FOXL2 represses the testis-specific enhancer of Sox9 to maintain ovary differentiation

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Für meine Eltern
I, Susanne Jakob, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

______________________________

Susanne Jakob
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List of Contents

Title page 1

Dedication 2

Declaration 3

Acknowledgements 4

List of Contents 5

List of Figures 9

List of Abbreviations 11

Abstract 13

1 Introduction 16

1.1 Mechanisms of sex determination ......................... 16

1.2 Overview of murine gonadogenesis ........................ 19

1.3 Early gonadal development ............................... 26

1.4 Taking the route towards testes ................. 29

1.4.1 Testis determination: Sry .......................... 29

1.4.2 Testis differentiation: establishment of Sertoli cells 36

1.5 Determined to develop as female ...................... 41

1.5.1 Ovarian determining or anti-testis genes .......... 41

1.5.2 Ovarian development ............................... 52

1.6 Outline of this thesis ............................... 55
2 Materials and Methods

2.1 Sequence analysis .................................. 57
2.2 Luciferase Assay ...................................... 58
  2.2.1 Cloning of constructs ............................. 58
  2.2.2 Cell culture ...................................... 59
  2.2.3 Transfection assays .............................. 60
2.3 Mouse Lines .......................................... 61
  2.3.1 Husbandry ....................................... 61
  2.3.2 Genotyping ...................................... 61
  2.3.3 Mouse lines ..................................... 62
  2.3.4 Harvesting of gonads ......................... 65
2.4 Immunohistochemistry ................................. 65
  2.4.1 Preparation of samples ......................... 65
  2.4.2 Histology ....................................... 66
  2.4.3 Immunostaining .................................. 66
  2.4.4 Imaging ......................................... 68
2.5 Protein interaction assays ............................ 68
  2.5.1 In vitro translation of $^{35}$S-SF1 ............... 68
  2.5.2 Preparation of GST-FOXL2 ..................... 69
  2.5.3 Co-Immunoprecipitation ....................... 70
  2.5.4 Nuclear protein extract ................. 70
  2.5.5 Electrophoretic Mobility Shift Assay (EMSA) .... 71

3 TESCO modulation by ovarian determining genes in vitro

3.1 Introduction ........................................ 73
3.2 Results ............................................. 76
  3.2.1 Sequence analysis of TESCO ................. 76
  3.2.2 Repression of TESCO in vitro ............. 78
3.3 Conclusions ........................................ 87
### 4 Possible mechanisms of TESCO modulation by FOXL2

#### 4.1 Introduction

#### 4.2 Results

- **4.2.1 Analyses of mutations in TESCO binding sites**
- **4.2.2 Analyses of interactions between FOXL2 and SF1**

#### 4.3 Conclusions

### 5 The effect of Foxl2 on TESCO activity in vivo

#### 5.1 Introduction

#### 5.2 Results

- **5.2.1 De-repression of TESCO in Foxl2 mutant mice**
- **5.2.2 De-repression of SOX9 in Foxl2 mutant mice**
- **5.2.3 The impact of oocyte depletion on the supporting cell lineage**

#### 5.3 Conclusions

### 6 The effect of Wnt4 on TESCO activity in vivo

#### 6.1 Introduction

#### 6.2 Results

- **6.2.1 The de-repression of TESCO in Wnt4 mutant mice**
- **6.2.2 SOXE expression in Wnt4 mutant mice**
- **6.2.3 De-repression of TESCO and endogenous Sox9 expression in Foxl2/Wnt4 double mutant mice**

#### 6.3 Conclusions

### 7 General Discussion

#### 7.1 The ovarian-promoting genes Dax1, Sox4 and Foxl2 repress TESCO activity in vitro

#### 7.2 FOXL2 represses TESCO activity synergistically with ERα

#### 7.3 FOXL2 interferes with SF1-mediated activation of TESCO in vitro
7.4 FOXL2 represses TESCO activity and endogenous Sox9 expression postnatally ........................................ 160
7.5 Sox9 expression is not up-regulated in oocyte-depleted ovaries ......................................................... 164
7.6 WNT4 signalling partially represses TESCO activity during embryonic development ......................... 166
7.7 Sox8 and/or Sox10 expression is de-repressed in embryonic Wnt4 mutant XX gonads ............................ 168
7.8 Additional factors must be involved in repressing Sox9 in embryonic XX gonads ............................... 171
7.9 Summary ........................................................................................................................................ 175

8 Appendices ........................................................................................................................................ 177
8.1 Plasmid maps .................................................................................................................................... 178
8.1.1 pcDNA-Foxl2 ............................................................................................................................ 178
8.1.2 pcDNA-Dax1 ............................................................................................................................ 179
8.1.3 pcDNA-Sox4 ........................................................................................................................... 180
8.2 PCR-protocols ................................................................................................................................ 181
8.2.1 CFP PCR ................................................................................................................................... 181
8.2.2 Foxl2 PCR ............................................................................................................................... 182
8.2.3 Wnt4 PCR .................................................................................................................................. 183
8.2.4 Cre PCR ...................................................................................................................................... 184
8.3 TESCO sequence alignment .............................................................................................................. 185
8.4 TESCO deletion constructs ............................................................................................................... 188

Bibliography ......................................................................................................................................... 189
List of Figures

1.1 The early development of testis and ovary ................. 25
1.2 Model of genetic interactions during testis development .... 35
1.3 Model for Sox9 regulation via TESCO in XY gonads .......... 39
1.4 Model of ovarian follicle development .................. 54
1.5 Model of distinct Sox9 regulation in XX and XY gonads during early development .......................... 56

3.1 Sequence analysis of TESCO ............................... 77
3.2 Repression of TESCO by DAX1 in vitro .................... 79
3.3 Repression of TESCO by SOX4 in vitro .................. 80
3.4 Repression of TESCO by FOXL2 and in vitro ................ 82
3.5 Repression of TESCO by ERα in vitro .................... 83
3.6 Effect of β-catenin and LEF/TCFs on TESCO activation in vitro ................. 85
3.7 Effect of β-catenin on FOXL2 mediated repression of TESCO in vitro ................. 86

4.1 The FOXL2 protein ........................................... 93
4.2 Effect of FOXL2 on TESCO with 2 mutated FOX sites .... 96
4.3 Additional consensus FOX sites in the TESCO sequence .... 98
4.4 Repression effect of FOXL2 on TESCO deletion constructs .... 99
4.5 Repression effect of FOXL2 on TESCO with mutations in all consensus FOX sites .......... 100
4.6 Repression effect of FOXL2 + ERα on TESCO with all FOX/ERE sites mutated ................. 100
4.7 Effect of excess amounts of vector on FOXL2 mediated repression of TESCO .......................................................... 102
4.8 Effect of FOXL2 on SF1-mediated activation .......................................................... 104
4.9 Direct physical interaction between FOXL2 and SF1 .......................................................... 104
4.10 FOXL2 interferes with SF1 binding to DNA .......................................................... 105
5.1 CFP expression in the TESCO:CFP reporter line in vivo ................................................. 113
5.2 Morphology of XX Foxl2^{LacZ/LacZ} gonads .......................................................... 116
5.3 TESCO activity in TESCO:CFP; Foxl2^{LacZ/LacZ} gonads during embryogenesis ............................... 117
5.4 TESCO activity in postnatal TESCO:CFP; Foxl2^{LacZ/LacZ} gonads ........................................... 119
5.5 Endogenous Sox9 expression in postnatal TESCO:CFP; Foxl2^{LacZ/LacZ} gonads ................... 121
5.6 Co-localisation of SOX9 and β-galactosidase .......................................................... 123
5.7 Sox9 expression in gonads of Zp3:Cre; R26DTA mice .................................................. 126
6.1 TESCO activity in TESCO:CFP; Wnt4^{-/-} gonads during embryogenesis ............................................... 138
6.2 FOXL2 and CFP do not co-localise in TESCO:CFP; Wnt4^{-/-} gonads ......................... 139
6.3 Endogenous Sox9 expression in TESCO:CFP; Wnt4^{-/-} gonads during embryogenesis ....................... 142
6.4 Endogenous SoxE expression in TESCO:CFP; Wnt4^{-/-} gonads during embryogenesis ....................... 143
6.5 TESCO activity in TESCO:CFP; Foxl2^{LacZ/LacZ}; Wnt4^{-/-} double mutant gonads at P0 .................. 145
6.6 Summary of in vivo data .................................................................................. 149
7.1 Model of possible interactions between FOXL2 and SF1 to regulate Sox9 expression in XX gonads .................................................................................................................. 159
7.2 A construct for conditional misexpression of Foxl2 ........................................................................... 164
7.3 Model of genetic interactions during ovarian development ...................................................... 174
7.4 Model of the regulation of gonadal Sox9 expression .......................................................... 176
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Δ</td>
<td>floxed out or deleted allele</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
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<tr>
<td>µm</td>
<td>micrometre</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BPB</td>
<td>bromphenol blue</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
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<tr>
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<td>Curie</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>di-amino-phenyl-indol</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>dpc</td>
<td>day(s) post coitum</td>
</tr>
<tr>
<td>dpp</td>
<td>day(s) post partum</td>
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DTA  diphtheria toxin fragment A
DTT  dithiothreitol
ECFP  enhanced cyan fluorescent protein
EDTA  ethylen diamine tetraacetic acid
EGFP  enhanced green fluorescent protein
EGTA  ethylen glycol tetraacetic acid
EMSA  electrophoretic mobility shift assay
ERT  estrogen receptor for tamoxifen
ESD  environmental sex determination
EYFP  enhanced yellow fluorescent protein
FCS  fetal calf serum
flox  flanked by loxP sites
GFP  green fluorescent protein
GoF  gain of function
GSD  genetic sex determination
GST  glutathion-S-transferase
H+E  haematoxylin-eosin
HA  hemagglutinin epitope tag
HEPES  4-2-hydrorxyethyl-1-piperazineethanesulfonic acid
HMG  high mobility group
hsp  heat shock protein
IgG  immunoglobulin G
IP  immunoprecipitation
kb  kilo base
LacZ  β-galactisodase gene
LB  Luria Broth
LoF  loss of function
loxP  locus of cross-over P
M  molar
<table>
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>NDS</td>
<td>normal donkey serum</td>
</tr>
<tr>
<td>neo</td>
<td>neomycin gene</td>
</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>OCT</td>
<td>optimal cutting temperature</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>ONPG</td>
<td>O-Nitrophenyl-β-D-galactopyranosid</td>
</tr>
<tr>
<td>pA</td>
<td>polyadenylation</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformadehyde</td>
</tr>
<tr>
<td>PGC</td>
<td>primordial germ cell</td>
</tr>
<tr>
<td>PGK</td>
<td>phosphoglycerine kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethysulphonyl fluoride</td>
</tr>
<tr>
<td>PTM</td>
<td>peritubular myoid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>ssc</td>
<td>side chain cleavage</td>
</tr>
<tr>
<td>TBE</td>
<td>tris borate EDTA</td>
</tr>
<tr>
<td>TES</td>
<td>testis specific enhancer of Sox9</td>
</tr>
<tr>
<td>TESCO</td>
<td>testis specific enhancer of Sox9 core element</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSD</td>
<td>temperature dependent sex determination</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>VP</td>
<td>vaginal plug</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume</td>
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</table>
Abstract

During normal testis development, SRY and SF1 up-regulate Sox9 expression via its testis-specific enhancer element (TESCO), which is crucial for the establishment of the male supporting cell lineage, the Sertoli cells. In contrast, during normal ovarian development, Sox9 expression needs to be down-regulated in the female supporting cells and failure results in XX female-to-male sex reversal. It was not known whether the repression of Sox9 expression is mediated via the TESCO regulatory element in XX gonads. FOXL2 is a forkhead transcription factor, expressed in the supporting cells of the ovary, but not testis, from 12.5 dpc onwards. Homozygous mutation of Foxl2 results in the up-regulation of Sox9 and other testis-specific genes in postnatal gonads, suggesting that FOXL2 plays an important role in proper ovarian development by maintaining the repression of Sox9. In this thesis, in vitro analyses demonstrate that FOXL2 can repress TESCO activity (as can other candidate ovarian determining genes, such as Dax1 and Sox4). The repressing effect of FOXL2 is more severe in the presence of ERα. Several forkhead factor binding sites and EREs are present in the TESCO sequence, however mutation analyses showed that the repression effect is not mediated only through these. Moreover, FOXL2 can interact with SF1 and interfere with its ability to activate TESCO in vitro. In vivo studies showed that homozygous loss of Foxl2 results in the de-repression of TESCO activity in the remaining granulosa-like cells in the XX gonads, which correlates with the expression of endogenous Sox9. However, this de-repression occurs only after birth, getting more severe with age, suggesting that Foxl2 is the critical factor to re-
press TESCO activity in the adult ovary. Moreover, evidence is provided that the de-repression of Sox9 is independent from oocyte-depletion and solely due to the loss of Foxl2 as targeted deletion of oocytes did not result in an up-regulation of Sox9 expression. Further in vivo analyses indicate the involvement of Wnt signalling in the repression of TESCO activity during embryonic development, as TESCO becomes partially de-repressed in Wnt4 homozygous mutant mice before birth.
Chapter 1

Introduction

1.1 Mechanisms of sex determination

During evolution, the complex living organisms on earth have evolved through generations of sexual reproduction, whereas asexual organisms mostly remain primitive and simple. The benefit of sexual reproduction comprises genetic variation, leading to the uniqueness of each individual, which is fundamental to the success of the species.

