-interacting myb-like protein". The sequenced regions seq. 1, seq. 2 and seq. 3 are shown in orange. The annealing region of the primer cDNA1 is reported in purple. The other colors were the same as in figure 79. The length of the segments is not to scale.

To confirm the reliability of the data produced, three sequences were performed using as template the PCR products obtained from different primer pair combinations. The sequence 1 was produced using, as template, the PCR product derived from the primer combination r52rev/r52Arev, whereas for sequences 2 and 3 the combination of r52Afwd/r52Erev was used. Fig. 54 reports the results of the homology of the three sequences (orange bars). The nucleotide sequences 1, 2 and 3 are reported below.

Seq. 1: 597 bp

(5′)- TCTGTCACT GACATGCGT TCACAATTT GAACTATGT ATGCTTCCA CCTAGTCCA
AACCACTGK CCTTTCCTA CTGTGCTGA CTCTAGCCC TTCTGCCCT BBAGAATGT
CTTSCTCAC AGCCACCAG AACAGTCCT GGTGATGAA AGTGAGCTT ATGCCATTT
TTCTGCTCA GAATCATCC AATGCCTCC TTTCTCGTC TTTGCTGCA GCCTGTGAG
GTCTCTTTC CGCTCTTTC TTCATCTGC TGTAACAAC AATGCACGG ATCACACTT
GTGCCAKTG TACGCGTGC CCTATCGCC AAGTTTTGT GCTTGTCTT TCCTTAAAG
TACGGTCCT GCAAGACTA GCTTCCTTC CTTTTCCCA AGTCGGATC ACCCTAGTT
CGTACCTAA AATTTGGGA CATACTGCC TCACTCTAC TCCATTCCA TTCCCTGCT
TTATCTTGT CCTTGGCTT ATATGAGTA TTAGCCAAC ATAGAATCC TGTCCATTT
CTAGACTAT AAGTCCCGT GAAAAAGTT TCCKACCTA GTATTCATG TCTGTTGAG
CAAATATGT GCAGTATAA TGATTGGTA AGAAGTGGG GGGGGGGGA
GGGGGAAGC AAG -(3′)

Seq. 2: 864 bp

(5′) - CGTGAAAGT TTCCTACCT AGTATTCAT GTCTGTTGA GCAAATATG TGCAGTATA
ATGATTGGT AAGACGTGG GTTTGGAAA TTAGATGAG CACATTTTC ACCGTGGAT
TTTAGTTTG GGTAATTAG ATCCTACAA TTACTACTG TGCAGATGA ATCATTATT
TTCTTGAAG TTTCAGAAA ATGATCAGA GCTTTGAGG TGACCATGA CGGCAACTA
CAGAGGTGG CAGATGATG AACTTTCTG AGGGAACTG TGACACAAA TTCAGGTAT
AGTACATAT TTTAAACTG GCTTCTGTG AGGAACATG GTTATKACA CACAACTTA
AGTAAAARA TGATAAGGA TRTACCTTG ACAACATAG TGTCAATAA AGCAAGGCC
CATCATCAC TGAGGTTTC TAGTCCCAA CATGTTGTA AGCAGAAAT AGATGCTCA
TTGTGCTCC CTGTAGAGA CCATGGTTG GAATAGGAA GTGATGGGT GTGCCTTCA
TCTGTGTCT AGAATCCTG CTTGGACTG TGAATAGCA GTGATTACC GTTGTGCTG
TTCCGTTGG TGCTTGTCG AGCTAGATA GGTTTTCTC ATATGCTGT GAGGCTGAC
TGGTAGCCA GGCCTTTCT GCTGTTCTT ACTGTGAAC TTCTTTTGC TGTCTTACA
TAGTTTGAA TCTAAAATG AGTATGTAT TCTAAATTT ATGTCTACC TGATTGTGC
ATCTTTTGC GTTTAGTTG GCTTATGCT TTTACTTCA TATCGTCAT TTAATGTCT

GAGANTTTT GAATTGAAA ATACTTGAA CATTTTTAT GAAGTATCA CAGTGCCCT
TTCGCTTAG GAAGTAWAA GACTTCCGG GGTTGAWAT GTAATADTA AGWACCGA
G - (3´)

Seq. 3: 660 bp

(5´)- GTAATATTA AGAACTGAA GAAGTAATA GAAAATTCT TTCTTCAGA TGAGGCTTA
TCAAATCAT TTCACCTTT TTAATATAA AAATTGTAT GAGCTTTAC TATAAAAAG
TCTTGTAAA TAAAATAAA GATCACAGA CCAAAGTTT ACCAGACTC CGTGCCTGT
GCATTTCAA AGAAGTTCT CGTAGCTAG GCCATTCTG CCGCTGTGG AGCCTCACA
CTGAGGCTT ACACTTTCA CTGCAGAGT TTGTGTTTA TACACATAA TCTACCACC
AACACTGAG TGCTCTCTG GCAGGAGGA GAGTGGGAC CCCAGCCTT ATATCCTGT
GTAACTCAT GTTCAGCAC CTGCATTCA GTCCTTCCT CATCCCTTA GAGTCAGCA
TTTCTTACT CTCTTTTCT GAGTTATAC TTTACTCAC ATGTATGCT CATGTTACA
TATATTATG GGCTGGATT CCGCATATG AAGGAGAAC ATGAAGTTT TTTTTCTTT
CTGAGATTA TATGATATA TATTCAAAG TCTATCCAT TTTCCTGAA ACTTTTGTT
ACTTCATTT TTTAGAGCT GAATAGTAT TCTAGTTTT TAAATTTTC ATCATTTAG
TATTATTTC AATTGATAC TGAAAGGGC ATTTGACAA AACTCACCA TGCCTTCAT
GATATAGTC CGG - (3´)

The combination of sequence A, 1, 2 and 3 (2284 nucleotides) is reported below. The sequences 1, 2 and 3 were represented in the reverse and inverted strands to follow the direction of sequence A

