Summary: pre-eclampsia

The parent-of-origin effect in pre-eclampsia

This thesis deals with the identification of STOX1, the pre-eclampsia susceptibility gene found in Dutch females. This gene is located on chromosome 10q22, a region subject to a parent-of-origin effect, described in Chapter 2.

One gene in the cluster of genes with downregulation in the androgenetic placenta, indicative of a parent-of-origin effect, was identified being imprinted, namely CTNNA3 (Chapter 3). The identification of the pre-eclampsia susceptibility gene itself is described in Chapter 4. Although STOX1 does not show differential methylation of its CpG island (Chapter 5), which would be a proof of imprinting of STOX1, and would explain the parent-of-origin effect, STOX1 is able to transactivate effector genes (Chapter 6). One of these effector genes is CTNNA3, imprinted in villous trophoblasts, the trophoblasts from which extravillous trophoblasts originate, but which are localized in the villi of the placenta. Therefore, instead of being imprinted itself, the parent-of-origin effect seen for STOX1 might be caused by imprinted genes downstream of STOX1 in the signal transduction pathway. Another possibility is that the differentially methylated region of STOX1 does not lie within its own CpG island, but is controlled from another region. This can be identified by investigating the acetylation and methylation status of histone H3 surrounding the STOX1 chromosomal location. Preliminary results did not show this kind of acetylation and methylation, but when the effects, like CTNNA3, are cell type-specific, imprinting of STOX1 itself cannot yet be ruled out [1].

Effectors of STOX1

Chapter 6 shows the cell type-specific, allele-dependent transactivational effects of STOX1 on CTNNA3 and LRRTM3 expression. The mutant form of STOX1, containing the predominant mutation (Y153H) found in pre-eclamptic patients, hereby shows a three fold increase in mRNA expression of these genes compared to the wildtype form of STOX1 in extravillous trophoblasts. These effects were not seen in term villous trophoblasts. CTNNA3 is involved in cell-cell adhesion via E-cadherin [2]. E-cadherin itself has been shown to be upregulated in pre-eclamptic placentas [3]. Leucine Rich Repeat proteins participate in many biologically important processes, such as hormone-receptor interactions, cellular trafficking and cell-cell adhesion. Therefore, LRRTM3 might have a function similar to CTNNA3 [4].

A third gene, identified by microarray analysis (Chapter 6), gives another indication for a pathway in which STOX1 is involved; the KLC1 gene has a role in microtubule cargo transport [5].

STOX1 regulation by the PI3K-Akt pathway

The regulation of STOX1 on transcription of CTNNA3 and LRRTM3 has been investigated as well (Chapter 6); similarities between STOX1 and members of the FOX (forkhead) genes pointed to the PI3K-Akt pathway. Evidence for involvement of this pathway in the activation of STOX1 was found by modifying possible Akt phosphorylation sites in the STOX1 gene. This showed reduction of nuclear expression as well as reduced transactivational activities.

STOX1 in the processing of amyloid precursor protein

Chapter 7 describes STOX1 in relation to late-onset Alzheimer’s disease (LOAD). STOX1 levels were increased in the hippocampus of LOAD patients. This implicates that STOX1 operates in the brain as well, i.e. in the promotion of APP (amyloid precursor protein) processing via increased LRRTM3 expression. This subsequently leads to increased BACE1 activity, one of the secretases responsible for APP processing giving rise to increased amyloid β levels. The transactivation effect of STOX1 on LRRTM3 expression in neuroblastomas was not dependent on the STOX1 allele carried (Y153 or H153) (unpublished observations), another indication of the cell type-specificity of the allele-dependent transactivation effects seen in extravillous trophoblasts. CTNNA3, like LRRTM3, was also upregulated in neuroblastomas after STOX1 transfection (unpublished observations). The APP protein, like CTNNA3, is furthermore involved in cell-cell adhesion [6]. Although the process of cell-cell adhesion itself most likely is not important in neurons, since they are not proliferative nor invasive, it does indicate there might be a function for APP in the placenta. Interestingly, Presenilin-1 (PS1), component of the γ-secretase complex responsible for the cleavage of βCTF to amyloid β, is also a stabilizing factor of the cadherin cell-cell adhesion complex [7]. The cell-cell adhesion complex also contains α-catenins, of which αT-catenin, transcribed by CTNNA3, is one of the members and upregulated after STOX1 transfection in the extravillous trophoblast and neuroblastoma cell lines. PS1 is also involved...
in the ubiquitination of β-catenin, another part of the cell-cell adhesion complex [8]. β-catenin can be found in different pools within the cell which are able to exchange the proteins [9], so this involvement of PS1 in the cell-cell adhesion might not be of great importance. PS1, however, not only stabilizes the complex; under conditions stimulating apoptosis, PS1 cleaves cadherin, together with α-secretase ADAM10, inducing disassembly of the cell-cell adhesion complex [7]. The upregulation of αT-catenin after STOX1 transfection, might influence the stability of the cell-cell adhesion complex and thereby stimulating apoptosis via PS1 and α-secretase cleavage.