The common precondition for sexual reproduction is the establishment of different sexes in one species. While in the majority of vertebrates all individuals are phenotypically identical at the beginning of embryogenesis, each embryo has to take the decision to develop into one sex, and not the other, at a specific time point during its development. This event is called sex determination. Sex determination happens in the gonads of the embryo, more precisely in the somatic cells of the gonad, and describes the decision of the originally bi-potential gonad to develop either into a testis or an ovary. Once this switch has been made, the
course is set for the rest of the embryo to develop either as a male or a female individual. However, many invertebrate species and some vertebrates, e.g. some fish species, develop as hermaphrodites which can produce both sperm and eggs either simultaneously or at successive life stages. In most species, sex determination is achieved during embryonic development, though there are examples of individuals, e.g. in some fish species, which are able change their sexual phenotype during adult life.

The triggers through which sex determination is achieved in different species are manifold. The switch mechanism can dependent on either environmental or genetic factors. Environmental sex determination (ESD) is based on the identical genetic makeup of all individuals and environmental events triggering distinct sexual phenotypes. In contrast, genetic sex determination (GSD) relies on the differences in chromosomal constitution (or even in a single gene) between individuals, that is the presence of distinct sex chromosomes.

A well studied form of ESD is temperature dependent sex determination (TSD) in which the temperature during a critical period of egg incubation determines the sex of the offspring, e.g. in crocodiles, alligators, most turtles and some lizards. For example, the red-eared slider turtle (*Trachemys scripta*) develops as male at an egg incubation temperature lower than 26°C and as female at temperatures higher than 31°C. Between 26°C and 31°C both sexes can develop with the percentage of females increasing with rising temperature ([Pieau et al., 1999](#)). Other ESD mechanisms have been described to depend on group dynamics including visual and hormonal signals, e.g. in Clown fish (*Amphiprion ocellaris*) (for review see [Gilbert, 2006](#)).
In contrast to ESD, GSD is determined by the genetic content or chromosomal constitution of each individual and has been found in, amongst others, worms, insects and most vertebrate species. In some species, the development of distinct sexual phenotypes depends on the presence of a haploid or a diploid genome (e.g. in bees and ants). In others, specific sex chromosomes have evolved leading to chromosomal differences between the sexes. Here, the sex determining switch can be controlled by a dosage-dependent mechanism (DSD) or the presence of a dominant gene on one of the sex chromosomes. DSD can be found in *C. elegans* and *D. melanogaster*, in which the sex determination switch is controlled by the ratio of X chromosomes to autosomes (although e.g. *Drosophila* has a Y chromosome which is required for male fertility but not for sex determination). Sex determining mechanisms which rely on sex-specific chromosomes have been described in the ZZ/ZW system (e.g. in some insects, fish, reptiles and birds) and the XX/XY system (e.g. in some fish, amphibians, reptiles and all known mammals). In the ZZ/ZW system, homogametic ZZ embryos develop as males whereas heterogametic ZW embryos develop as females. For a long time, two possible mechanisms of sex determination have been discussed in this system: a dosage-dependent effect of Z chromosome gene(s) and the activity of female-specific gene(s) from the W chromosome. Recently, it has been shown in chick that the Z-linked gene *Dmrt1* is essential for male sex determination ([Smith et al., 2009](#)) supporting the Z-dosage dependent hypothesis. However, it is still not known whether *Dmrt1* is sufficient to induce male development. Furthermore, it is also possible that the role of *Dmrt1* is to antagonise crucial gene(s) on the W chromosome which might be necessary for female development. In the mammalian XX/XY system, the Y
chromosome is the dominant male determinant and males are heterogametic XY whereas females are homogametic XX. The first hints for the importance of the Y chromosome for mammalian sex determination came from studies in human patients with Turner (45, X) and Klinefelter (47, XXY) syndrome, where XO individuals develop as females and XXY individuals develop as males (Ford et al., 1959; Jacobs and Strong, 1959). The same correlations were found in studies in mice (Welshons and Russell, 1959). These findings led to the proposal of the Y chromosome as the male determinant in mice and men. The subsequent quest for the specific testis determining factor (TDF) on the Y chromosome went on for 30 more years. Finally, in 1990, the analysis of several XX male patients led to the discovery of a conserved 35 kb region on the Y chromosome. Within this region, the male determining gene was identified by positional cloning and named SRY for ”sex-determining region of the Y chromosome” (Sinclair et al., 1990; Gubbay et al., 1990).

1.2 Overview of murine gonadogenesis

In mice, SRY stands at the top of a sex determination cascade, being the switch between female and male development. Before going into detail about how the presence or absence of SRY triggers the molecular events that will eventually result in the formation of either a testis or an ovary, a closer look should be taken at the morphology of the embryonic gonad as the place were primary sex determination takes place. The morphological development of murine gonads can be divided into two phases: the development of a bi-potential gonad which is
morphological identical in both sexes and the subsequent sex-specific differentiation into either a testis or an ovary following the time point of sex determination around 11.5 dpc (days post coitum).

In the early embryo, the undifferentiated genital ridge can be recognised as a thickening of the coelomic epithelium adjacent to the mesonephros around 10.0 dpc. Shortly afterwards, primordial germ cells (PGCs) start to colonise the gonads (Figure 1.1). PGCs emerge as a cluster in the region posterior to the primitive streak at 7.25 dpc and then proliferate and migrate through the gut mesentery to enter the genital ridge between 10 and 11 dpc (Ginsburg et al., 1990). PGCs are indifferent between the sexes when they enter the genital ridge, and the special environment of the gonad is needed for adequate differentiation into either spermatogonia or oogonia (Saitou et al., 2002). In the male, germ cells then enter mitotic arrest in G0/G1 around 13.5 dpc until perinatal stage, whereas in the female they enter meiotic prophase as oocytes around 13.5 dpc and will then arrest in diplotene phase around the time of birth (McLaren, 1984).

While the germ cells originate outside of the genital ridge, the somatic cells of the gonad are thought to arise from proliferation of the coelomic epithelium. The somatic cells in the developing gonad are categorised into three major cell lineages based on their cell fate after sex determination. Supporting cell precursors develop into Sertoli cells in the male and follicle (granulosa) cells in the female. This cell lineage was named after their function to support and maintain the germ cells and is most crucial for gonad differentiation in both sexes. Steroidogenic cell precursors develop into Leydig cells in the male and theca cells in the female.
Finally, the connective cell lineages which are involved in the formation of the organ as a whole show distinct testicular and ovarian patterns of differentiation. At the time of sex determination in XY gonads, Sry is expressed exclusively in supporting cell precursors, which originate from the coelomic epithelium, and directs their fate to become Sertoli cells (Koopman et al., 1990; Karl and Capel, 1998). Directly after initiation of Sry expression, proliferation of coelomic epithelial cells increases, a phenomenon specific to XY gonads (Schmahl et al., 2000). During this male-specific proliferation, cells from the epithelium delaminate and give rise to Sertoli cells and an interstitial cell population (Karl and Capel, 1998; Schmahl et al., 2000). The differentiating Sertoli cells then enclose the germ cells and form testis cords. These eventually give rise to the seminiferous tubules characteristic of the testis (Figure 1.1) (Brennan et al., 2002).

Leydig cells differentiate in the interstitium between the testis cords around 12.5 dpc. The function of Leydig cells is to produce testosterone and thereby guide the masculinisation of the whole embryo. In contrast to Sertoli cells, the origin of fetal Leydig cells is not clear yet. Some evidence suggests that Leydig cell precursors could arise from the coelomic epithelium (Brennan et al., 2003), whereas other data suggests that Leydig precursor cells originate from the mesonephros and migrate into the gonad before 11.5 dpc where they remain undifferentiated until 12.5 dpc (Jeays-Ward et al., 2003). As cell division is rarely detected among differentiated Leydig cells, it has been proposed that the increase in the fetal Leydig cell population depends on an undifferentiated pool of progenitor cells in the gonadal interstitium which continuously proliferates and differentiates (Ortl, 1987). Recent studies strongly support this model.
and show that Notch signalling is involved in the regulation of the maintenance of these fetal Leydig progenitor cells (Tang et al., 2008). Furthermore, signalling molecules from Sertoli cells have been implicated to be involved in the differentiation of fetal Leydig cells, such as Dhh (Desert hedgehog) and PDGF signalling (Platelet-Derived Growth Factor) (Yao et al., 2002; Brennan et al., 2003; Schmahl et al., 2008). The fetal Leydig cell population is lost at birth and replaced by an adult population which forms from interstitial mesenchymal cells during puberty (Davidoff et al., 2004). It has been hypothesised that the adult Leydig cells might differentiate from the same Leydig cell progenitor cell pool as the fetal Leydig cells (Habert et al., 2001; Tang et al., 2008).

In the XY gonad, testis-specific peritubular myoid (PTM) cells can be found, which surround the testis cords and thereby separate the cords from the interstitium (Figure 1.1). Originally these cells were thought to emerge from the mesonephros (Merchant-Larios and Moreno-Mendoza, 1998), but recent studies have shown that the PTM cells do not migrate from the mesonephros into the gonad between 11.5 dpc and 12.5 dpc (Cool et al., 2008; Combes et al., 2009). It has then been hypothesised that PTM cells and other interstitial lineages (except endothelial cells) are induced within the gonad.

Endothelial cells start migrating into the XY gonad from a large vascular plexus in the mesonephros around 11.5 dpc (Coveney et al., 2008). These individual cells migrate directly towards the coelomic epithelial domain of the gonad where they aggregate and from the male-specific coelomic vessel around 12.5 dpc. The migrating endothelial cells have been suggested to direct the formation of the developing testis cords (Combes et al., 2009). It has also been suggested that
testicular blood vessels promote efficient export of testosterone out of the developing testis, as well as supplying oxygen to the rapidly growing tissue (Ross and Capel, 2005).

In the XX gonad, supporting cell precursors differentiate into follicle (granulosa) cells whereas the steroidogenic cell lineage gives rise to theca cells (Figure 1.1). Both cell types are involved in the production of estrogens. Although endothelial cells migrate from the mesonephric vascular plexus into the gonad from 11.5 dpc, no vascular pattern is obvious in the XX gonad at 12.5 dpc (Coveney et al., 2008). It has been suggested that the developing vasculature in XX gonads results from proliferation and extension of the primordial gonadal vasculature (Coveney et al., 2008). In the ovary, most of the morphological reorganisation occurs after birth. However, some morphological changes already start at the time of the entry of the germ cells into meiotic prophase around 13.5 dpc. At this time, oocytes become associated in clusters which are called germ cell nests or cysts (Gomperts et al., 1994; Pepling and Spradling, 2001). Each cluster of oocytes is surrounded by a layer of pre-granulosa cells. The cysts break down around the time of birth and while approximately two-third of the oocytes undergo programmed cell death, the remainder individually become surrounded by somatic pre-granulosa cells and form primordial follicles (Pepling and Spradling, 2001).

The internal reproductive tract and associated organs are derived from a pair of genital ducts, the Müllerian and Wolffian ducts, which are initially present in both sexes (Figure 1.1). The Wolffian ducts develop from the intermediate mesoderm and are completed by 10.5 dpc. In XY mice, they differentiate into
vas deferens, epididymis and seminal vesicles, whereas they regress in XX. The Müllerian ducts start to develop around 11.5 dpc. They give rise to oviduct, uterus and the upper parts of the vagina in XX mice, whilst they degenerate in XY due to the presence of anti-Müllerian hormone (AMH) made by Sertoli cells. It has been reported that the Müllerian duct needs the presence of the Wolffian duct to form (Gruenwald, 1941). However, the Wolffian duct does not contribute cells to the Müllerian duct, but is thought to provide essential (though still unknown) signals to guide its development (Orvis and Behringer, 2007).
The early development of testis and ovary.

(a) The genital ridge forms as a thickening of the coelomic epithelium around 10.0 dpc and primordial germ cells (pink) enter between 10.0 and 11.0 dpc. Both, the Müllerian and the Wolffian duct form initially in the mesonephros underlying the bi-potential gonad. In XY gonads, Sry is expressed between 10.75 and 12.5 dpc and initiates testis development: differentiation of supporting cell precursors to Sertoli cells (green) which, together with the peritubular myoid cells (orange), encircle the germ cells (purple) to form testis cords. In between the testis cords develop the male steroidogenic cell lineage: the Leydig cells (blue). The male-specific vasculature (red) forms and vessels branch between the testis cords. In contrast, no significant morphological changes are visible in XX gonads during this period. Adapted from [Ross and Capel (2005)](https://www.nature.com/articles/nature02638). 