(5') - **(seq. 3)** ATWTCAACC CCGGAAGTC TTWTACTTC CTAAGCGAA AGGGCACTG
TGATACTTC ATAAAAATG TTCAAGTAT TTTCAATTC AAAANTCTC AGACATTAA
ATGACGATA TGAAGTAAA AGCATAAGC CAACTAAAC GCAAAAGAT GCACAATCA
GGTAGACAT AAATTTAGA ATACATACT CATTTTAGA TTCAAACTA TGTAAGACA
GCAAAAGAA GTTCACAGT AAGAACAGC AGAAAGGCC TGGCTACCA GTCAGCCTC
ACAGCATAT GAGAAAACC TATCTAGCT CGACAAGCA CCAACGGAA CAGCACAAC
GGTAATCAC TGCTATTCA CAGTCCAAG CAGGATTCT AGACACAGA TGAAGGCAC
ACCCATCAC TTCCTATTC CAACCATGG TCTCTACAG GGAGCACAA TGAGCATCT
ATTTCTGCT TACAACATG TTGGGACTA GAAACCTCA GTGATGATG GGCCTTGCT
TTATTGACA CTATGTTGT CAAGGTAYA TCCTTATCA TYTTTTACT TAAGTTGTG
TGTMATAAC CATGTT **(seq. 2)** CCTCACAGA AGCCAGTTT AAAATATGT
ACTATACCT GAATTTGTG TCACAGTTC CCTCAGAAA GTTCATCAT CTGCCACCT
CTGTAGTTG CCGTCATGG TCACCTCAA AGCTCTGAT CATTTTCTG AAACTTCAA
GAAAATAAT GATTCATCT GCACAGTAG TAATTGTAG GATCTAATT ACCCAAACT
AAAATCCAC GGTGAAAAT GTGCTCATC TAATTTCCA AACCCACGT CTTACCAAT
CATTATACT GCACATATT TGCTCAACA GACATGAAT ACTAGGTAG GAAACTTT
(seq. 1) TTCACGGGA CTTATAGTCT AGAAATGGA CAGGATTCT ATGTTGGCT
AATACTCAT ATAAGCCAA GGACAAGAT AAAGCAGGG AATGGAATG GAGTAGAGT
GAGGCAGTA TGTCCCAAA TTTTAGGTA CGAACTAGG GTGATCCGA CTTGGGAAA
AGGAAGGAA GCTAGTCTT GCAGGACCG TACTTTAAG GAAAGACAA GCACAAAAC
TTGGCGATA GGGCACGCG TACAMTGGC ACAAGTGTG ATCCGTGCA TTGTTGTTA
CAGCAGATG AAGAAAGAG CGGAAAGAG ACCTCACAG GCTGCAGCA AAGACGAGA
AAGGAGGCA TTGGATGAT TCTGAGCAG AAAAATGGC ATAAGCTCA CTTTCATCA
CCAGGACTG TTCTGGTGG CTGTGAGSA AGACATTCT VVAGGGCAG AAGGGCTAG
AGTCAGCAC AGTAGGAAA GGMCAGTGG TTTGGACTA GGTGGAAGC ATACATAGT
TCAAATTGT GAACGCATG TCAGTGACA **(seq. A)** GAACCGGTA TGATTTGAA
AACAAACAA GATGTGTGA GAAATTGGA AAGATCAAG ATGACTGCT TTGCCCCCT
TCTTTTTAA ATCTAAGCA ACCGGAATA GACTACCAT ACAGCATCT GTGAATTTT
CAGGCAGTG CAGTCACTT TTACATACA TGACTGAGA CAGCTACTA AACACCAAG
AGTTCTGGG AAAAATCTG AAGGTGGCT CTCTGTACC AAGCAGTTC TAAGAGAAG
TTTGGCTAA AATTAGTAA GACCATCAT CACCAGAAA AAAAA - (3')

4.1.2 Searching for the 5' end of the fragment

To try to find the complete sequence of this fragment, a liver cDNA library was screened using the "r52" primer pair of the real time RT-PCR as a probe, unfortunately it was not possible to find any sample that contained this fragment. It was not possible to recognize if the negative result was due to the absence of this fragment in the library, or from its low presence in the cDNA library, below the limit of the real time RT-PCR.

After this unsuccessful approach with the cDNA library I attempted to get the complete cDNA of the fragment r52 with specific reverse transcription of the fragment r52 (described in Materials and methods, 5.2), the primer cDNA1 anneals with the "r52" fragment as shown in fig. 54. After reverse transcription with the specific primer for r52 the resulting cDNA was tested by real time RT-PCR with different primer pairs: 1A, C3 and r52. A signal of amplification was expected only in the r52 sample, for the presence of a single cDNA population, used as template, consisting of only the r52 fragment. All samples gave a signal of amplification, show the experiment to lack specificity.

4.1.3 "In silico" analysis

At the same time a different "in silico" analysis of the sequence A and her boundary region was performed.

The first step was the search for an ORF (Open Reading Frame). Usually an ORF indicates a transcribed region of the genomic DNA. This was done to validate the theory that the sequence r52 was part of a gene encoding a protein. Towards this goal the software "ORF finder" present on the web site of the NCBI was utilized using sequence A as the investigated sequence. This search gave negative results. Looking for an ORF in the 10.000 nucleotides up stream of the r52Hrev primer showed the longest ORF to be 540 bp and not including the r52 sequence (sequence A). The first ten matching ORFs did not give suitable results: invertebrates, plants or unicellular organisms proteins. The search for an ORF in the region between r52Lfwd and r52Hrev was also unsuccessful. The combination of the two sequences together, r52Lfwd-r52Hrev + 10.000 bp upstream r52Hrev, did not yield better results.

The 10.000bp up stream of the r52Hreverse primer was investigated for the presence of a known promoter using various software packages for the promoter analysis (listed in the Materials and methods 5.5), none of them delivered a positive result.

Another possibility was that the sequence r52 has a function without to be transcribed into a protein, as regulatory RNA. Towards this goal the folding of the RNA sequence was studied. To study the folding of the RNA of r52 into a possible secondary structure, the sequence A

was tested using the software “Mfold”. The resulting secondary structure is shown in fig. 55. The resulting (-dG) was -48,43, to have an order of magnitude lys-tRNA has a (-dG) of –26,28, almost the half, indicating its stability.

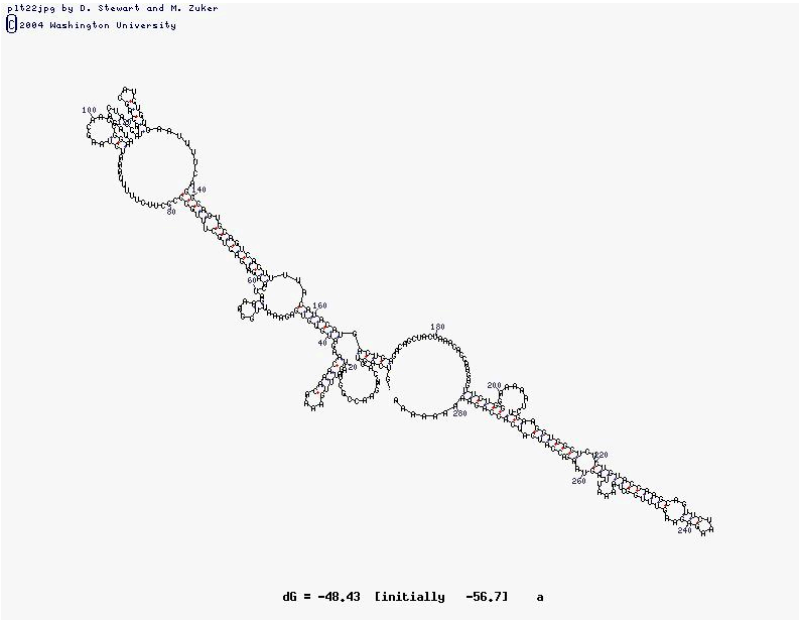

Fig. 55: Secondary structure of the sequence A RNA according to the software “Mfold”.

4.2 Gene expression analysis of r52 by real time RT-PCR

To verify the interesting behavior of the sequence r52 in response to estrogen treatment, its expression was studied in two tissues of ovariectomised rats *in vivo*: the uterine tissue and the liver, treated by estrogens, E2 and EE, and phytoestrogens, Gen, Dai, 6DMAN and 8PN.

The results shown in fig. 56 to 61 came from a single animal experiment and a single RNA extraction. Every result derived from the pooled RNA of at least 6 animals and is presented as the average of at least three independent cDNA synthesis and two independent real time RT-PCR analysis per cDNA synthesis. The results are reported according to the animal experiment performed.