**STOX1 in microtubuli**

*Chapter 4* describes the finding of abundant STOX1 expression in the nuclei of polyploid cells. These cells have undergone endoreduplication whereby the chromosomes have multiplied without the cells dividing. In trophoblast cells this leads to cells changing from an invasive to a non-invasive phenotype [10,11]. In the light of pre-eclampsia, this is an indication that STOX1 is involved in changes in invasive extravillous trophoblasts leading to less invasion. This indicates that normal STOX1 expression, indirectly, inhibits polyploidization of extravillous trophoblasts. The STOX1 expression in the microtubuli of dividing cells, found in both trophoblasts and neuroblastomas (*Chapter 7*), is compatible with this process. The microtubule association is also described in *Chapter 6*, where the gene from the microarray analysis showing significant regulation by STOX1, *KLC1*, functions in the transport of cargos along the microtubules [5]. This gene has furthermore been linked to LOAD; a SNP (single nucleotide polymorphism) within intron 13 was significantly associated with Alzheimer’s disease [12]. Interestingly, endoreduplication is also mentioned in relation to Alzheimer’s disease; neurons containing amyloid β are, under influence of DNA polymerase β, able to go into endoreduplication [13]. This causes neuronal cell death leading to Alzheimer’s disease. The STOX1-microtubuli connection can also be based on the cytoplasmic STOX1 expression in hippocampal neurons containing tau tangles (*Chapter 7*), representing inactive STOX1. Tau itself is a microtubule component, phosphorylated in neurofibrillary tangles [14]. This finding indicates an interaction between STOX1 and tau.

![Figure 1: Summarizing model of the functions of STOX1 and the pathways in which STOX1 operates.](image-url)
STOX1 as a marker in pre-eclampsia and LOAD

The STOX1 protein already showed upregulated expression in people not yet diagnosed with dementia (Chapter 7). This shows that STOX1 can be used to recognize early patho-physiological changes in the development of Alzheimer’s disease. STOX1 does not seem suitable as a general biomarker for pre-eclampsia; preliminary data could not detect differences in expression levels between normal and pre-eclamptic term placentas or early placentas in correlation with the STOX1 Y153 genotype. This lack of difference in STOX1 expression levels is most likely due to cell type-specific effects, already seen in the transactivation effect on CTNNA3 and LRRTM3 (Chapter 6). These effects could only be detected in the early extravillous trophoblast cell line. The transactivated genes themselves, for this reason, will also be no suitable pre-eclampsia early detection markers. To function as a biomarker, STOX1, or its effector genes, must be measured in the cell type in which the influence of the Y153H mutation and the influence on effector genes is detected to circumvent the masking effects of other cells.

A model of STOX1 function

Below a model can be found summarizing the functions of STOX1 and pathways in which STOX1 operates, all described in this thesis and summarized above (Fig. 1).

DIRECTIONS FOR FUTURE RESEARCH

Imprinting of STOX1

To investigate if STOX1 itself is subject to imprinting, the methylation and acetylation status of histone H3 of the chromosomal area of STOX1 must be studied. To be certain if imprinting occurs, since preliminary data could not detect different methylation and acetylation in whole placenta samples, histone H3 status must be studied in individual cell types, most importantly extravillous trophoblasts.

Identifying STOX1 effector genes and pathways

Up to now three genes have been identified being effector genes of STOX1, i.e. CTNNA3, LRRTM3 and KLC1. Since the microarray data obtained in Chapter 6 were not corrected for transfection efficiency their usability was limited. To identify more genes up- and downstream in the pathway of STOX1, a new microarray must be performed that uses stable transfected trophoblast cells to obtain data that can be studied in more detail. Another possibility is using chromatin immuno precipitation (ChIP). This can yield, next to effector genes of STOX1, also a general DNA binding sequence used by STOX1. When by either method more genes are identified, more insight is given in the pathways in which STOX1 operates, as well as genes downstream of STOX1 responsible for the parent-of-origin effect seen for STOX1. The genes found may coincide with genes on other pre-eclampsia susceptibility chromosomal loci found in other populations. This might even lead to finding the other pre-eclamptic factors contributing, thereby explaining the complete pre-eclamptic phenotype in patients with the STOX1 Y153H mutation since this mutation is not fully penetrant.