(b) The distribution of germ cells (purple, marked by STELLA) and supporting cells (green, marked by FOXL2 in XX gonads and SOX9 in XY gonads) in the embryonic gonad at 13.5 dpc. Scale bar = 100μm
1.3 Early gonadal development

In the indifferent gonad, several genes are expressed in both sexes from 9.5 dpc onwards, including Wilms’ tumour suppressor gene 1 (Wt1), steroidogenic factor 1 (Sf1), LIM homeodomain protein 9 (Lhx9), M33 and Emx2. In mice, functional mutations of these genes cause gonadal agenesis, indicating that they are required for the early formation of the bi-potential gonad prior to the time of sex determination.

Wt1 was originally identified as a gene involved in infant kidney cancer and mutations and deletions of the gene are associated with WAGR (Wilms’ tumour/aniridia/genitourinary abnormalities/mental retardation), DDS (Denys-Dash syndrome) and FS (Frasier syndrome). The gene is composed of ten exons, which undergo alternative splicing and show different translational start sites, resulting in a total of 24 different protein isoforms (Reddy and Licht, 1996; Sharma et al., 1994), which share a characteristic domain of four zinc fingers in the C-terminus. Of particular interest for gonadal development are two splice variants which differ in the insertion or omission of the three amino acids KTS between the third and fourth zinc finger (+KTS and -KTS isoforms). The two protein isoforms vary in their DNA binding activity in vitro (Laity et al., 2000) and only the +KTS isoform is able to bind and stabilise RNA (Bor et al., 2006). Unlike human patients, mice with a heterozygous null mutation of Wt1 do not show any gonadal phenotype. Mice with homozygous Wt1 null mutations die between 13 dpc and 15 dpc, due to a failure of kidney development, and lack both gonads and adrenal glands (Kreidberg et al., 1993). Targeted deletion of either the
+KTS or -KTS isoforms revealed their distinct function during gonadal development. While WT1(+KTS) seems to be more important in testis development, WT1(-KTS) is required for formation and survival of the gonadal primordium in both sexes, as WT1(-KTS\(^{-\Delta}\)) mice show an increased number of apoptotic cells in the early gonad (Hammes et al., 2001).

Steroidogenic factor 1 (Sf1, Nr5a1) is a member of the nuclear receptor family and was first identified as an activator of genes involved in steroidogenesis (Parker and Schimmer, 1997). SF1 contains a DNA-binding domain composed of highly conserved zinc fingers and recognises (C/T)CAAGG(T/C)(C/T) and (A/G)(A/G)(A/G)AGGTCA DNA sequences (Morohashi et al., 1992). In human patients, heterozygous mutation of SF1 is associated with primary adrenal failure, XY male-to-female sex reversal including different degrees of gonadal dysgenesis and persistent Müllerian structures (for review see Lin and Achermann, 2008). In contrast to humans, mice with a heterozygous mutation of Sf1 do not display any gonadal phenotype (Luo et al., 1994). Mice carrying a homozygous null mutation of Sf1 (Sf1\(^{-/-}\)) start to develop the genital ridge correctly and migration of PGCs appears normal. However, somatic cells undergo apoptosis around 11 - 11.5 dpc, which results in the complete lack of gonads and adrenals, indicating an essential role for Sf1 in regulating the survival and proliferation of the adrenal and somatic gonadal cells (Luo et al., 1994).

Lhx9 is a member of the LIM homeobox transcription factor family with two N-terminal LIM domains and a homeobox domain. The LIM domain is a cysteine-rich domain consisting of two zinc fingers which mediates protein-protein inter-
actions, whereas the homeobox domain is involved in DNA binding. Mice with a homozygous null mutation of \( Lhx9 \) (\( Lhx9^{-/-} \)) show failure of somatic cell proliferation in the genital ridge, resulting in the absence of gonads (Birk et al., 2000). Sf1 expression is significantly reduced in \( Lhx9^{-/-} \) mice, indicating that LHX9 modulates Sf1 activation. Indeed, it has been shown in vitro that LHX9 and WT1(-KTS) interact and cooperatively activate the Sf1 promoter (Wilhelm and Englert, 2002).

The \( M33 \) gene (also known as \( Cbx2 \)) is a member of the polycomb (PC) group of proteins which were first identified in \( Drosophila melanogaster \). Mice with homozygous deletion of the M33 C-terminus (\( M33^{cterm/cterm} \)), which is highly conserved among \( Drosophila \) Pc proteins (Pearce et al., 1992), show retardation in the formation of the genital ridge in both sexes (Katoh-Fukui et al., 1998). In adult XX \( M33^{cterm/cterm} \) mice, ovaries are either small or residual, whereas XY \( M33^{cterm/cterm} \) mice show varying degrees of male-to-female sex reversal with the appearance of ovarian-like structures containing follicles. Further analyses of the \( M33 \) mutant mice indicated a reduced expression of Sf1 (Katoh-Fukui et al., 1998). Moreover, M33 directly binds to the Sf1 gene locus in vitro, suggesting that M33 may be involved in the regulation of Sf1 expression (Katoh-Fukui et al., 2003). Recently mutations in \( CBX2 \) were identified in a human patient with XY male-to-female sex reversal (Biason-Lauber et al., 2009).

\( Emx2 \) is a homeobox gene which was first identified due to its homology to the \( Drosophila \) gene empty spiracles (Simeone et al., 1992). Mice carrying a homozygous null mutation of \( Emx2 \) (\( Emx2^{-/-} \)) show a complete absence of gonads due
to a failure in thickening of the coelomic epithelium at 11.5 dpc. Moreover, the genital tract system is absent in both sexes in the $Emx2^{-/-}$ mice as the Müllerian ducts never form and the Wolffian ducts start to degenerate around 11.5 dpc in XY $Emx2^{-/-}$ mice ([Miyamoto et al.], 1997).

In summary, before 11.0 dpc the gonad is indistinguishable between XY and XX embryos. In both sexes, the same set of regulatory genes is expressed setting the stage for the changes to come and the gonad bears the potential to differentiate into either a testis or an ovary. And then along comes Sry and activates a cascade of gene activity which will eventually lead to the establishment of testis development in XY gonads.

1.4 Taking the route towards testes

1.4.1 Testis determination: Sry

SRY was shown to be critical for male sex determination through both loss- and gain-of-function studies. Analysis of human XY sex-reversed female patients discovered a de novo frameshift mutation in the Y-linked gene SRY, which indicated that it was necessary for testis development ([Jäger et al.], 1990). Subsequently, many XY females have been found with point mutations in Sry (for review see Sekido, 2010). Parallel studies confirmed the importance of Sry for testis determination in mice. Loss-of-function (LoF) experiments demonstrated that XY mice with a 11 kb deletion of Sry, which results in a lack of a functional Sry gene, develop ovaries and become female ([Gubbay et al.], 1990). Gain-of-function
(GoF) studies showed that XX mice carrying a Sry transgene develop testes and become male, although they are infertile \(\text{(Koopman et al., 1991)}\). These data demonstrate that Sry is both sufficient and necessary for testis development and therefore is only gene on the Y chromosome required for male development.

Studies in chimeric mice demonstrated that more than 90% of Sertoli cells in the testes were XY, in contrast to other cells which did not show any bias, suggesting that Sry acts in the male supporting cell lineage \(\text{(Palmer and Burgoyne, 1991b)}\). However, some of the cells were XX indicating that a non-cell autonomous factor can recruit XX cells to become Sertoli cells. By variation of the XX:XY cell ratio in the chimeric mice, a threshold level of 20% Sry-expressing cells was found to be sufficient for testis development \(\text{(Burgoyne and Thornhill, 1993)}\). Subsequent studies confirmed that Sry expression is restricted to cells that differentiate into Sertoli cells \(\text{(Sekido et al., 2003; Wilhelm et al., 2005)}\). These data substantiate the idea that the critical role for SRY in sex determination is to establish the Sertoli cell fate decision in the supporting cell lineage in the early gonad.

In the mouse, Sry is expressed in the Sertoli cell precursors of the XY gonad in a centre to pole wave \(\text{(Bullejos and Koopman, 2003; Sekido et al., 2003; Albrecht and Eicher, 2001)}\). Both mRNA and protein analyses showed that Sry is only present during a narrow time window between 10.75 dpc and 12.5 dpc in the nucleus of Sertoli cell precursors \(\text{(Koopman et al., 1990; Sekido et al., 2003)}\). Sry expression needs to reach a critical threshold per cell and also in a sufficient number of cells in order to initiate and maintain Sertoli cell differentiation \(\text{(Palmer and Burgoyne, 1991b; Burgoyne and Thornhill, 1993)}\). Recently, ex vivo studies
showed that XX gonads carrying an inducible Sry transgene only initiate correct Sertoli cell differentiation and thus testis development when the transgene was expressed in a 6 hour time window between 11.0 dpc and 11.25 dpc (Hiramatsu et al., 2009). These data show that if Sry is expressed at a critical time during gonadal development, it acts as a genetic switch to shift the development of the bi-potential gonad towards to male pathway.

Several factors have been proposed to be involved in the activation of Sry expression in the XY gonad, including SF1, WT1(+KTS), GATA4/FOG2, M33 and members of the insulin receptor (IR) family (Figure 1.2). Cell fate mapping analyses showing that Sry-expressing Sertoli precursor cells indeed originate from Sf1-expressing coelomic epithelial cells, suggests the importance of SF1 in Sry regulation and Sertoli cell differentiation (Schmahl et al., 2000). In support of this hypothesis, it has been shown that SF1 binds and activates both human and pig Sry promoters in vitro (de Santa Barbara et al., 2001; Pilon et al., 2003) and Sry expression can not be detected in gonads of XY Sf1−/− mice (S. Guioli, unpublished). FS patients lacking the WT1(+KTS) isoform also show XY sex reversal (Gubler et al., 1999). Recently, it has been shown that WT1(+KTS) is involved in the cell-autonomous regulation of Sry expression as gonads of XY (+KTS∆/∆) mice show reduced Sry expression per cell as well as reduced proliferation of somatic cells and failure of Sertoli cell differentiation (Bradford et al., 2009).

Mice carrying a homozygous null mutation of the zinc finger transcription factor Gata4 (Gata4−/−) die before 10.5 dpc due to severe defects in heart development (Molkentin et al., 1997). However, a hypomorphic mutation of Gata4 (Gataki) which abolishes the interaction between GATA4 and its co-factor FOG2, or the
null mutation of Fog2 (Fog2<sup>-/-</sup>), both resulting in embryonic lethality after the time of sex determination, cause XY male-to-female sex reversal (Tevosian et al., 2002). Sry transcript levels are significantly decreased in XY Fog2<sup>-/-</sup> gonads (Tevosian et al., 2002). Recent studies revealed that GATA4 interacts with both WT1 isoforms (+KTS and -KTS) to activate the mouse, human and pig Sry promoters <i>in vitro</i> (Miyamoto et al., 2008). Combined triple deletions of IR genes, including IR itself, insulin-related receptor (IRR) and insulin-like growth factor 1 receptor (IGF1R), also result in a reduction of the Sry transcript level, associated with XY sex reversal (Nef et al., 2003). It is thought that this phenotype is due to a reduced proliferation of the somatic cells resulting in fewer Sry-expressing cells. However, an alteration of Sry transcription itself cannot be excluded. Recently, the involvement of MAPK (mitogen-activated protein kinase) signalling in the regulation of Sry expression has been described (Bogani et al., 2009). Loss of MAP3K4 activity results in XY sex reversal due to a failure in the up-regulation of Sry expression. However, it is not clear yet whether this failure of proper Sry expression is due to transcriptional regulation of Sry by the MAP3K4-signalling pathway or due to insufficient numbers of Sertoli cell precursors. Taken together, although a lot of genes have been implicated in Sry activation, little is still known about the exact transcriptional regulation of Sry.

Recent studies on the evolution of Sry have revealed that the 5’ sequence upstream of Sry is partially conserved in human, pig, bovine and goat, including conserved binding sites for transcription factors, whereas the mouse sequence was found to be considerably different (Ross et al., 2008). In contrast to the transient expression of Sry in mouse, the expression of human and pig Sry persists
throughout later stages of development, which might suggest different regulatory mechanisms (Hanley et al., 2000; Daneau et al., 1996).