4.2.1 Gene expression of the fragment r52 in uterus

The influence of the estrogens, E2 and EE, and the phytoestrogens: Gen, Dai, 6DMAN and 8PN were tested in rat uterotrophic assay. The results shown in this section, derived from the analysis of the same RNA material used for the experiment reported in fig. 3, 4, 10 and 11 of this chapter

In fig. 56 the results of treatment with E2, EE, and Gen for 7, 24 and 72 h, and Dai for 72 h are reported. E2 and EE induced a down regulation of the r52 gene expression with an increase of the effect according to progression of the duration of treatment, which after 72 h reached 20 % of the control level. Gen treatment resulted in a light down regulation of r52, with only a reduction in the gene expression level to 60 % of the control level after 24 h, which is statistical significant. Dai did not influence r52 expression.

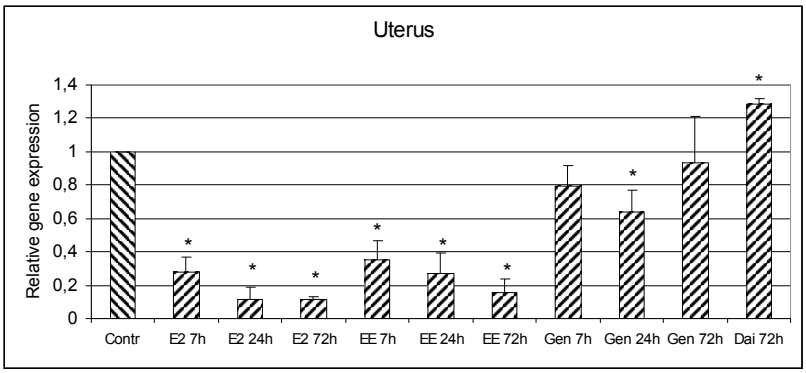

Fig. 56: Gene expression analysis of r52 using real time RT-PCR, correlated to control (set to one), in rat uterus in a time dependent manner (7-24-72h). Treatment (s.c. injection): EtOH (Contr), E2 (3µg/kg/day), EE (3µg/kg/day), Gen (10mg/kg/day) and Dai (20mg/kg/day). Significance: Student's t-test *: $p<0,05$.

The effects of E2 and 8PN after a 72 h treatment are shown in fig. 57. It was possible to show that both substances induced a strong down regulation of gene expression of r52.

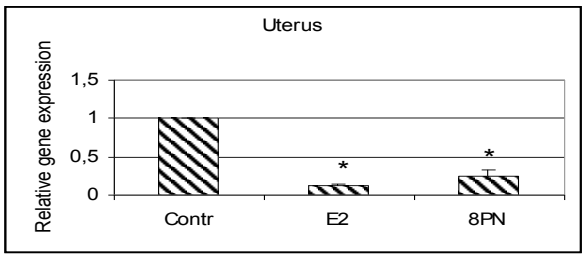

Fig. 57: Gene expression analysis of r52 using real time RT-PCR, correlated to control (set to one), in rat uterus after 72h treatment. Treatment (s.c. injection): EtOH (Contr), E2 (30µg/kg/day) and 8PN (10mg/kg/day). Significance: Student's t-test *: $p<0,05$.

In fig. 58 the results of treatment by E2 and Dai for 7 and 24 h and Gen for 24 h on r52 gene expression in uterus are shown. In this case the gene expression of r52 was studied using two different real time RT-PCR primer pairs. One of these anneals in the original "r52" fragment, and another one that anneals to a new identified region, r52E. The choice to study the gene expression of two primer pairs designed in the same sequence derived from the need to verify that the amplified region of r52 (indicate in azure in fig. 53) was really the same sequence and not a chimera.

In this case E2 and Gen triggered a clear down regulation of the relative gene expression of r52 detected by both primer pairs with statistical significance. Dai did not produce any effect. A striking similarity between the results could be seen with primers, “r52” and “r52E”.

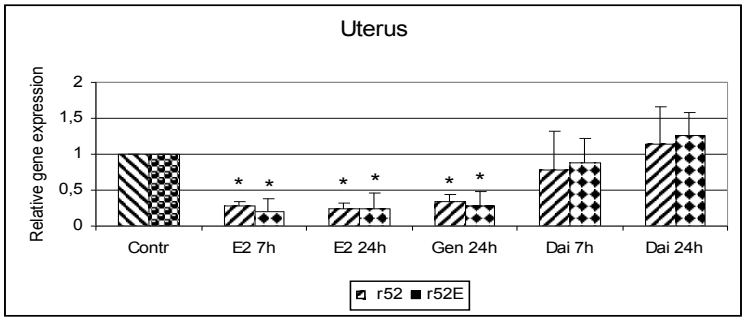

Fig. 58: Gene expression analysis of r52 and r52E using real time RT-PCR, correlated to control (set to one), in rat uterus in a time dependent manner (7-24h). Treatment (s.c. injection): EtOH (control), E2 (4µg/kg/day), Gen (10mg/kg/day) and Dai (40mg/kg/day). Significance: Student’s t-test *: p<0,05.

The effects of E2, 6DMAN and 8PN for 7 and 24 h are shown in fig. 59. The gene expression of r52 was studied using the same primers pair used in the previous experiment. After 7 and 24 h of treatment (fig. 59a and b) it was possible to observe that E2 induced a strong repression of gene expression reported by both primers pair. 8PN mimicked the same effect, but less strongly, at both times and using different primers. After 7 h of treatment 6DMAN did not seem to influence the regulation of r52, delivering also a not statistical significant result with both primers. After 24 h of 6DMAN administration the primer pair “r52” showed a down regulation to 50 % of the control level, the primer pair “r52E”, after the same treatment showed a down regulation to 20 % of the control level, both results were statistical significant.

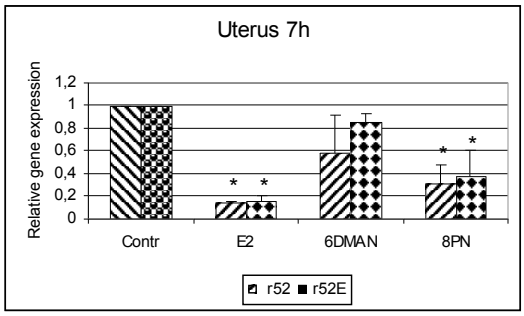

Fig 59a

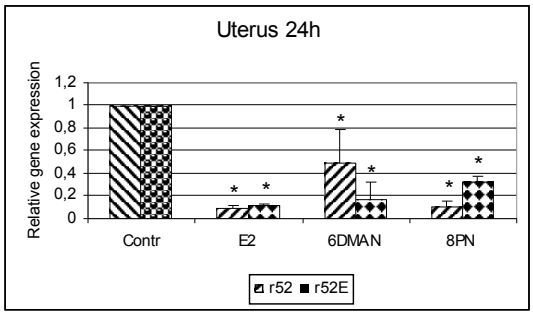

Fig. 59b

Fig. 59: Gene expression analysis of r52 and r52E using real time RT-PCR, correlated to control (set to one), in rat uterus after 7 (a) and 24 h (b) of treatment. Treatment (s.c. injection): EtOH (Contr), E2 (10µg/kg/day), 6DMAN and 8PN (15mg/kg/day). Significance: Student’s t-test *: p<0,05.