STOX1 regulation

Although it seems that STOX1 is regulated by the PI3K-Akt pathway, other regulatory mechanisms most likely exist as well. Either as other upstream pathways or for instance as regulation by microRNAs [15]. MicroRNAs can be either located within the STOX1 gene, thereby regulating STOX1 expression or the expression of other genes, or microRNAs can be found on other chromosomal loci that directly act on STOX1 expression. Over the last years, different prediction programs have been published that make it possible to predict microRNA target sides within genes [16]. STOX1 does contain potential microRNA target sequences for microRNAs [17], of which some are preferentially expressed in placenta [18], making them interesting candidates to study in more detail, especially because differential expression of several microRNAs has been identified in pre-eclamptic placentas [19]. Also algorithms have been developed which predict possible microRNAs located within DNA [16]. No potential microRNAs within the STOX1 DNA sequence have been identified up to this moment, but this has been done for the CTNNA3 gene. Two pre-microRNAs, the precursors of mature microRNAs, were identified by us, but their mature microRNAs have not yet been found due to problems related to the low expression of the pre-microRNAs in combination with the amount of sequencing of cloned small RNA products needed.

STOX1 in the cell cycle

To investigate the involvement of STOX1 in microtubuli, it is interesting to look at the influence of STOX1 on the cell cycle. The most interesting phases in the cell cycle regarding STOX1 to investigate are the anaphase of the mitosis, because of the STOX1 expression in microtubuli, and the skipping of mitosis leading
to polyploid trophoblast cells. Studies can be performed using siRNA knockdown of cell cycle regulators to determine their influence on STOX1 expression, or by knockdown of STOX1 to find out how this changes different checkpoints in the cell cycle. This can be analyzed by either expression studies or by using FACS (fluorescent-activated cell sorting) which can distinguish the different phases of the cell as well as identify polyploid cells.

Allele-dependent cell type-specific transactivation
The cell type-specific transactivational effects of STOX1 could only be identified in an in vitro cell system. An in vitro model (i.e. a trophoblast-decidua co-culture) that accounts for cellular interactions that occur in vivo in the early placenta would therefore be highly informative and important [20]. This will provide insight into the in vivo impact of the STOX1 Y153H mutation regarding its effects on the expression of CTNNA3, LRRTM3, KLC1 and other effector genes of STOX1 yet to be identified. This model can also be used to study cell type-specific effects by using the possibility to isolate different cell types by laser-capture microdissection.

STOX1 in trophoblast invasion
An in vitro model as described above can also be used to investigate the influence of STOX1 on trophoblast invasion. The advantage of this type of in vitro model compared to immunostaining of placental coupes is the possibility to manipulate conditions. This can be accomplished by down- or upregulation of STOX1 expression using siRNA or STOX1 transfection, respectively, but also by introducing different substances influencing migration and invasion of trophoblasts. Influence of STOX1 on trophoblast invasion can furthermore be accompanied by an altered epithelial-mesenchymal transition (EMT). These studies will yield information on the direct effects of STOX1 in the etiology of pre-eclampsia.

Animal models
Animal models can function as valuable tools in identifying phenotype changes caused by knockout of specific genes. For genes functioning in the placenta, like STOX1, there is a limitation to animal models, because the human placenta organisation is different from placentas of other species. The mouse is however frequently used as a knockout model, but it must first be investigated if STOX1 expression and functions are the same in mice as in the human placenta. When its functions do not correlate, a mouse model would not yield results that can be extrapolated to the human STOX1 function. If STOX1 functions do seem to be similar, it will be interesting to investigate how STOX1 knockout in the placenta influences trophoblast invasion and other pre-eclamptic phenotypes. Another interesting option is looking at STOX1 upregulation in vivo. This can be accomplished by micro-injection of a tagged STOX1 protein into a mouse placenta [21]. To study the influence of STOX1 in the hippocampus of mice a general knockout of STOX1 can be used. This will yield information regarding the functions of STOX1 in Alzheimer’s disease.

STOX1 in Alzheimer’s disease
Another focus for the future can be laid upon the influence of STOX1 in Alzheimer’s disease; is it just a partner in the processing of APP or can it influence the disease progression itself, for instance by mutations in STOX1 or mutations in members associated with the STOX1 pathways occurring in neurons? It does not seem, however, that the predominant pre-eclampsia mutation Y153H is involved in the pathiology of LOAD. It would, however, be interesting to look in more detail at the occurrence of severe early onset pre-eclampsia and the occurrence of LOAD in the population to see if one disease gives a predisposition for the other disease or might act as a protection.

Early detection markers based on STOX1
Finally, to obtain a non-invasive method for the early detection of pre-eclampsia, the expression levels (protein, RNA) ideally should be measurable in maternal serum or plasma [22]. For STOX1 or its effector genes to function as a biomarker in Alzheimer’s disease, their detection in CSF or blood is needed [23]. The identification of early detection markers will be simplified by elucidating the pathways and learning more about the functions of STOX1 regarding cell-cell adhesion and trophoblast invasion, its involvement in APP processing, its expression in microtubuli and its correlation with phosphorylated tau. This hopefully will eventually lead to points of action for treatment of these diseases.
REFERENCES