SRY proteins usually consist of three different regions: N-terminal domain, HMG box and C-terminal domain (Sekido, 2010). Interestingly, mouse SRY has a more unusual structure compared to other species. Due to a very short N-terminal domain, the HMG box is essential N-terminal. Moreover, the protein contains a large glutamine (Q) repeat region at the C-terminus which is connected to the HMG box by a bridge domain. In vitro SRY binds to the DNA consensus sequence (A/T)ACAA(T/A) via its HMG box and thereby bends DNA up to an angle of 90° (Ferrari et al., 1992; Giese et al., 1994). Outside the highly conserved HMG box domain, the conservation of the protein sequence is poor amongst mammals. The vast majority of point mutations in SRY in cases of human XY male-to-female sex reversal occur within the HMG box which suggests that this is the most critical part of the protein (Sekido, 2010). However, both the N- and C-terminal domains might play a role in Sry function, as point mutations in these regions have been found in a few cases of XY sex reversal (Shahid et al., 2004). The importance of the mouse-specific Q-stretch in male sex determination was suggested in transgenic mouse experiments, as Sry constructs lacking the Q-stretch were not able to induce testis development in XX embryos (Bowles et al., 1999). However, it was not possible to ascertain whether any mutant protein was actually made or stable in these mice. In contrast, the SRY proteins from M. mus. domesticus (Y^DOM) or M. mus. poschiavinus (Y^POS) contain a truncation of this Q-stretch. When Y^DOM or Y^POS chromosomes were transferred onto the M. mus. musculus background C57BL/6, ovarian tissue developed, indicating that these
Y chromosomes function as weak alleles, possibly due to the reduction of the Sry Q-stretch (Eicher et al., 1982). Recent studies indicated the involvement of the Q-stretch in interference with the female-promoting Wnt/β-catenin pathway (see later) (Tamashiro et al., 2008). In contrast to mouse SRY, human SRY seems to be unable to inhibit the Wnt/β-catenin, possibly due to the lack of the C-terminal Q-stretch domain. However, human SRY fused to the murine Q-stretch domain is able to interfere with this pathway (Tamashiro et al., 2008). Interestingly, even though human and mouse SRY are distinct in their C-terminus, hSRY expressed under the control of mouse regulatory sequences, can still induce testis development in the mouse (Lovell-Badge et al., 2002), as does the expression of goat Sry (Pannetier et al., 2006).

Both human and mouse SRY is able to directly interact with other proteins, such as SRY-interacting protein (SIP-1) and Krüppel-associated box only protein (KRAB-O). SIP-1 was identified by an in vitro screen for human SRY interacting proteins (Poulat et al., 1997). Subsequent analyses revealed that SIP-1 interacts with the bridge domain between the HMG box and the Q-stretch of mouse SRY and with a specific motif (TKL-motif) at the C-terminus of human SRY (human SRY does not contain a bridge domain) (Thevenet et al., 2005). KRAB-O interacts with the bridge domains of mouse SRY and a domain adjacent to the C-terminus of the human SRY HMG box, but no clear motif has been identified (Oh et al., 2005). A recent study showed that ablation of KRAB-O expression in vitro via RNAi, results in a failure to up-regulate the SRY target gene Sox9 (see later) (Polanco et al., 2009). However, targeted deletion of KRAB-O in mice did not impair testis development in vivo, which might be due to a possible
redundancy between members of the large KRAB gene family.

Interestingly, in the adult mouse testis, a circular Sry transcript has been found (Capel, 1998). This circular RNA is not translated and no function has been associated with it so far. It has not been found in humans or other mammals and might represent a mouse-specific mechanism to disable Sry function in the adult.

In summary, Sry must be expressed at the right time of development and in the right place in order to act as a molecular switch by activating downstream target genes which ensure that the supporting precursor cells become Sertoli cells and thereby setting the development of the bi-potential gonad towards testis development.

![Diagram of genetic interactions during testis development](image)

**Figure 1.2: Model of genetic interactions during testis development.**
Possible genetic interactions in the XY gonad resulting in the establishment of Sox9 expression in the supporting cell lineage. The testis-determining genes also have to ensure the inhibition of the ovarian-determining genes in order to maintain the Sertoli cell fate XY gonads.
1.4.2 Testis differentiation: establishment of Sertoli cells

As SRY acts only transiently in testis development, other genes, which act downstream of SRY, must be involved in the establishment of Sertoli cells. Several studies indicated the autosomal gene Sox9 (Sex determining region Y, box 9) as the a crucial downstream target of SRY in testis development (Chaboissier et al., 2004; Sekido et al., 2004; Sekido and Lovell-Badge, 2008). Sox9 is an Sry-related gene belonging to the Sox E family of HMG box proteins (together with Sox8 and Sox10). Like SRY, SOX9 can bind and bend DNA but it also contains a transactivation domain at the C-terminus, suggesting a role as a transcriptional activator (Bell et al., 1997; Lefebvre et al., 1997; Südbäck et al., 1996). In contrast to SRY which is found only in mammals, SOX9 is highly conserved amongst vertebrates.

Human patients with SOX9 haploinsufficiency display a severe skeletal malformation syndrome, Campomelic dysplasia (CD), which is associated with XY male-to-female sex reversal in about 75% of all cases (Foster et al., 1994; Wagner et al., 1994). On the other hand, duplication of the chromosomal region containing SOX9 (17q23-24) results in XX female-to-male sex reversal (Huang et al., 1999). In the mouse, homozygous null mutation of Sox9 (Sox9−/−) is embryonic lethal around the time of sex determination (Bi et al., 2001). However, when genital ridges from 11.5 dpc XY Sox9−/− mice were cultured in vitro, they neither formed testis cords nor expressed Amh (Chaboissier et al., 2004). Conditional deletion of Sox9 via the Cre/LoxP system, using Sf1:Cre to delete Sox9 specifically within the gonad (Sox9Δ/Δ), can result in XY male-to-female sex reversal.
(Chaboissier et al., 2004; Barrionuevo et al., 2006). Moreover, misexpression of Sox9 in XX mice induces female-to-male sex reversal (Bishop et al., 2000; Vidal et al., 2001). These results mimic the LoF and GoF studies of Sry, respectively, implying that Sox9 is the only critical downstream target of SRY and thereby is both necessary and sufficient for normal testis development (Figure 1.2). However, a possible functional redundancy between Sox9 and other members of the SoxE family, such as Sox8 or Sox10, has been suggested in testis differentiation (Chaboissier et al., 2004; Barrionuevo et al., 2006).

Sox9 is initially expressed at low levels in the gonad of both sexes around 10.5 dpc, and thus before the onset of Sry expression (Morais da Silva et al., 1996). Furthermore, Sox9 expression persists in Sertoli cells throughout life after transient Sry expression has ceased, indicating that other factors are involved besides Sry in regulating Sox9 expression. Several studies have suggested that Sf1 is particularly important for testis differentiation and might be involved in the early regulation of Sox9 expression. Indeed, early sex-independent expression of Sox9 is abolished in the gonads of XY Sf1−/− mice (Sekido et al., 2004). Moreover, ubiquitous misexpression of Sry in XX gonads only results in the initiation of Sox9 expression in SF1 positive cells (Kidokoro et al., 2005). Recently, the regulatory element responsible for the testis-specific expression of Sox9 has been identified 13 kb upstream of the transcription start site, and named testis-specific enhancer of Sox9 (TES) including a 1.3 kb core region (TESCO) (Sekido and Lovell-Badge, 2008). This element is highly conserved between mouse, rat, dog and human. Chromatin immunoprecipitation (ChIP) analyses demonstrated that both SF1 and SRY bind to the TESCO enhancer sequence, confirming Sox9 as
a direct target of SRY. Subsequent analyses with transgenic mice carrying mutations in either SRY or SF1 binding sites showed little effect on the enhancer activity but simultaneous mutation of all the binding sites completely abolished TESCO activity (Sekido and Lovell-Badge, 2008). In vitro co-transfection experiments demonstrated that SF1 on its own can activate TESCO weakly, whereas the combination of SF1 and SRY results in a stronger activation. These results indicate that SF1 can indeed initiate TESCO (and therefore Sox9) expression on its own and the synergistical effect of SF1 and SRY results in the up-regulation of its expression. Moreover, the ChIP assays revealed that SOX9 itself can bind to TESCO via the same binding sites as SRY or via fragments containing SF1 binding sites (Sekido and Lovell-Badge, 2008). This result suggests that SF1 and SOX9 might physically interact to regulate TESCO activity. Indeed, a direct interaction between the N-terminal domain of SOX9 and the C-terminal region of SF1 was previously described for the regulation of the Amh promoter (De Santa Barbara et al., 1998). Subsequent in vitro co-transfection assays showed that the TESCO element can be activated robustly by the combination of SF1 and SOX9, indicating a positive feedback mechanism in which SOX9 ensures the maintenance of its own expression. All these data led to the proposal of a new model of Sox9 regulation via the TESCO element: (i) SRY-independent initiation, (ii) SRY-dependent up-regulation and (iii) SRY-independent maintenance (Sekido and Lovell-Badge, 2008) (Figure 1.3).
Figure 1.3: Model for Sox9 regulation via TESCO in XY gonads.
Sox9 expression in the developing XY gonad is regulated via the TESCO element in three phases: (i) SF1 is binding to the enhancer at 10.5 dpc and initiates a low expression of Sox9, (ii) Sry is activated around 11.5 dpc and up-regulates Sox9 expression (iii) SOX9 itself can bind to its own enhancer and maintains its expression via this autoregulation and other feedback loops involving FGF9 and PGD2 signalling.

A number of other factors have been implicated to be involved in the regulation Sox9 expression. For example, Sox9 expression is lost in both Gata$^{ki}$ and Fog2$^{-/-}$ XY gonads (Levosian et al., 2002), indicating that the GATA4/FOG2 complex might be required directly for Sox9 transcription. Recent findings also show that FGF9 (fibroblast growth factor 9) plays an important role in maintaining Sox9 expression (Figure 1.2). Fgf9 is originally expressed in the gonad of both sexes and then becomes XY specific in Sertoli cells after Sry expression (Colvin et al., 2001). In XY mice carrying a homozygous null mutation of Fgf9 (Fgf9$^{-/-}$), the expression of Sry is not affected and Sox9 starts to be expressed normally at 11.5 dpc, which suggests that FGF9 is not involved in the initial up-regulation of Sox9 expression (Kim et al., 2006b). However, SOX9 is not detectable anymore in the XY mutant gonads after 12.5 dpc which results in XY male-to-female sex reversal, indicating that FGF9 is involved in maintaining Sox9 expression. This correlates with the finding that the misexpression of Fgf9 alone does not result in
up-regulation of Sox9 in XX gonads in chicken (Yoshioka et al., 2005). Similar to Fgf9−/−, mice carrying a homozygous null mutation in the main receptor of FGF9 signalling, FGF receptor 2 (Fgfr2−/−), show XY sex reversal due to a loss of Sox9 expression after 11.5 dpc (Kim et al., 2007; Bagheri-Fam et al., 2008). Moreover, human patients with a deletion of the region containing FGFR2 (10q26) display XY sex reversal (Wilkie et al., 1993). All these data suggests the importance of FGF9/FGFR2 signalling in the maintenance of Sox9 expression. Moreover, it has been shown that conditional XY Sox9Δ/Δ mice have decreased levels of Fgf9 at 11.5 dpc (Kim et al., 2006b), indicating that SOX9 is, in turn, necessary for Fgf9 expression. Taken together, FGF9/FGFR2 signalling seems to be crucial for normal testis development by acting on Sox9 expression via a positive feedback loop.

Another target of SOX9 is the prostaglandin D synthase gene (Ptgds) which is involved in the synthesis of Prostaglandin D2 (PGD2). SOX9 can directly bind and activate the Ptgds promoter whereas active PGD2 signalling in turn enhances import of SOX9 from the cytoplasm into the nucleus in an autocrine and paracrine manner (Wilhelm et al., 2005). Moreover, ex vivo analyses have shown that addition of PGD2 to XX gonads resulted in up-regulation of Sox9 expression and partial masculinisation (Adams and McLaren, 2002; Wilhelm et al., 2005). This suggests that PDG2 signalling is sufficient to up-regulate Sox9 expression in cells which did not express Sry. This Prostaglandin signalling pathway might be involved in the recruitment of XX cells to become Sertoli cells as seen in XX-XY chimeric mice (Palmer and Burgoyne, 1991b). On the other hand, mice carrying a targeted homozygous null mutation for Ptgds do not show any signs
of sex reversal indicating that PGD2 signalling is a back-up mechanism to ensure efficient Sertoli cell differentiation of all cells of the supporting cell lineage (Moniot et al. 2009).

In summary, SOX9 is the crucial factor which define Sertoli cell phenotype and function. This includes the direct or indirect repression of genes characteristic of the supporting cell precursors of the bi-potential gonad, as well as the repression of genes involved in ovarian development (see later). Thus, maintenance of Sox9 expression is indispensable in the XY gonad in order to develop as a fully functional testis.

1.5 Determined to develop as female

1.5.1 Ovarian determining or anti-testis genes

Female development in mammals has often been considered as the default pathway of development (Jost, 1952). The bi-potential gonad develops into an ovary in the absence of Sry, suggesting the testis pathway to be the active pathway of gonadal formation. However, it has also been proposed that a gene in XX gonads (the Z gene) acts as a negative regulator of male sex determination (McElreavey et al., 1993). Although no exclusive female-determining gene has been found so far, several cases of partial XX female-to-male sex reversals have been described in the absence of Sry, in both mice and humans. In some cases the relevant genes have been identified, for example in LoF mutations in Wnt4 (a member of the wingless family of genes), Rspo1 (R-spondin1) and Foxl2 (forkhead box
factor L2). This indicates that an active repression of the testicular pathway is necessary for normal ovarian development, rather than this depending entirely on a passive default pathway. Indeed, it seems that a "battle of the sexes" is happening in the developing gonad with different genes involved in tipping the balance either towards testis and male development or towards ovary and female development. The genes involved in this battle would activate other genes in their developmental pathway and simultaneously repress genes which are important for the opposite pathway.