4.2.2 Gene expression in liver

To investigate if the interesting expression pattern of the fragment r52 in response to estrogen and phytoestrogen detectable in uterine tissue was present also in other tissues, its gene expression was tested in liver as well.

The RNA material used in these experiments was the same as already analyzed in fig. 21 and 22 of this chapter.

The effects of E2, EE and Gen after treatment for 7, 24 and 72 h and Dai for 72 h are reported in fig. 60. The treatment with E2 induced a doubling of the relative gene expression of r52 after 72 h of treatment, but without statistical significance. EE showed in opposite a repression of the gene expression to half of the control level after 7 h, without a statistical significance. No clear effects were obtained for both of the other durations of treatment. Gen and Dai showed a tendency to repress the gene expression to values near to 50 % of the control, as Gen after 7 and 72 h of treatment showed statistical significance.

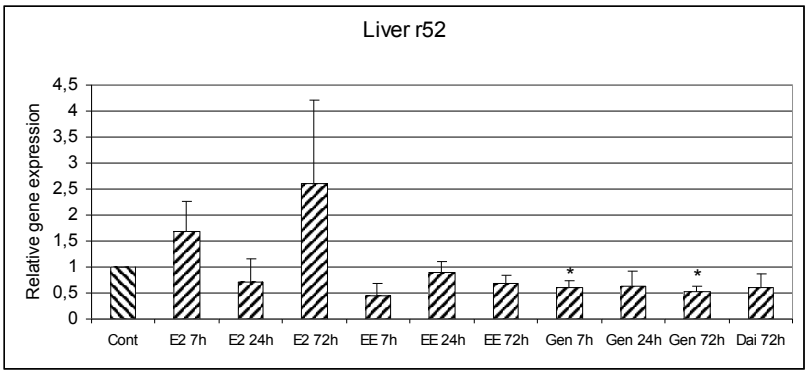

Fig. 60: Gene expression analysis of r52 using real time RT-PCR, correlated to control (set to one), in rat liver in a time dependent manner (7-24-72 h). Treatment (s.c. injection): EtOH (Cont), E2 (3µg/kg/day), EE (3µg/kg/day), Gen (10mg/kg/day) and Dai (20mg/kg/day). Significance: Student's t-test *: $p<0,05$.

The effects of E2 and 8PN treatment for 72 h in liver were not relevant of r52 gene expression (data not reported).

Discussion

1. Project overview

Beneficial or potential adverse impacts of xenoestrogens on human health are still controversially debated.

My thesis tries to contribute to this discussion by creating a methodology capable of performing a fast, cheap and high throughput mode of analysis to characterize different substances for their estrogenical activity using gene expression profiling. Towards this aim I used *in vitro* and *in vivo* rat uterus and liver models.

First I conducted a study using real time RT-PCR to investigate the response of estrogen/xenoestrogens treatment of some genes, C3, Clu, IGFBP1 and CaBP9k that are classical targets of estrogens in the studied tissues. The effects of those substances on proliferation were controlled by gene expression profiling of PCNA. Subsequently, I investigated, for the first time, the effect of estrogen/xenoestrogenes exposure on rat tissues, liver and uterus, of CA2, a classical target of estrogens in bone tissues. CA2 showed an interesting patter of answer to some of those substances also in these tissues. I characterized the response to estrogen/xenoestrogens in liver and uterus of a newly identified rat sequence as well individuated by our collaborator Dr. Patrick Diel.

At the same time I established, for the first time, a microarray setup in our laboratory. The method consisted in a complete “in house” procedure from the spotting of the samples on glass slides to the analysis of the results; this allowed control of every single step of the procedure and to optimize it better to fit the goal of the thesis.

Finally, I produced a microarray DNA chip with 16 genes that are estrogen sensible and were tested in a rat hepatic cell line after exposure to xenoestrogens: 17α-ethynilestradiol and nonylphenol. Those results were compared to those obtained from real-time RT-PCR analysis showing a similar expression pattern.

2. Analysis of estrogen sensitive genes in uterus and liver models, by real time RT-PCR

2.1 Estrogen sensitive genes in uterus models (C3 and Clu).

The genes C3 and Clu present an already proven responsiveness to estrogens exposure in both *in vitro* and *in vivo* rat uterus models (Sundstrom *et al.,* 1989; Diel *et al.,* 2001, 2004; Zierau *et al.,* 2004).

Using RUCA-I cell culture I showed that C3 and Clu gene expression is influenced only when the cells are cultured on a layer of ECM (Result: fig. 1 and 2), as already shown by morphological difference and gene expression analysis by RT-PCR (Vollmer *et al.,* 1995).

In uterus C3 presents a strong response to E2 treatment (Results: fig. 3, 5, 10 and 11), presenting a time depending up regulation following the length of the treatment, as already shown (Diel *et al.,* 2004). The treatment with EE presented the same results (Results: fig. 3), as when treating the rats with the same amount of drug. EE can be considered as a pure agonist in uterus (Diel *et al.,* 2004; Tinwell *et al.,* 2002). The treatment with the phytoestrogen Gen resulted in a clear stimulation of C3 expression only after 24 h of treatment (Results: fig 4 and 10). It did not completely mimic the effect of E2, resulting always an order of magnitude weaker than E2 in stimulation C3 expression, even if used at 3.000 times higher doses. In this case Gen can be considered a weak agonist in the uterus confirming already published results (Diel *et al.,* 2001, Rickard *et al.,* 2003).

The other isoflavone Dai never showed any influence on C3 expression, also at doses 6000 times higher than E2. With this result I show a case of no agonistic activity of Dai in rat uterus. The behavior of Dai in uterus is still debated because other evidences report its weak agonist activity (Hopert *et al.,* 1998; Diel *et al.,* 2000).

The agonist activity of the two flavones 8PN and 6DMAN in uterus (Zierau *et al.,* 2004; Diel *et al.,* 2004, Humpel *et al.,* 2005) was confirmed (Results: fig 5 and 11). Both substances act as agonist, mimicking the effects of E2, but their lower potency has to be underlined, the same effect is reached with a 300 times higher dose.

2.2 Estrogen sensitive genes in liver models (IGFBP1 and CaBP9k)

The genes IGFBP1 and CaBP9k are affected in their expression by estrogen treatment in liver models (Diel *et al.,* 1995, 2004; Geis *et al.,* 2005). Hepatic cell line, Fe33, exposed to E2 presents a clear up regulation of both IGFBP1 and CABP9k (Results: fig. 12, 13, 14, 17, and 18).

The same cell line treated by EE shows in both cases the pure agonistic effect of this substance, reproducing, in a stronger way, the stimulatory effect of E2 administration (Results: fig. 13 and 14). EE stimulates IGFBP1 and CaBP9k expression in both time and dose dependant manners, 10^{-7}M can be considered the most active dose. The larger effect is present after 7, for IGFBP1, and 24 h, for CaBP9k.

Moving the attention to an *in vivo* model the administration of E2 in rat liver triggered a doubling of IGFBP1 expression after 72 h (Results: fig. 21 and 22) confirming the responsiveness of IGFBP1 to estrogen in rat liver (Geis *et al.,* 2005).