The first significant molecular event happening in XX gonads is the down-regulation of Sox9 expression in the precursors of the supporting cell lineage shortly before 11.5 dpc. Thereupon, these cells differentiate into female granulosa cells instead of male Sertoli cells. An important question is therefore which genes are directly responsible for the repression of Sox9 at this stage and for maintaining its repression throughout life in granulosa cells. A number of genes have been proposed to act as anti-testis or ovarian-determining genes, including Wnt4, Rspo1, β-catenin, Dax1 (Dosage-sensitive sex-reversal (DSS) adrenal hypoplasia congenita (AHC) critical region on the X-chromosome, gene 1), Sox4, Foxl2 and ERs (estrogen receptors).

The Wnt family (Wingless-type MMTV integration site family) is a conserved group of secreted glycoproteins, which was named after the Drosophila segment polarity gene (wingless) and the first discovered vertebrate homolog (integrated). The extra-cellular WNT ligand can stimulate different intra-cellular transduction cascades: the canonical β-catenin dependent pathway and two non-canonical
pathways (planar cell polarity pathway and Wnt/Ca\textsuperscript{2+} pathway). In the canonical pathway, WNT ligands bind to the transmembrane receptor frizzled (Fz) and the co-receptors LDL-receptor related protein 5 and 6 (Lrp5/6) and the signal is then transduced to the cytoplasmic protein Dishevelled (Dsh). In the absence of WNT ligand \(\beta\)-catenin is phosphorylated by a multiprotein complex which leads to its ubiquitination and subsequent degradation via the proteosome. Upon binding of WNT, Dsh prevents \(\beta\)-catenin degradation by disrupting the protein complex needed for its phosphorylation. Stabilised \(\beta\)-catenin translocates into the nucleus and interacts with T-cell factor/Lymphocyte enhancer factors (TCF/LEF). This protein complex then binds to cis-regulatory elements of target genes and thereby activates Wnt-responsive genes (for a more detailed review see Komiya and Habas, 2008). On the other hand, it has been shown that LEF/TCF proteins can physically interact with co-repressors, such as Groucho and CtBP, which leads to the repression of their target genes (Roose et al., 1998).

\textit{Wnt4}, is particularly crucial for female development. In human patients, a duplication of the chromosomal region including \textit{WNT4} (1p35) has been associated with XY sex reversal (Wieacker et al., 1996), whereas a point mutation within the \textit{WNT4} coding region (E226G) has been reported as a LoF mutation which causes masculinisation in an XX patient (Biason-Lauber et al., 2004). In the mouse, \textit{Wnt4} expression starts in the early gonad in the mesenchyme along the length of the mesonephros from 9.5 dpc onwards. At 11.0 dpc it is expressed in the mesenchyme of the indifferent gonad in both sexes and then becomes female-specific due to its down-regulation in the XY gonad around 11.5 dpc. \textit{Wnt4} is strongly expressed in the mesenchymal cells of the developing Müllerian duct
but not the Wolffian duct (Vainio et al., 1999). XX embryos carrying a targeted null mutation of Wnt4 (Wnt4−/−) show partial masculinisation of the gonad, including some Leydig cell differentiation and the formation of a coelomic vessel. Moreover, the Wolffian duct is maintained in these embryos, whereas the Müllerian duct is absent (Vainio et al., 1999; Jeays-Ward et al., 2004). However, misexpression of Wnt4 in XY transgenic embryos (Sf1:Wnt4) does not cause male-to-female sex reversal, although it does interfere with the normal formation of the coelomic blood vessel (Jeays-Ward et al., 2003; Jordan et al., 2003) (more detailed discussion of the Wnt4 mutant phenotype in Chapter 6).

R-spondin 1 (R-SPO1) belongs to a family of secreted ligands and can interact with Fz/Lrp receptors via its two furin-like domains (Kim et al., 2006a). It might activate the same intracellular canonical pathway as WNT ligands, leading to a stabilisation of β-catenin (Kazanskaya et al., 2002). Thus, RSPO1 is thought to potentiate canonical WNT signalling although it could also act independently to achieve a similar effect. In human patients, homozygous LoF mutation of R-SPO1 (R-SPO1−/−) can lead to complete XX female-to-male sex reversal in the absence of SRY (Parma et al., 2006). R-SPO1 does not seem to be necessary for testis differentiation because XY R-SPO1−/− patients develop normal and functional testes (Micali et al., 2005). In the mouse, Rspo1 is expressed in somatic cells of the indifferent gonad in both sexes at 10.5 dpc and then becomes female-specific around 12.5 dpc (Parma et al., 2006). Mice with a targeted null mutation of Rspo1 (Rspo1−/−) show partial XX female-to-male sex reversal with formation of the male-specific coelomic vessels and the appearance of several seminiferous tubules (Chassot et al., 2008; Tomizuka et al., 2008). Moreover, adult
XX Rspo1\(^{-/-}\) mice show production of testosterone, masculinised external genitalia and persistence of both, Wolffian and Müllerian ducts. However, expression of testis differentiation markers such as Sox9, Fgf9 and Dhh cannot be detected in the mutant embryos at the time of sex determination, although expression of ovary differentiation marker genes, such as Wnt4, Fst and Bmp2, is reduced (Chassot et al., 2008; Tomizuka et al., 2008). Epistatic analysis showed that Axin, a known target gene of \(\beta\)-catenin is not expressed in XX Rspo1\(^{-/-}\) embryos, while misexpression of a stable form of \(\beta\)-catenin rescues the XX Rspo1\(^{-/-}\) phenotype (Chassot et al., 2008). These data indicates that Rspo1 acts upstream of \(\beta\)-catenin. On the other hand, Rspo1 expression is not affected in Wnt4\(^{-/-}\), suggesting that Rspo1 acts either upstream of, or in parallel to, Wnt4 (Chassot et al., 2008). Taken together, these data suggest that both Wnt4 and Rspo1 play an important role in female sex determination by ensuring appropriate levels of stable \(\beta\)-catenin.

Stabilised \(\beta\)-catenin, as the downstream effector of WNT4 signalling and perhaps RSPO1, has been shown to rescue the phenotype of Wnt4\(^{-/-}\) gonads in organ culture and the gonadal phenotype of R-spo1\(^{-/-}\) mutant mice in vivo (Maatouk et al., 2008; Chassot et al., 2008). In XY gonads from a transgenic mouse carrying a dominant stabilised form of \(\beta\)-catenin (Catnb\(^{ex3}\)), Sry and early Sox9 expression are unaffected and both the formation of the coelomic vessels and the early proliferation of the coelomic epithelium, which is specific for XY gonads, occur normally. However, Sox9 expression rapidly decreases after 12.5 dpc resulting in a disruption of testis cord formation and up-regulation of female-specific markers (Bmp2, Foxl2, Dax1, Wnt4, Fst) in somatic cell lineages, indicating the transi-
tion from male to female development (Maatouk et al., 2008; Chang et al., 2008). On the contrary, Sertoli cell-specific deletion of β-catenin, by either Amh:Cre or Sf1:Cre, does not lead to any abnormalities in testis development, implying that it is not essential for Sertoli cell differentiation (Chang et al., 2008; Liu et al., 2009). However, in XX gonads, conditional deletion of β-catenin in SF1-positive somatic cells results in the appearance of testis-specific vasculature, maintenance of the Wolffian duct and loss of germ cells, closely resembling the phenotype of both Wnt4−/− and R-SPO1−/− mutant mice (Liu et al., 2009). Taken together, the data suggest that the role of β-catenin is to antagonise testis development and to maintain rather than initiate female development. It has been shown in chondrocyte differentiation that SOX9 and β-catenin are able to repress each other's effects on transcriptional activation in a DNA binding independent manner. As the C-terminal transactivation domain of Sox9 physically can interact with the Armadillo repeats in β-catenin, SOX9 can compete with LEF/TCF factors for binding to β-catenin. The resulting SOX9/β-catenin complex is eventually degraded via the proteosomal machinery (Akiyama et al., 2004). Therefore, Sox9 can act as a de-stabiliser of β-catenin causing a reduction in the expression of β-catenin target genes or vice-versa. Similarly, there is some evidence that human SRY is able to inhibit β-catenin by direct protein interaction in vitro (Bernard et al., 2008). It is not known which part of the SRY protein mediates the interaction with β-catenin, only that the SRY HMG-box on its own is not sufficient. Other studies indicate a role of β-catenin in estrogen production in granulosa cells where β-catenin is able to directly interact with SF1 and thereby modulate SF1 activity, e.g. in the regulation of Cyp19A1 in vitro (Parakh et al., 2006).
Dax1 (also known as Nr0b1) is an X-linked gene encoding an unconventional member of the nuclear receptor (NR) superfamily. It lacks the classic zinc finger DNA binding domain of NRs and instead contains three and a half repeats of a novel domain composed of the LXXLL-motif (Ito et al., 1997), which appears to be involved in interactions between DAX1 and other NRs. DAX1 binds to SF1 through this motif and represses SF1 activity by the recruitment of a nuclear co-repressor NcoR (Ito et al., 1997; Crawford et al., 1998). On the other hand, the Dax1 promoter contains binding sites for SF1 and mutation of these sites abolishes Dax1 expression. Moreover, Dax1 expression is significantly reduced in Sf1−/− mice (Hoyle et al., 2002) implying that Dax1 is regulated by SF1 in the developing gonad. Thus, the two genes interact in a complex fashion which is not yet fully understood. A consensus DNA binding sequence for DAX1 has not yet been described. It might bind DNA directly, like other nuclear receptors (e.g. SF1), but it has also been suggested that DAX1 binds to DNA secondary structures (Zazopoulos et al., 1997) or that it does not bind to DNA, but to RNA (Lalli et al., 2000). Human XY patients with duplications of a region of Xp21, containing the DAX1 gene, display a male-to-female sex reversal (Bardoni et al., 1994). This suggested that DAX1 can act as an anti-testis gene and that it might be is important in ovary development. In mice, Dax1 is expressed in the indifferent genital ridge of both sexes and is down-regulated in XY gonads during testis development (by 12.5 dpc) whereas the expression persists in the somatic cells of XX gonads throughout ovarian development (Swain et al., 1998). Misexpression of Dax1 in mouse strains carrying a weak Sry allele (Y^POS) or with a delayed onset of Sry expression causes XY sex reversal (Swain et al., 1998).
Moreover, targeted deletion of Dax1 in mice did not result in XX sex reversal (Meeks et al., 2003). On the other hand, mice carrying this Dax1 deletion show XY male-to-female sex reversal in the presence of Y\textsuperscript{POS} (Meeks et al., 2003) or on a specific genetic background (C57BL/6HJ) (Bonma et al., 2005), indicating that Dax1 is required for testis development. This is in contrast to the human cases where LoF mutations in DAX1 do not affect testis development in XY AHC patients (Zanaria et al., 1994; Muscatelli et al., 1994). The molecular mechanisms underlying this paradox are not understood yet, but it is possible that Dax1 is needed for both testis and ovarian development depending on timing and dosage of its expression. It is possible that the deletion might not be a true null mutation as only the second of the two Dax1 exons was deleted and a truncated DAX1 protein (containing only the N-terminal region) could still retain some functional activity (Ludbrook and Harley, 2004). It might interact with SF1 via its remaining N-terminus and could act as a dominant negative with respect to SF1 activity (maybe by reducing the pool of SF1 protein available to activate the male pathway).

Sox4 belongs to the SOX family of transcription factors (Gubbay et al., 1990), subgroup C, together with Sox11. Sox4 is expressed in a number of tissues including neural tissue, heart, lung and thymus (van de Wetering et al., 1993). Moreover, in mice Sox4 has been found to be expressed specifically in the ovary and not in the testis at the time of sex determination (P. Koopman, personal communication) suggesting a potential role in ovarian development. Mice with a homozygous null mutation of Sox4 (Sox4\textsuperscript{−/−}) die at 14.0 dpc due to heart malformation (Schilham et al., 1990) but the gonadal phenotype of those mutant mice
has not yet been analysed. It is known that SOX proteins are transcriptional regulators that can activate or repress target genes according to the cellular context (Chew and Gallo, 2009). Hence, it is possible that SOX4 could act as a repressor of Sox9 expression in the developing XX gonad. Moreover, it has been shown that SOX4 directly interacts with β-catenin and TCF/LEF proteins and that SOX4 can enhance β-catenin activity in colon carcinoma cells in vitro (Sinner et al., 2007).

Foxl2 encodes for a member of the forkhead family of transcription factors. It was originally identified as a candidate for a gene involved in ovarian development in goats. In these animals, an 11.7 kb deletion 200 kb upstream of the Foxl2 gene causes a dominant hornless phenotype that is associated with a recessive XX female-to-male sex reversal, known as polled/intersex syndrome (PIS) (Vaiman et al., 1996). In human patients, LoF mutations of FOXL2 cause the dominant disorder blepharophimosis/ptosis/epicanthus inversus syndrome (BPES), characterised by small palpebral fissures (blepharophimosis), drooping eyelids (ptosis), a small skinfold inwards from the lower lid (epicanthus inversus) and a broad nasal bridge (Crisponi et al., 2001). Two distinct types of BPES have been described: BPES type I which is associated with premature ovarian failure (POF) in female patients and BPES type II where patients remain fully fertile. Mutations within the coding sequence of FOXL2 resulting in a truncated FOXL2 protein cause BPES type I, while frameshifts or duplications further downstream in the sequence produce an extended protein which is associated with BPES type II. Some BPES patients show intact coding sequences for Foxl2, but carry mutations about 100 kb upstream of the gene itself (Uhlenhaut and Treier, 2001). In the
mouse, Foxl2 is, amongst other tissues, expressed in the granulosa cells of ovarian follicles. The gonadal expression is female-specific and starts in the supporting cell precursors of the ovary around 12.5 dpc and is maintained in adult granulosa cells (Schmidt et al., 2004). Foxl2 is also expressed in some theca cells in the adult ovary (Uhlenhaut et al., 2009). Mice with a heterozygous mutation of Foxl2 display no phenotype, whereas homozygous loss of Foxl2 results in a number of defects resembling the human BPES condition (Uda et al., 2004; Schmidt et al., 2004). This includes XX infertility and the postnatal up-regulation of testis-specific genes (more detailed description of the Foxl2 mutant phenotype in Chapter 6). Only a few target genes of FOXL2 have been described so far, including the gonadotrophin releasing hormone receptor (GnRHR), the human steroidogenic acute regulatory gene (StAR), which is involved in the rate-limiting step of steroidogenesis, and Cyp19 which encodes for an aromatase involved in converting androgens into estrogens (more detailed discussion of FOXL2 target genes in Chapter 4). Moreover, it has been reported that forkhead factors can compete with TCFs for interaction with β-catenin in vitro and direct interaction of forkhead factors and β-catenin leads to the enhancement of forkhead transcriptional activity (Hoogeboom et al., 2008).

Estrogen receptors (ERs) are members of the nuclear receptor superfamily which transmit 17β-estradiol (E₂) signals into the nucleus and activate target genes by binding to estrogen response elements (ERE). EREs are palindromic sequences of the estrogen receptor half site GGTCA (Klinge, 2001). Two ERs have been identified in mammals which are expressed in many tissues including the ovaries, where ERα is expressed in stroma, theca and granulosa cells and ERβ is expressed
predominantly in granulosa cells (Fitzpatrick et al., 1999). Single mutations of either ERα or ERβ cause distinct defects. In XX ERβ−/− mice, follicular development is partially arrested and follicular maturation occurs at reduced efficiency resulting in smaller ovaries and sub-fertility whereas XY males are fully fertile (Krege et al., 1998). XX ERα−/− mice are infertile with a block of folliculogenesis and a decrease in granulosa cell proliferation, severe uterine and vaginal hypoplasia and unovulatory adult ovaries with accumulation of large cystic follicles. Moreover XY ERα−/− mice are infertile indicating that estrogens might play an important role in male as well as in female development (Lubahn et al., 1993; Dupont et al., 2000). Mice carrying double homozygous mutations of both estrogen receptors (ERαβ−/−) display a more severe phenotype than the single mutants (Conse et al., 1999; Dupont et al., 2000). Similar to ERα−/− males, XY ERαβ−/− mice are infertile due to reduced numbers of sperm, while the reproductive tract appears normal. XX ERαβ−/− mice are also infertile and show vaginal hypoplasia, large ovarian cysts, absence of mature corpora lutea and proliferation defects in granulosa and theca cell layers. These mice also show partial sex reversal with appearance of seminiferous tubule-like structures and Sertoli-like cells expressing Sox9 and Amh. However, this phenotype only occurs after puberty (Conse et al., 1999). It has also been shown that forkhead transcriptional regulators are essential for estrogen receptor DNA binding, e.g. in breast cancer cell lines (Carroll et al., 2005), suggesting a possible link between FOXL2 and ERs.
1.5.2 Ovarian development

The germ cells in the XX genital ridge enter meiotic prophase at 13.5 dpc and arrest in diplotene at the time of birth. It has been proposed that they follow an intrinsic clock to enter meiosis, as even germ cells assembled in lung aggregates enter meiosis about 13.5 dpc in vitro (McLaren and Southee, 1997). However, it has also been shown that not all germ cells in the ovary enter meiosis at the same time but in an anterior-to-posterior pattern (Menke et al., 2003; Bullejos and Koopman, 2004). Moreover, there is now substantial evidence that suggests that retinoic acid, synthesised by the mesonephros, diffuses into the early ovary to trigger entry of the germ cells into meiosis, probably by induction of Stra8 expression (Bowles et al., 2006; Koubova et al., 2006). In the early testis, CYP26B1, which is produced by Sertoli cells and probably a target of Sox9, degrades retinoic acid and thus prevents meiosis (Bowles et al., 2006; Koubova et al., 2006).

Morphological changes in XX gonads are not obvious until late gestation when the ovary can be divided into two main regions: the cortex and the medulla. Around 16.5 dpc many germ cells in the medulla undergo apoptosis resulting in an accumulation of germ cells in the cortex (Yao et al., 2004). The germ cell loss correlates with the breakdown of germ cell cysts and the start of folliculogenesis (Pepling and Spradling, 2001). At this stage, the pre-granulosa cells extend cytoplasmic processes and enclose individual oocytes in a single layer of squamous cells, forming primordial follicles (Figure 1.4). Oocytes express Figα (factor in germ line α), which is involved in the recruitment of pre-granulosa cells. Although germ cells of mice with a homozygous null mutation in Figα enter meiosis cor-
rectly, they fail to form primordial follicles resulting in the depletion of oocytes directly after birth (Soyal et al., 2000). During normal ovarian development, the primordial follicles enlarge after birth due to an increase in the size of the oocyte and the transition of the squamous granulosa cells into cuboidal granulosa cells, resulting in the formation of primary follicles (Hirshfeld, 1991). The granulosa cells then proliferate to form multiple layers around the oocyte and thereby form pre-antral follicles, which are enclosed by an outer layer of theca cells. During the following antral stage, the granulosa cells secrete follicular fluid (containing serum proteins and steroid hormones) which fills the emerging antral cavity. Most of the antral follicles will undergo atresia, but the ones remaining will become pre-ovulatory follicles under the influence of follicle-stimulating hormone (FSH). After ovulation, the remaining granulosa and theca cells form the corpus luteum which secretes steroid hormones to promote implantation of the embryo. In the adult ovary, follicles of each stage can be found, which is due to some primordial follicles entering a temporary state of quiescence to ensure the continuous production of ovulatory follicles (Figure 1.4).

The function of the female steroidogenic cell lineage, the theca cells, is the production of estrogens. Theca cells express Cyp11a, 3β-HSD (HSD3B2) and Cyp17 which are required for the conversion of cholesterol into androgens. Granulosa cells express Cyp19 and 17β-HSD (HSD17B1) which converts these androgens into estradiol. Thus, both cell types are required for estrogen production in the ovary (Magoffin, 2005).
Figure 1.4: Model of ovarian follicle development.

(a) Folliculogenesis starts in the embryo with a pool of germ cells and somatic cells (granulosa and theca cells). The model shows the development of primordial follicles which grow to primary, pre-antral and antral stages before they become pre-ovulatory follicles. The granulosa cells (green) surround the oocyte (pink) and proliferate to several layers of supporting cells. The theca cells (blue) start to surround the follicles at the pre-antral stage. The antral cavity which contains the follicular fluid is shown in yellow. Adapted from Barnett et al. (2006).

(b) H+E staining illustrating the morphology of an adult ovary. Scale bar = 100μm.
1.6 Outline of this thesis

Sox9 plays an essential role during embryonic development as its presence or absence determines the fate of the gonadal supporting cell lineage and thereby controls the establishment of either a testis or an ovary. The aim of this thesis is to investigate the effect of potential ovarian-promoting or anti-testis genes on the expression of Sox9 via the TESCO element, both in vitro and in vivo. Therefore, the repressing potential of DAX1, SOX4, FOXL2, ERα and WNT4-signalling on TESCO activity was individually analysed in transfection assays in vitro. Subsequently, the consequences of loss of either Foxl2 or Wnt4 individually or in combination was assessed in vivo, focusing on the potential de-repression of TESCO and the male pathway in XX gonads during embryonic development and adult stages. Finally, different possible mechanisms by which FOXL2 could mediate repression of TESCO were examined in vitro.
Figure 1.5: Model of distinct Sox9 regulation in XX and XY gonads during early development.

In the bi-potential gonad, SF1 binds to the TESCO enhancer of Sox9 and initiates a low Sox9 expression in both sexes. In XY gonads, SRY binds to TESCO and up-regulated Sox9 expression, which is maintained by SOX9 itself and other factors such as FGF9 and PGD2 signalling. In contrast, the initial Sox9 expression is actively down-regulated in XX gonads and the repression is maintained throughout life. Several anti-testis or ovarian-determining genes have been proposed to be involved in this regulation of Sox9 expression, although no direct interactions have been shown yet.
Chapter 2

Materials and Methods

2.1 Sequence analysis

TESCO sequences from different species were compiled from the Ensemble database (http://www.ensembl.org): mouse sequence (ENSMUSG00000000567), rat sequence (ENSRNOG0000002607), human sequence (ENSG00000125398) and dog sequence (ENSCAFG0000004374). The mouse TESCO sequence was analysed for potential transcription factor binding sites using the MatInspector program (http://www.genomatix.de). To analyse the homology between the TESCO elements of different species, sequences were aligned and compared using the BLAST software from NCBI (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi).
2.2 Luciferase Assay

2.2.1 Cloning of constructs

To analyse the effect of several candidate genes in co-transfection assays, the cDNAs of those genes were individually cloned into a CMV expression vector (for plasmid maps see Appendix). Existing plasmids containing the cDNA of interest were cut by enzymatic digestion following standard protocols. For Foxl2, the gene was amplified from whole ovarian DNA by PCR (KOD Hot Start Polymerase, Novagen) following standard protocols. The DNA fragments were then purified using the GeneClean II kit (Qbiogene) and ligated into the linearised pcDNA3 vector (Invitrogen) for 30 minutes at 16°C. Subsequently, constructs were transformed into chemically competent TOP-10 cells (Invitrogen) by adding the ligation mix to 50μl of TOP-10 cells, incubating on ice for 20 minutes followed by an incubation at 42°C for 1 minute. After resting for 5 minutes on ice, 500μl of LB medium was added to the cells, which were then incubated at 37°C for 1 hour. 100μl of transformed cells were streaked onto LB plates containing ampicillin (50μg/ml) and incubated at 37°C overnight. The next day, single bacterial colonies were picked and incubated in 2ml LB broth containing ampicillin (100μg/ml) at 37°C in a Brunswick Scientific Shaking Incubator for 6 hours at 350rpm. Plasmid DNAs were extracted via Miniprep following standard protocols and successful cloning was determined by restriction digest. For large-scale DNA preparations, 100ml LB broth containing ampicillin (100μg/ml) were inoculated with 100μl of the remaining Mini-culture from the correct clones and incubated overnight at 37°C. The plasmid DNAs were extracted via Maxipreps.
using the Nucleobond Xtra kit (Macherey-Nagel) and stored at 4°C. From each clone, 1ml of culture was kept and 0.5ml 50% glycerine was added to generate glycerine stocks which were stored at -80°C.

For analyses of transcription factor binding, several base pairs were mutated in the TESCO element. This was done using modified oligonucleotides in a PCR amplification of the TESCO sequence using the KOD polymerase, resulting in specific point mutations at the sites of interest. The TESCO sequence with all FOX/ERE sites mutated was generated by de novo DNA synthesis (GENEART, Germany). The altered TESCO sequences were then cloned into the Luciferase vector (p51LUCII) between BamHI and SalI. All constructs were sequenced (Cogenics) and analysed to determine the accuracy of the mutations and cloning.