The administration of EE at the same dose had similar effects, confirming its agonistic activity *in vivo* as well. In this way EE, confirmed its agonistic potency in the liver (Geis *et al.,* 2005).

Studying the gene expression of IGFBP1 and CaBP9k of hepatic cells, Fe33, treated by NP it could be possible to notice its weak agonistic activity (Results: fig. 17 and 18). NP reached the same effect as E2 in stimulating the gene expression of both genes with a concentration 100 to 1000 fold higher. The mimicking effects were presents just in the first 3 h of treatment indicating a fast time course of the substance also. In this way the weak estrogenic potency of NP already proven in other in vitro tests was confirmed (Shelby *et al.,* 1996; Wober *et al.,* 2002), but for the first time showed in a hepatic cell line focusing on gene expression.

The isoflavone Gen in cell culture mimicked E2 effect only after 7 h of treatment (Results: fig. 12), at a dose 100 times higher than the positive control E2, indicating its weak estrogenic activity. The influence of Gen on IGFBP1 *in vivo* at a 3.000 times larger dose was similar to E2 for the first 7 h of treatment, after 72 h the effect was totally inverted (Results: fig. 21). This result confirms the weak agonistic activity of Gen (Dopp *et al.,* 1999; Diel *et al.,* 2001).

Dai in cell culture did not show any particular effect on both IGFBP1 and CaBP9k expression (Results: fig. 12), even if used 100 times more concentrated. *In vivo* the effect of E2 was mimicked after 72 h but always using a 6.000 times larger dose. It is not possible to define if this result stems from its agonistic weakness or that Dai does not behave exactly as an agonist in the liver.

The flavone 8PN showed its potency as estrogen agonist in cell culture stimulating the IGFBP1 expression after 7 h of treatment at a 100 times larger dose (Results: fig. 12). *In vivo* 8PN used at a dose 300 times larger than E2 performed an even larger stimulation of IGFBP1 expression (Results: fig. 22). The clear agonistic activity of 8PN was proven in liver as well.

2.3 Safety towards proliferation (PCNA)

The effects of the different substances on cell and tissue proliferation were studied through the analysis of PCNA expression. This result has to be considered in the context of cancers.

In RUCA-I cell culture, after 24 h of E2 treatment, the proliferation was not affected. This result may be due to the length of the E2 stimulation, in cell culture an effect on proliferation has to be studied at an earlier time point.

In rat uterus, E2 presented a time dependent stimulation of PCNA expression with the higher effect after 24 h (Results: Fig. 6). This result is perfectly comparable to the uterotrophe assay, where a constant increase of uterine wet weight is shown (fig. 1). This shows clearly the necessity to avoid the use of E2 in HRT, because of its proliferative effect it may result in a uterus over proliferation.

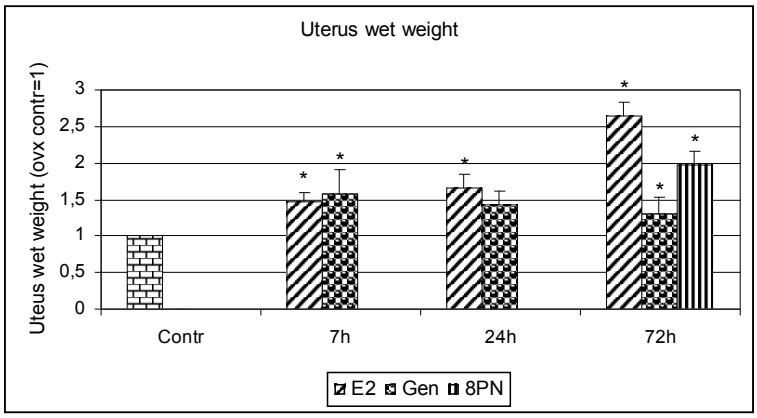

Fig. 1: Effects of s.c. administration of Gen and 8PN on the uterine wet weight of ovariectomised Wistar rats. Uterine wet weights of ovariectomised (ovx) Wistar rats after administration of 10 mg/kg/BW Gen, 7 and 24 h after a single s.c. administration and after repeated administration of Gen 10 mg/kg/BW day, or 8PN 10 mg/kg/BW day for 3 days. E2 (4 µg/kg/BW) served as reference compound. The uterine wet weight is indicated as relative uterine wet weight defining control (Contr) as 1. Significance: Student's t-test<0.05. (Data kindly provided by Dr. Patrick Diel already published "Diel *et al.,* 2003, 2004").

In the cell culture of hepatic cells, Fe33, a time dependent stimulation of PCNA was present with the higher effect after 1 and 3 h of treatment (Results: fig. 15 and 19). This result can explain the reason because the cell proliferation of RUCA-I resulted unaffected after 24h of E2 treatment, the duration was simply too long.

In liver, E2 administration showed an anti-proliferative effect on the tissue indicated by the time dependent down regulation of PCNA (Results: fig. 23). This result can be controversial in human health because from one site in HRT, E2 does not stimulate liver proliferation,

showing its safety in the context of hepatic cancer. On the other site the repression of the normal liver proliferation could lead to other diseases.

EE confirming its pure agonistic behavior mimicked the activity of E2 perfectly. In uterus, there was an identical result (Results: fig. 6). Also, in this case, the use of EE in HRT has to be deeply reconsidered, for the possible uterus over proliferation that can result after EE administration in menopause. In liver, EE was always comparable to E2 (Results: fig. 23). Also in this case the same consideration of E2 and its potential dangerous activity in liver are valid for EE.

NP showed a stimulation of PCNA only after the first 3 h of treatment confirming its short term activity (Results: fig. 19). Stimulation of PCNA after 24 h of treatment mimicking the stimulatory effects of E2 at the same concentration or ten times more diluted, should be carefully considered, they are exactly at the border of doubling of PCNA expression. As reported in fig. 15 of the results the same treatment by E2 did not show any particular effect. NP shows a proliferative effect in Fe33 as already observed by Olsen in MCF-7, breat cancer cell line (Olsen *et al.,* 2005), this has to be considered in the case of NP exposure, further analysis is needed.

Gen administration in uterus did not show any activity on proliferation (Results: fig. 6). This result has to be compared to the uterotrophe assay (fig.1), also considering the effect of E2. After 7 and 24 h both compounds increased the uterus wet weight, after 72 only E2 continued to increase the uterus weight. This result compared to the gene expression analysis shows that the first 24 h increase of uterus wet weight is not a real proliferation but just water imbibitions. This result is confirmed by Diel (Diel *et al.,* 2004) where immunohistological staining of uterine tissue shows that PCNA is up regulated only in the epithelium and not in the whole uterus. This result adds evidence to the safety to Gen environmental exposure. PCNA triggers only an epithelial proliferation of the uterus. In liver Gen did not affected the PCNA expression, resulting in this way as a "safe" compound in the case of cancer risk, corroborating the thesis of safety of Gen in cases of exposures.

Dai did not show any particular activity in both uterus and liver. It did not have any influence on the PCNA expression. This compound like gen, could be considered "safe" with respect to influence on tissue proliferation.