2.2.2 Cell culture

COS7 cells (ATTC #CRL-1657) were cultured in Dulbecco’s modified Eagle medium (DMEM, Sigma) with 10% fetal calf serum (FCS, Gibco), 1% L-glutamine (Gibco) and 1% Penicillin/Streptomycin (Gibco) at 37°C and 5% CO₂. When the cells were 70-80% confluent, they were washed with PBS (calcium and magnesium free, Gibco) and incubated with Trypsin-EDTA (Gibco) until they detached from the bottom of the flasks. Culture medium was added to stop trypsinisation and the cells were centrifuged at 1000rpm for 5 minutes at 4°C. The cell pellet was then resuspended in fresh culture medium and the cells re-plated at a lower density. For stock freezing, cells were re-suspended in 0.5ml of DMEM containing 20% FCS and 10% DMSO (Sigma) and stored in liquid nitrogen.
2.2.3 Transfection assays

COS7 cells were plated in normal culture medium at 5x10^4 cells per well in 24-well plates 24 hours before transfection. The cells were then transfected with 0.5µg Luciferase vector containing the TESCO element (either wild-type or with inserted point mutations) and 0.1µg of the expression vectors containing Sfl, Sox9 or Sry to activate Luciferase activity. To analyse potential repression effects, the same amount of expression vectors containing Foxl2, ERα, Dax1, Sox4, β-catenin, Tcf1, Tcf3, Tcf4 or Lef1 were added individually in different co-transfection assays. To determine dosage-dependent effects, increasing amounts of these effector genes were transfected (20ng, 60ng, 180ng, 540ng). The amount of expression vector was equalised by adding pcDNA3 vector, the amount of DNA was adjusted using pBluescript vector. For normalisation 0.3µg pmiwZ (Suemori et al., 1990) was added. The DNA and 4µg Lipofectamine (Invitrogen) were mixed in serum-free OptiMEM (Gibco), incubated at room temperature for 20 minutes and then added to the cells in fresh DMEM. Luciferase activity was measured 48 hours after transfection using the Luciferase assay system (Promega) and normalised to β-galactosidase activity using 2.5µg of o-Nitrophenyl-β-D-galactopyranosid substrate (ONPG, Sigma) per ml of LacZ buffer (10mM KCl, 1mM MgCl₂, 40mM NaH₂PO₄, 60mM Na₂HPO₄, 1mM DTT). All co-transfection assays were performed in triplicates, error bars indicate the standard deviation from three biological replicates and p-values were calculated using the GraphPad Software t-test calculator (www.graphpad.com/quickcalcs/ttest1.cfm).
2.3 Mouse Lines

2.3.1 Husbandry

Animals were kept on a 12 hour light-dark cycle, water and food were provided *ad libitum*. For embryonic studies, the morning of vaginal plug (VP) detection was defined as 0.5 days *post coitum* (dpc), assuming the time of conception was in the middle of the dark phase. For postnatal studies, the day when pups were born was defined as postnatal day zero (P0), followed by the first day *post partum* (dpp). All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986 under the project licence 80/1949A and the personal licence 80/10095.

2.3.2 Genotyping

Ear pieces were lysed overnight at 55°C in lysis buffer (0.5M Tris-HCl pH 8.5, 5mM EDTA, 0.1M NaCl, 2.5% SDS) with 1mg/ml proteinase K (Roche). The DNA was then purified by Phenol extraction using an equal amount of Phenol-Chloroform-Isoamylalcohol (25:24:1) followed by precipitation with NaOAc (1/10 volume) and Isopropanol (1x volume). Finally, the DNA was resuspended in 50μl TE and used in PCRs. For genotyping of embryos or newborn pups, a small piece of tail was used and the DNA extracted as described above.
2.3.3 Mouse lines

TESCO:CFP

The 1.3 kb TESCO sequence was cloned into the Asshsp68LacZpA vector, containing the hsp68 minimal promoter and in which the \textit{LacZ} gene had been replaced by ECFP. The TESCO element drives CFP expression specifically in Sertoli cells of the testis (Sekido and Lovell-Badge, 2008). The mice were kept on an MF1 background and the presence of the transgene was detected by standard PCR with primers located in the ECFP: 5’-GACCTGAAGTTCATCTGCAC-3’ and 5’-GTGGCTGATGTAGTTGTACTC-3’ (for protocol see Appendix S2).

TESCO pAECFPhsp68

\textit{Foxl2}^{\textit{LacZ}}

\textit{Foxl2}^{\textit{LacZ}} mice were originally generated and described by Schmidt et al. (2004). \textit{Foxl2} is a single exon gene and encodes for a protein of 375 amino acids. Upon homologous recombination at the \textit{Foxl2} locus, the amino acids 62 to 375 were replaced by the \textit{LacZ} gene, generating a functional null allele (see diagram below). The mice were first kept on a C57BL/6 background, but later bred onto an MF1 background. Homozygous offspring were obtained by inter-crossing heterozygous males and females. The \textit{Foxl2}^{\textit{LacZ}} mice were genotyped by detecting the wild-type \textit{Foxl2} allele with the primer set (5’-CAGATGATGGCCAGCTACCCCGAGC-3’ and 5’-GTTGTGGCAGGATGCTATTCTGCCAGCC-3’) and the mutant allele
with a third primer (5'-GTAGATGGGCGCATCGTAACCGTGC-3') which is located within the LacZ sequence and the forward primer described above (for protocol see Appendix S2).

**Wnt4<sup>neo</sup>**

*Wnt4<sup>neo</sup>* mice were originally generated and described by *Stark et al.* (1994). Homologous recombination at the *Wnt4* locus resulted in a deletion of about 2.2 kb including the coding sequence for the WNT4 amino acids 106 to 196 and replacement by a PGK-neo selection cassette, resulting in a functional null allele (see diagram below). The mice were kept on an 129/Sv background and homozygous offspring were generated by inter-crossing heterozygous mice. The wild-type *Wnt4* allele was detected with the primers 5'-CTTCACAACAACGAGGCTGGCAGG-3' and 5'-CACCCGCATGTGTGTCAAGATGG-3', whereas the targeted *Wnt4<sup>neo</sup>* allele was identified by a primer located within the sequence of the PGK-neo cassette 5'-GCATTGTCTGAGTAGGTGTCATTC-3' and the reverse primer described above (for protocol see Appendix S2).
R26DTA mice were originally generated by Ivanova et al. (2005) and contain a GFP/PGK-neo cassette between two LoxP sites followed by the diphtheria toxin fragment A (DTA) in the ROSA26 locus. In the absence of Cre recombinase, GFP is expressed and DTA is not active. Upon Cre recombination, the GFP/PGK-neo cassette located between the two LoxP sites (pink triangles in the diagram below) is excised resulting in the activation of DTA. When expressed, the subunit A of the diphtheria toxin mediates the termination of all protein synthesis which eventually leads to apoptosis of the cell (Maxwell et al., 1986), therefore resulting in cell depletion specifically in the cell type expressing the Cre. Homozygous R26DTA mice were kept on the C57BL/6 background and bred to mice carrying a Cre recombinase under the control of the oocyte-specific promoter of the zona pellucida 3 gene (Zp3:Cre, Fvb background) to generate Zp3:Cre, R26DTA mice. The presence of the Zp3:Cre allele was detected by PCR using the primer set 5’-GGCGGATCCGAAAAGAAAA-3’ and 5’-CAGGGCGCGAGTTAGTAGC-3’ (for protocol see Appendix 8.2).

After ZP3cre recombination

Chr6, ROSA26 locus

GFP PGK-neo pA DTA pA

After ZP3cre recombination

Chr6, ROSA26 locus

DTA pA
2.3.4 Harvesting of gonads

For the analysis of embryonic gonads, pregnant mice were harvested using a schedule 1 method at different time points after VP. Embryonic gonads (including the mesonephros at stages earlier than 16.5 dpc) were then dissected into PBS and cleaned from all other tissues. In addition, a small piece of embryo tail was collected and used for genotyping. For analyses of postnatal stages, pups and adult mice were harvested at different ages using a schedule 1 methods and ovaries and testes were then dissected into PBS as described above.

2.4 Immunohistochemistry

2.4.1 Preparation of samples

Embryonic gonads were fixed in 4% PFA (w/v in PBS) for 30 minutes (from 12.5 dpc to 14.5 dpc) or 1 hour (from 16.5 dpc to P0) at 4°C. Postnatal gonads were fixed overnight at 4°C. After fixation, gonads were washed with PBS and equilibrated in 30% sucrose (w/v) at 4°C overnight. Samples were then embedded in OCT compound (VWR) in Dispomoulds and frozen using dry ice. Frozen samples were sectioned at a thickness of 8μm using a Leica cryostat (model CM3050S) and the sections were then collected onto Superfrost Plus slides (Thermo Scientific). Slides were stored at -80°C until further processing.
2.4.2 Histology

For Haematoxylin-Eosin (H+E) staining, postnatal gonads were fixed in Bouin’s solution (Sigma) overnight at 4°C. After fixation, samples were washed in 70% ethanol and then stored in fresh 70% ethanol at 4°C. Samples were subsequently embedded in wax, sectioned at a thickness of 5μm and processed for H+E staining (Histology Service, NIMR).

2.4.3 Immunostaining

Cryosections were thawed at room temperature and then washed twice with PBS to remove the remaining OCT compound. Adult samples were additionally fixed in 4% PFA for 10 minutes at room temperature and then washed again twice with PBS. Afterwards, sections were blocked with 1% normal donkey serum (Sigma) in PBS/0.05% triton for 1 hour at room temperature. Subsequently, sections were incubated with primary antibodies diluted in blocking solution in a humidified chamber overnight at 4°C. The following day, sections were washed three times with PBS/0.1% triton and then incubated for 1 hour with the appropriate fluorescent secondary antibody (Molecular probes) diluted in blocking solution in a humidified chamber in the dark at room temperature. After the slides were washed with PBS/0.1% triton three times, sections were incubated with DAPI solution (Sigma, 1:10000 v/v in PBS) at room temperature for 5 minutes. Finally, sections were rinsed with PBS, air dried and mounted with Aqua PolyMount solution (Polysciences).

For co-staining, sections were washed three times in PBS/0.1% triton after incu-
bation with the secondary antibody and then fixed again in 4% PFA for 10 minutes at room temperature. After rinsing with PBS, sections were blocked for 1 hour at room temperature and the second primary antibody was added in blocking solution and incubated overnight at 4°C. The following day, sections were processed as described for single staining.

**Primary antibodies**

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**Secondary antibodies**

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</table>
2.4.4 Imaging

Pictures of H+E stainings were obtained with a light microscope (Leica CTR-MIC) using a 40x lens and OpenLab software (Improvision, UK). Immunofluorescent pictures were taken with a Leica SP5 confocal using a LAS software (Leica, Germany). Images were taken with either 40x or 100x lenses and processed using Adobe Photoshop 5.5 (Adobe Systems).

2.5 Protein interaction assays

2.5.1 In vitro translation of $^{35}$S-SF1

$^{35}$S-labelled SF1 was generated following the instructions of the TNT coupled reticulocyte lysate system from Promega. The pcDNA3-Sf1 plasmid was used as DNA template (0.5μg/μl). For the reaction mix, 25μl TNT rabbit reticulocyte lysate, 2μl TNT reaction buffer, 1μl RNA Polymerase T7, 1μl 1mM amino acid mixture (without methionine), 1μl RNase Inhibitor, 2μl DNA template and 1μl $[^{35}\text{S}]$methionine (10.25mCi/ml) were incubated in a total volume of 50μl at 30°C for 90 minutes. The reaction was then stopped by transfer to ice and a fraction of the in vitro translated protein was subsequently analysed on a 6% acrylamide gel whereas the remaining sample was aliquoted and stored at -80°C. The gel was run at 170V for 2-3 hours, dried afterwards and the presence of radioactivity was detected by film (Kodak) exposure overnight at -80°C.
2.5.2 Preparation of GST-FOXL2

The Foxl2 cDNA was cloned into the GST gene fusion vector pGEX-2T (Addgene) via BamHI and EcoRI. The construct was transformed into bacterial cells and the following day clones were picked and inoculated in 5ml LB broth at 37°C overnight. Cultures were then transferred to 150ml fresh LB broth and further incubated at 30°C until it reached an OD between 0.5 - 1.0 after approximately 4 hours. Protein production was induced by adding 0.2mM IPTG followed by incubation at 30°C for 2 hours. Subsequently, cultures were centrifuged at 5000rpm for 10 minutes at 4°C. Pellets were resuspended in 5ml PBS (including 100μM ZnCl₂, 1mM PMSF) and sonicated (duty cycle 30%, output control 5, 30 seconds on, 1 minute off, 7 cycles). Afterwards Tween-20 and DTT were added to a final concentration of 1% and 4mM, respectively. Samples were then centrifuged at 4000rpm for 15 minutes at 4°C. The supernatant was incubated with 0.25ml Glutathione Sepharose-4B beads (Pharmacia, 50% w/v in PBS) by mixing gently for 10 minutes at 4°C. The beads were then washed three times with PBS (containing 100μM ZnCl₂, 1mM PMSF) and centrifuged at 800rpm for 2 minutes at 4°C. Afterwards, the beads were resuspended in buffer G (50mM Tris-HCl pH9.5, 5mM Glutathione S-transferase (Sigma)) and mixed gently for 15 minutes at 4°C. Finally, the beads were collected by a short spin up to 5000rpm at room temperature and the supernatant was transferred into a dialysis tube. The protein extracts were dialysed against 10mM Tris-HCl pH 8.0, 100mM ZNSO₄, 1mM PMSF overnight at 4°C. The final protein extracts were aliquoted and stored at -80°C.
2.5.3 Co-Immunoprecipitation

5μl 35S-SF1 and either 3μg GST-FOXL2 or GST control protein were incubated in CoIP buffer (20mM HEPES pH7.9, 100mM KCl, 10% Glycerol, 0.1% Triton, 1mM DTT, 1mM PMSF) at 4°C for 2 hours. Samples were subsequently incubated with 20μl Glutathione Sepharose-4B beads (50% w/v in PBS) for 30 minutes at 4°C. The beads were then washed three times with CoIP buffer and centrifuged at 800rpm for 2 minutes at 4°C. Afterwards, the beads were resuspended in 20μl 2x protein sample buffer (Invitrogen) and boiled at 95°C for 5 minutes. 10μl of each sample and 5μl input control (35S-SF1 protein) were run on 4-12% pre-made gels (Invitrogen) for 1 hour at 170V and 70mA. Afterwards, the gels were dried for 30 minutes and the presence of radioactive sample was detected by film exposure overnight at -80°C.