8PN exposure in uterus performed a slight up regulation of PCNA (Results: fig. 7). This result has to be kept in mind in case of 8PN exposure, consuming food containing this flavone. This result confirms the finding already shown on the effect of 8PN in the uterotrophe assay

(fig. 1). 8PN 300 higher concentrated performs perfectly the same effect of E2, increasing the uterus wet weight and stimulating PCNA expression, as reported by Diel (Diel *et al.,* 2003).

2.4 Characterization of novel response genes (CAII and r52)

The gene CAII already has shown its estrogen responsiveness mainly in the bone tissues, as reported in the introduction 3.1. Here I showed that CAII also presents an interesting gene expression pattern in response to estrogenic treatment in uterus and liver, as already published (Caldarelli *et al.,* 2005).

In uterus the treatment by E2 resulted in a time dependent down regulation according to the progression of the time (Results: fig. 8), showing a complete opposite behavior to that of C3, this may be due to the different promoter region of these genes. CAII does not contain an ERE in its promoter, but two AP-1 interaction sites and 4 for the inhibition of NFκB (McGowan *et al.,* 1997), in opposite C3 contains a triple ERE (Fan *et al.,* 1996). The administration of EE lead to the same result (Results: fig. 8), confirming its sensitivity to pure agonistic substances. The administration of the phytoestrogens in uterus, Gen, Dai and 8PN, resulted in a light repression of CAII (Results: fig 8 and 9). Comparing those results to that of C3, I show an opposite behavior of the two genes. In this case CAII gene expression analysis shows that 8PN does not behave as a pure agonist in uterus, also when used at a concentration 300 times higher that E2. In the cancer cell line RUCA-I it was not possible to perform a gene expression analysis of CAII due to the apparent absence of expression in this cell line (Results 1.1). This particular result could derive from the absence of CAII in uterus cancer, but no studies are present so far on CAII expression in uterine cancer.

The CAII expression results are influenced by estrogen treatment in liver models as well. In cell culture, Fe33, a time dependent stimulation of expression with the larger effect after 7 h of treatment due to E2 or EE administration is present (Results: fig. 16). The result of E2 or EE administration in liver on CAII expression is surprising, compared to the liver cell line. Here a time dependent down regulation is reported with the larger effect after 72 h of treatment. This may be due first from the different conditions a whole organism, that include more complex effects, compared to a single cell line. Another point as in the case of uterus and RUCA-I could be the different kind of tissues, a normal tissue in the in vivo experiment compared to a cancer cell line. This result adds more evidence to the interesting aspects of CAII behavior.

CAII results are not influenced by the xenoestrogens in both *in vitro* and *in vivo* models (Results: fig: 12, 20, 24 and 25), showing in this way its lower sensitiveness, compared to

IGFBP1 or CaBP9K to weak estrogenic compound in liver. This may be due to the different promoter regions of the three genes; CAII presents only an AP-1 binding site instance compared to CaBP9k which presents an imperfect ERE in the promoter that differs by only one nucleotide.

The newly identified sequence r52 presents a really interesting expression patter in uterus in response to estrogenic substances exposure. In the case of E2 and EE administration a time dependent down regulation of r52 is shown (Results: fig. 56). This result compared to C3 expression and the uterotrophic assay showed a perfect reverse picture. The interesting aspect is the response of r52 to the phytoestrogens. In the case of the flavones 8PN and 6DMAN, generally considered pure agonist (Zierau *et al.,* 2002, 2004; Diel *et al.,* 2004), a strong repression of r52 expression is present (Results: fig. 57 and 59), as shown for E2 and EE administration. The response to Gen administration should be better characterized. In a first experiment the administration of Gen in uterus did not influence the r52 expression (Results: fig. 56) indicating the potency of r52 to distinguish between a pure agonistic substance, as E2, EE, 8PN or 6DMAN, to substances showing organ-selective response pattern, a SERM (Selective Estrogen Response Modulator) as Gen is indicated from several authors (Diel *et al.,* 2001) some of them showed even a weak estrogenic activity of Gen and Dai to ERα and a pure agonistic activity with ERβ using a reporter gene (Salvatori *et al.,* 2003). A second animal experiment conducted with the same amount of Gen (10mg/kg/day) delivered a different result (Results: fig. 58). In this second case Gen behaved as a pure agonist mimicking perfectly the activity of E2. Further experiments are indeed to better characterize r52 expression profile in response to substances with estrogenic potency. In liver, E2 administration resulted in a complex response pattern, however without any statistical significance. EE and Gen presented similar action repressing r52 expression. This fragment does not present a particular response pattern in this tissue.

At the end of the gene expression profiling, different genes were proven for their utility to assess the risks of exposure to different substances with potential estrogenic effects. C3 and Clu confirmed their estrogen sensitivity in uterine models, as also IGFBP1 and CaBP9k for the liver ones. The information derived from PCNA expression profiling results is crucial to assess the risk of different substances on the proliferation site. Two novel genes, CAII and r52, were studied for their interesting response to estrogenic treatment in uterus and liver.

At the end of this section is it also possible to summarize the activities of the different xenoestrogens in the models used.

EE acted always as pure agonist, mimicking the activity of E2 in both tissues and the gene expression pattern of every studied gene. NP also presented an agonistic activity but with lower potency, for this reason it can be considered a weak agonist. Gen presented a more complex pattern of activities. Gen presents a similar activity of E2 in response gene in uterus, without affecting cell proliferation, this last aspect shown not only in gene expression but also in uterotrophe assay. In liver Gen does not present a marked agonistic activity, this may be due to the absence of ERβ in liver (Gustaffson 1999) and the weak agonistic activity with ERα (Salvatori *et al.,* 2003). From this evidence it could be considered a SERM, but further studies are needed (Diel *et al.,* 2001, Salvatori *et al.,* 2003).

Dai behaves generally similarly to Gen but with an even weaker agonistic potency.

8PN presents a marked agonistic activity at a concentration around 100-1000 higher than E2 in both uterus and liver tissue, from this evidence it could be described as pure agonist.

6DMAN presented also in uterus an agonistic activity, generally mimicking the effects produced by E2 administration but at a 100 time larger dose.

3. Set up of the microarray technology and application for gene expression analysis

The establish of the microarray technology in the laboratory has been a long and complicated process. In literature, little is reported on it at the level of optimization for every single step. Authors report just the final conditions used during the experiment, as for other methods. In some cases I tested different conditions reported from authors, as in the case of the labeling of the targets. The direct (Yu *et al.,* 2002) and indirect (Badiee *et al.,* 2003; Hui *et al.,* 2003) labeling of the cDNA samples was tested delivering completely different results from those reported in the literature. In my case the indirect labeling of the samples was a disaster. In others cases I just tested the different options proposed by the producer, as in the case of coupling of the oligonucleotide on the slide, comparing the results of U.V. exposure to warm bath. In this case the last option was the most favorable.

At the end of the established procedure I can report that as for other techniques that present the hybridization of probes to samples, like the northern southern or western blots, immunostaining and many others, every protocol is laboratory specific. Generally using the same material, it is possible to reach similar results using similar procedure, but a fine optimization has to be performed in every case.

The reagents are also “producer specific” which means that a product usually “likes” to be used together in combination with others produced from the same company, as example for the case of spotting buffer, the slides, and the hybridization buffer, all of them should be from

the same producer. In my case, after the closure of GeneScan I was obliged to find another hybridization buffer.

For the normalization of the data deriving from the array, I chose to normalize the signal to an internal control (housekeeping genes), that was a reasonable choice due to the size of the array itself, it presented less than 1000 spots, so a normalization on the overall signal was not possible. The positive results stemming from the experiment of dye swap confirms the quality of this choice.

3.1 Reproducibility of the results obtained by the microarray

The gene expression analysis using the microarray was performed just once for every sample, hence a direct comparison of the same RNA material is impossible. In both cell culture experiments evaluated by the microarray, treatment by EE or NP at different doses and time length (Results: 3.1 and 3.2), the same positive control was always present, the treatment by E2 10^{-8}M. The gene expression of IGFBP1, CaBP9k and CAII resulted influenced by E2 in both experiments, producing in this way a sort of duplicate of the same experiment. In both experiments, E2 produced an up regulation of all three genes only after 7 h of treatment Results: fig. 41-43 and 45-47). This is a good indication of reproducibility of the method.

3.2 Comparison of the gene expression results produced by real time RT-PCR and microarray

The gene expression results produced by the microarray at the end of the establish process were compared to those obtained using a robust and routinely used method for our laboratory: the real time RT-PCR, set up by Renate B. Geis. They are reported in the Results section at the point 3.3.1. The use of real time RT-PCR to validate the gene expression data produced by microarray analysis was already successfully used by other groups (Mutch *et al.,* 2001; Hwang *et al.,* 2002) and also in those cases the real time RT-PCR showed an higher sensitivity to gene expression change. Here I showed that the microarrays are able to repeat the gene expression result produced by the real time RT-PCR with lower sensitivity.

At the end of the process of setting up microarray technology I can be satisfied by the performance reached by this method. The reproducibility of the results showed with E2 treatment in two different cell culture experiments (EE and NP group), the dye swap, and the comparison of gene expression results to the real time RT-PCR, indicate the stability and the reliability reached by this method in our laboratory.

The next step is to increase the number of genes present on the array so as to have a more general overview on the estrogenic effects of the different substances on different samples (cell culture and tissues).

4. Characterization of the r52 sequence

The sequence r52 presents a really interesting gene expression pattern in response to estrogens and particularly phytoestrogens in rat uterus as discussed above.

The GC content (39,4 %) of the r52 is low for coding sequences. This is a first evidence that it could be something else then a protein coding mRNA. It is also uncommon that it localizes in an intronic region of a gene and in the complementary strand (Results: fig. 52). This sequence is also clearly not at the starting point of the poly A sequence present at the 3' end of a mRNA, according form the results of the PCR product obtained using a primer pair that amplify the region downstream the r52, the primer pair formed by r52fwd and r52Lfwd (Results tab.8 and fig. 53). The PCR products got by combining the primer pairs, L to H, show also that this sequence should interrupt at the end of the intron between the exon 5 and 6 (Results fig. 53). This result leads to imagine that the r52 putative mRNA ends at that point. The research of an ORF, which indicates a transcribed region, for approximately 5000 bp up stream the r52 seq. A did not produce positive result. No promoters were present in the same region as well. The three sequences performed starting from the sequence r52 confirm the continuity of the fragment until 1998 nucleotides up stream the end of already knew sequence (Results fig 53 and seq. 1, 2 and 3). Another evidence that r52 is not at the 3' end of an mRNA is confirmed by the absence of poly adenilation signal (AAUAAA). Regrettably every attempt to get a complete cDNA sequence failed, first using a cDNA library and then a gene specific reverse transcription (Results 4.1.2). To explain why r52 was not found in the cDNA library, I can propose that this sequence is not exactly what was thought at the beginning: an mRNA coding for a protein. A cDNA library is produced starting from mRNA, for this reason that library may not contain r52. The attempt of gene specific reverse transcription lacked specificity. This negative result could be explained with a not perfect purification of the mRNA after the RNA isolation and DNAse digestion, to eliminate the present DNA that usually contaminate RNA isolation. Also the use of mRNA specific purification kit, did not help for this intend. In the resulting mRNA used for reverse transcription could be present, even in very low concentration, some short oligonucleotides, coming either from RNA or digested DNA, that during the reverse transcription can act as random primer and reverse

transcribed also other genes. For this reason at the end also the primer pair for 1A and C3 could deliver a PCR product.

After those results it is hard to define what exactly the sequence r52 is. In the case that this putative gene is not coding for a protein it could be also a regulatory RNA, a non coding RNA (ncRNA). The dogma DNA→ RNA→ Protein could be challenged by this find out in the past years. It is already known that some transcribed RNAs are not translated to proteins, as tRNA and rRNA. In the last 7-8 years the interest around those transcripts increased. An overview of the possible origin of ncRNA was already reviewed by Mattick (Mattick, 2003). A ncRNA can be transcribed as a protein coding gene, or derive from the introns of another gene, i.e. the HBI36 small nucleolar RNA (snoRNA) present in the serotonin receptor 5-ht2c gene (Cavaille *et al.,* 2000). Another class of ncRNAs intron derived are miRNA (Ying &Lin 2005), they are short single strand RNAs (around 21 nucleotides) involved in post transcriptional regulatory functions. For those reasons it is also clear the interesting possibility that this r52 sequence could be an ncRNA that is somehow affected in its expression by E2 treatment in rat uterus. The sequence found by Dr. Diel presents already a considerable stable secondary structure. The secondary structure predicted using the software Mfold delivered a structure that present the –dG, free energy set free by folding, of -48,43 J. To understand the meaning of this result, the –dG set free folding the lys-tRNA correspond to -26,28 J and this tRNA has a stable secondary structure that allows to take part to the transcriptional machinery. So the r52 itself present a considerable stability in a secondary structure. Further studies to identify the complete sequence of r52 are indeed. To better characterize the r52 sequence another experiment to perform could be to produce a BAC (bacterial artificial chromosome) of this gene cloning at its end a fluorescent protein (like eGFP) as fusion gene to allow experiment of microscopy and localize the protein. This modified BAC can be used also for a fast gene expression analysis avoiding performing real-time RT-PCR experiment but just measuring by flow cytometry the intensity of the GFP signal is response to different compounds.

5. Conclusions

At the end of my work I investigated the estrogenic potency of different Xenoestrogens that commonly occur in our daily life, in rat cells and tissue using well known estrogen sensitive genes like C3, Clu, IGFBP1 and CaBP9k. I focused on their effect on cell proliferation, studying PCNA expression. For the first time sensitivity of the gene CA2 was proofed in liver and uterus. A new identified mRNA sequence, r52, was characterized for its sensitivity to

estrogenic exposure. This sequence was investigated at the molecular level expanding the known nucleic sequence.

I produce a microarray chip with 16 genes to investigate the estrogenic potency of different compounds. As proof of principle of the microarray method completely produced in house I compared the result of gene expression obtained by the chip to that obtained by real time RT-PCR finding a similarity of results. This new established method is less sensitive than the real-time RT-PCR but allows a high throughput of gene expression analysis producing at the end a more complete picture of the expression signature of a compound.

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Abbreviations

E2: 17ß-estradiol

EE: 17α-ethinylestradiol

NP: nonylphenol

EtOH: ethanol

Gen: genistein

Dai: daidzein

8PN: 8-prenylnaringenin

6DMAN: 6-(1,1-dimethylallyl)naringenin

ICI: ICI 182,780 (Faslodex)

min: minute

h: hour

RT: room temperature

bp: base pair

M: Molar

aa-dUTP: amino-allyl dUTP

IGFBP1: insulin like growth factor binding protein 1

CaBP9k: calcium binding protein 9k dalton

PCNA: proliferating cell nuclear antigen

CAII: carbonic anhydrase 2

C3: component complement C3

ERα: estrogen receptor alpha

ERß: estrogen receptor beta

Clu: clusterin

Cox-2: cyclooxigenase-2

IGF1: insulin growth factor 1

HSP70: heat schock protein 70k dalton

Ki-67: Ki-67

1A: cythocrome-c oxigenase subunit 1A

HK1: hexokinase 1

Ppia: cyclophilin

ß-act: beta actin

DMEM/12F: Dulbeccos's modification of Eagle's medium F12

DCC: dextran-coated charcoal

SSC: Sodium Chloride Sodium Citrate solution

SDS: Sodium dodecyl sulfate

PBS: phosphate buffered saline

OVX: ovariectomised

RUCA-I: rat uterus adenocarcinoma cells

PMT: Photon multiplier tube

List of publications

"Effect of phytoestrogens on gene expression of carbonic anhydrase II in rat uterus and liver"
Caldarelli A, Diel P, Vollmer G
Journal of Steroid Biochemistry and molecular Biology 97 (2005) 251-256

"The differential ability of the phytoestrogen Genistein and of Estradiol to induce uterine weight and proliferation in the rat is associated with a substance specific modulation of uterine gene expression."
Diel P, Geis RB, Caldarelli A, Schmidt S, Leschowsky UL, Voss A, Vollmer G.
Mol Cell Endocrinol. 2004 Jun

"Regulation of gene expression by 8-prenylnaringenin in uterus and liver of Wistar rats."
Diel P, Thomae RB, Caldarelli A, Zierau O, Kolba S, Schmidt S, Schwab P, Metz P, Vollmer G.Planta Med. 2004 Jan

Dresden 12 October 2006

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Declaration

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from the 3rd of December 2001 to the 31th December 2004 under the supervision of Prof. Dr. Günter Vollmer at the department of Biology, Institute for Zoologie, at the Technical University of Dresden.

Dresden 12 October 2006

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Aknowledgment

Many many people supported me during those years, may be I'll forgot someone.

Zuerst ich möchte sehr herzlich mich bedanken beim Prof Dr. Günter Vollmer, ohne Ihn, seine Betreuung, Gedult und Freundschaft ich hätte nie diese Doktorarbeit angefangt und am ende gebracht.

Vielen dank noch zum die Vollmer AG, für alles das in dieses 3 und mehr Jahr passiert ist, besonderaweise ich möchte mich bedanken beim Dr. Jannette Woeber für die Betreuung und Unterstuzung; Mohre für was sie getan hat und ich nie vergessen werde; Annette Illing für die besondere Freundschaft, die diplomanden: Anita, Thomas, Kerstin, Bianca, Kristin & Kerstin, damit manchmal ich habe vergessen, nicht in meine Heimat zu sein.
Georg und Renate gute Kollege; Germain pour n'avoir pas oublie' mon francais; Antje und Suse die haben sich gezeigt als eckte freundinen; Hagen weil mit seine Entscheidung hat mir die Möglichkeit gegeben dieses Doktorarbeit zu schreiben; Sebastian für die Deutsche männliche Freundschaft und die unterstuzzung wahrend die Etablierung von die Microarray; Jens das erste Person die ich direkt betreue habe.

Liang, gut das gibt Leute wie hin.
Nicola ed Enrico per un pezzetto d'Italia e italianità a Dresden
Alexander perché c'e'!
Liane für die Freundschaft
Alessio, Gaspare e Laura perché la Sicilia non e' poi cosi lontana
Davide quasi Papa Tonelli, per le chiacchierate sull'RNA e compagnia
Giuseppe testa, perché un giorno potrò vantarmi: "A me Giuseppe Testa una volta mi ha fatto una crepe", e poi non si può dimenticare che lui crede in me più di quanto ci creda io stesso
Stefano Salvucci uno che ha "visibilmente" cambiato la mia vita! Non Sara mai ringraziato abbastanza.
Camilleri e Crescenzo per il piacere di leggere all'estero sulla propria patria.
La società italo tedesca Dante alighieri, per avermi indicato quale via non devo percorrere
Wie könnte ich Marie und Karsten vergessen???
Frau Voß und Frau David weil sie mich sehr viel geholfen haben mit mein deutsche!!

All those people that I met and lost because I got something from them and at the end I didn't lose that much.

Ilonka wie eine Mutter, Andre' wie ein Bruder, Jürgen weil wenn ich ihn anckuche ich sehe mich in 35 Jahr.

Roman und Robert weil für den ich bin ein Fußballgott!!

Vineeth e Charles to gave an English form to this thesis

Vins per quanto ci siamo scritti

Luigino e Catia per il legame alla terra

Marco Mauro e Emanuela compagni di viaggio a distanza

Mauro perché l'amicizia quand´é vera e sincera fa anche male

Non ringraziare Eleonora sarebbe un delitto, dopo tutto quello che ha fatto per me

E che Simona ce la vogliamo dimenticare?

Barbara il mio contatto con la realtà marchigiana.

Luana che mi a fatto una sorpresa super per il mio compleanno

Beatrice??? Certo grazie Bea! Un 'altra Amelie...

E che ci volgiamo dimenticare Fabrizio Lara e Sara???? Suvvia non siamo stupidi

Skevos, parce que je t'ai perdu

Davide Aldo e Stefano che una volta sono venuti a Dresda e ancora sto a ride

Davide per quando tornavo in italia, e poi che dire di uno che fa il fratello al posto tuo?

Andrea per aver alimentato i miei sogni di gloria

Moreno perché per me ci sarà sempre un posto da gommista, almeno spero.....

E che dire di Gabriele e Cristiana??? Due che ti vengono a prendere a casa!!

Il mio-nostro gatto Max, perché sta li sempre tranquillo ogni tanto rompe e spesso fa le fusa

Sara l´altra gatta perché il primo amore.....

Zio Valerio perché: "se ci sono problemi, chiama!"

Per le seguenti persone potrei scrivere capitoli di ringraziamenti tanto il mio cuore tracima di amore e riconoscenza nei loro confronti, ma sarei ancora pi mieloso di quello che giá sono stato fin ora, e poi loro giá sanno...

Aldo che dire di uno che oltre a essere mio fratello ti sostituisce anche come figlio?

Mamma perché é mamma

Papà perché é papà

Nonna Gagliarda perché é sempre gagliarda nonostante l'etá, ve ce vorrei vedé a voi!

Particularly I want to thanks all the people that hading know about my condition they suggested me to give up!

Si dice che ogni grande uomo ha accanto a se una grande donna, io non credo di essere un grande uomo, al massimo un alto, ma ciò nonostante al mio fianco c´é davvero una grande donna, che é la causa di tutto questo, ma proprio di tutto, grazie a Dio c´é Yvonne

E poi infine....

Grazie speciale a Dio che c´é e se scrivo questo dottorato ne é la prova, una delle tante....

There would be many many other people to thanks and other things to say but let stop it.