2.5.4 Nuclear protein extract

COS7 cells were plated at a density of 5x10⁴ cells per 10 cm² dish and incubated at 37°C and 5% CO₂. The following day, 24μg of either pcDNA-Foxl2 or pcDNA-Sf1 were transfected using 60 μg Lipofectamine in fresh medium. After 48 hours, the cells were washed twice with PBS and then centrifuged for 15 seconds at 13000rpm at 4°C. The cells were resuspended in 600μl ice cold buffer A (10mM HEPES pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 1x proteinase inhibitor cocktail) and incubated on ice. After 15 minutes, 37.5μl 10% NP-40 were added to the cells, which were vortexed for 10 seconds and then centrifuged for 30 seconds at 13000rpm at 4°C. The supernatant, which contains
the cytoplasmic proteins, was removed and the pellet resuspended in 50µl ice cold buffer C (20mM HEPES pH7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1x proteinase inhibitor cocktail). After incubation at 4°C for 15 minutes, the samples were centrifuged for 5 minutes at 13000rpm at 4°C. The supernatant containing the nuclear cell extract was then aliquoted and stored at -80°C.

2.5.5 Electrophoretic Mobility Shift Assay (EMSA)

Two complementary DNA sequences of a length of 43 nucleotides, each containing a 5’ overhang including a GTP, were annealed to form a double strand. 600pmol of each oligonucleotide (MWG) were incubated in 100µl STE (100mM NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA) for 10 minutes at 95°C, followed by 30 minutes at 65°C, 1 hour at 37°C and overnight at 4°C. The double stranded sequences were then labelled by filling the 3’ gaps of each strand with nucleotides including \([\alpha\textsuperscript{32}P]dCTP\) by Klenow polymerase in a total volume of 50µl for 30 min at 37°C (50ng DNA, 5µl 10xbuffer, 1µl 5mM d(GAT)P, 2µl d\textsuperscript{32}P-\alphaCTP (10mCi/ml), 1µl Klenow DNA polymerase I (5U/µl)). The reaction was stopped by phenol-chloroform extraction and 50µl STE were added before the labelled probes were purified over a Sephadex G50 column. The radioactive incorporation was determined via the Cherenkov method and samples with greater than 10^6 cpm/μg DNA were used in subsequent shift assays. Binding reactions for SF1 and FOXL2 using nuclear protein extracts were carried out in DNA binding buffer (20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 20% Glycerol, 1 mM DTT, 50 µg/ml poly dI-dC, 2.5 µg/ml salmon sperm DNA, 50 µg/ml BSA) using 1 µl 32P end-
labelled probe and 1 μl nuclear protein extract in a total of 20μl for 30 minutes at room temperature. The incubations were stopped by transfer to ice and 2μl 10x loading dye (0.2M HEPES pH7.9, 0.5M KCl, 10mM EDTA, 0.25% BPB) were added to each sample. 10μl of each sample was subsequently run on 6% acrylamide gels in 0.5x TBE for 4 hours at 100V. The gels were then dried for 30 minutes and analysed by film exposure overnight and for 1 week at -80°C.
Chapter 3

TESCO modulation by ovarian determining genes in vitro

3.1 Introduction

The key element in the early gonad for ovarian development is the down-regulation of Sox9 expression in the supporting cell lineage by 11.5 dpc. If Sox9 fails to be down-regulated and is instead maintained, XX female-to-male sex reversal occurs. This has been shown in XX human patients with duplication of the chromosomal region containing SOX9 (17q23-24) and by transgenic misexpression of Sox9 in XX mice (Huang et al., 1999; Bishop et al., 2000; Vidal et al., 2001). However, it was not known which gene(s) are responsible for the initial down-regulation of Sox9 expression in XX gonads and the maintenance of this repressed state throughout adult life. Several genes have been proposed to act as ovarian promoting or anti-testis genes and might be involved in the initial down-regulation of Sox9 and/or in the maintenance of its repression in the XX gonad.

Recently, the 1.3 kb enhancer sequence TESCO was identified as the regulatory
element for sex-specific Sox9 expression in the XY gonad (Sekido and Lovell-Badge, 2008). Moreover, in vitro studies in COS7 cells using a reporter assay approach in which the mouse TESCO element drove the expression of a Luciferase reporter gene, showed that TESCO could not be activated by SRY or SOX9 by themselves. However, it could be activated about 5-fold by SF1, about 10-fold by combination of SF1 and SRY and a robust activation (30-fold) was detected by a combination of SF1 and SOX9 (Sekido and Lovell-Badge, 2008). When the consensus binding sites for both SF1 and SOX proteins were mutated in the TESCO sequence, no activity could be detected in the co-transfection assays, furthermore indicating a synergistic effect between SF1 and SRY/SOX9 in regulating TESCO activity. Studies in mice carrying a CFP reporter transgene under the control of the TESCO element showed that the enhancer activity recapitulates the gonadal expression of Sox9 in vivo. While high levels of TESCO activity could only be detected in the presence of endogenous SOX9, some reporter activity was also seen in the absence of SOX9 at 11.5 dpc in vivo, indicating that SRY is required for TESCO activation. Furthermore, corresponding to the in vitro results, mutation of the SF1 and SOX binding sites abolished TESCO:CFP expression in the transgenic mice (Sekido and Lovell-Badge, 2008). All these data let to the proposal that the TESCO element is necessary to activate and maintain Sox9 expression in the XY gonad.

On the other hand, it is also possible that the TESCO element plays a role in the regulation of Sox9 in the XX gonad, either in its initial down-regulation and/or the maintenance of its repression. This hypothesis raises the question whether any of the proposed ovarian determining or anti-testis genes could be involved in the
regulation of TESCO activity in the XX gonad. These genes have been suggested to be important for ovarian development due to their sex-specific expression in XX gonads both during the time of sex determination and in the adult. Thus, the aim of the study presented in this chapter was to analyse whether the ovarian promoting genes could indeed regulate TESCO activity.

To determine whether DAX1, SOX4, β-catenin, FOXL2 and ERα could possibly repress Sox9 expression via the TESCO element, the TESCO sequence was first analysed for potential binding sites of the proposed transcription factors. Subsequently the individual effect of each factor on the TESCO element was analysed by in vitro co-transfection assays.
3.2 Results

3.2.1 Sequence analysis of TESCO

The 1.3 kb TESCO sequence was analysed for putative binding sites of forkhead factors (FOXL2), EREs, nuclear receptors (DAX1) and LEF/TCF factors (as effectors of Wnt4-signalling) using the MatInspector program (Quandt et al., 1995).

The search revealed two possible forkhead factor binding sites, three ERE half sites, two LEF/TCF binding sites and three nuclear receptor sites (Figure 3.1). In addition to the sites identified in this study, the TESCO sequence also contains six SF1-specific binding sites and three sites for SOX proteins (Sekido and Lovell-Badge, 2008). From the Ensemble database, the TESCO sequences were compiled for mouse (ENSMUSG00000000567), rat (ENSRNOG0000002607), human (ENSG00000125398) and dog (ENSCAFG0000004374) and then analysed by the NCBI-BLAST software. A comparison between those species showed high conservation for most of the predicted binding sites (see Appendix 8.3), suggesting a potential role for these sites in Sox9 regulation.

To test if FOXL2, ERα, DAX1, SOX4 or the β-catenin/LEF/TCF complex could indeed exert a repressive effect on the TESCO element, in vitro co-transfection assays were carried out.
Figure 3.1: **Sequence analysis of TESCO.**

The TESCO sequence contains several binding sites for forkhead factors (FOXL2), EREs, nuclear receptor sites (DAX1) and binding sites for LEF/TCF factors, as well as SF1 and SOX binding sites.

- **FOXL2 binding sites**
  - G/A T/C C/A A A C/T A

- **Estrogen response half site**
  - GGTCA

- **TCF/LEF-1 binding sites**
  - C/T C T T G A/T A/T

- **SF1 binding sites**
  - T/C A A G G T/C C G/A

- **SOX binding sites**
  - A G A A C A A T G G

- **possible DAX1 binding sites**
  - secondary structure
3.2.2 Repression of TESCO in vitro

To investigate the possible repressive effects of DAX1, SOX4, WNT4, FOXL2 and ERα, each of the genes was cloned into the same expression vector used for SF1 and SRY. Each construct was then expressed in COS7 cells together with the TESCO reporter construct and combinations of SF1, SF1 + SOX9 and SF1 + SRY, respectively. Dosage-dependent effects were analysed by co-transfection of different amounts of the candidate gene upon TESCO activation by SF1 + SOX9.

Addition of DAX1 resulted in 74% reduction of TESCO activation when activated with SF1, 85% when activated with SF1 + SOX9 and 86% when activated with SF1 + SRY (Figure 3.2a). Increasing amounts of DAX1 showed a dosage-dependent reduction of TESCO activity down to background levels (Figure 3.2b).

Addition of SOX4 did not have any significant effect on TESCO activation by SF1, but co-transfection of SOX4 with SF1 + SRY or SF1 + SOX9 resulted in a reduction of around 10% (Figure 3.3a). Increasing amounts of SOX4 correlated with a greater repression of TESCO activity, up to a reduction of about 60% of the original activation by SF1 + SOX9 (Figure 3.3b).
Figure 3.2: Repression of TESCO by DAX1 in vitro.
SF1, SF1 + SOX9 or SF1 + SRY mediated TESCO activation was robustly repressed in vitro by co-transfection of DAX1 [a]. This effect was dosage-dependent as higher amounts of Dax1 resulted in a greater repression of TESCO [b]. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
Figure 3.3: Repression of TESCO by SOX4 in vitro.
SOX4 did not have any significant effect on the activation of TESCO by SF1 alone, but in combination with SF1 + SOX9 or SF1 + SRY resulted in a minor repression of TESCO activation. Increasing amounts of SOX4 revealed a dosage-dependent repression effect on SF1 + SOX9 mediated TESCO activation. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with * $p < 0.05$, ** $p < 0.01$, ns = not significant.
The co-transfection assays with FOXL2 were first done using an EGFP-Foxl2 N-terminal fusion plasmid (gift from M. Treier). These assays showed a reduction of TESCO activity by 32% when activated with SF1, by 35% when activated with SF1 + SOX9 and a reduction by 43% when activated with SF1 + SRY (Figure 3.4a). This effect was dosage-dependent as higher amounts of FOXL2 resulted in greater TESCO repression, up to a reduction of about 72% of the original activation by SF1 + SOX9 (Figure 3.4b). However, because EGFP can dimerise and this might compromise the activity of the linked FOXL2, the co-transfection assays were repeated using a different construct containing only the Foxl2 gene. The same results were obtained: reduction of TESCO after activation by SF1, SF1 + SOX9 or SF1 + SRY and a dosage-dependent repression effect (data not shown).

To investigate a possible interaction between FOXL2 and estrogen receptors during sex determination, the effect of ERα on TESCO activity was analysed on its own and in combination with FOXL2. ERα alone showed no significant effect on TESCO activation by SF1 + SOX9. However, when ERα was co-transfected with FOXL2 it enhanced the FOXL2-mediated repression of TESCO activation, down to around 10% of the original activation by SF1 + SOX9. This synergistic repression effect of ERα seemed to be independent of the amount used (Figure 3.5).
Figure 3.4: Repression of TESCO by FOXL2 in vitro.
TESCO activation was repressed in vitro by co-transfection of FOXL2 to SF1, SF1 + SOX9 or SF1 + SRY, respectively [a]. This effect was dosage-dependent as higher amounts of Foxl2 resulted in a greater repression of TESCO [b]. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
Figure 3.5: Repression of TESCO by ER$\alpha$ in vitro.
ER$\alpha$ on its own did not have any effect on SF1 + SOX9 mediated activation of TESCO, but it enhanced the repression effect mediated by FOXL2. This enhanced repression was independent of the amount of ER$\alpha$ used. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with *** $p < 0.001$, ns = not significant.
Next, the effect of WNT signalling on TESCO activation was assessed. WNT4 itself did not activate the WNT-reporter plasmid TOPflash (Molenaar et al., 1996) in COS7 cells (data not shown), which suggests that the WNT4 signalling pathway is not active in this cell line. Thus, the effectors of the signalling pathway, such as β-catenin and LEF1, TCF1, TCF3, TCF4 were used in the co-transfection assays to test the implication of WNT signalling on TESCO activity. As the β-catenin/LEF/TCF complex usually acts as a transcriptional activator, it was theoretically possible that this complex could activate TESCO on its own or enhance the activation mediated by SF1 or SF1 + SOX9. However, co-transfection assays with β-catenin alone or in combination with any LEF/TCF factor did not activate TESCO in the absence of SF1 + Sox9 (Figure 3.6a). Moreover, TESCO activation by SF1 + Sox9 was not significantly altered by addition of β-catenin alone, any of the LEF/TCF factors on their own or by combinations of β-catenin and any of the LEF/TCF factors (Figure 3.6b). For both assays, the results are shown for TCF3 though the same results were obtained for TCF1, TCF4 and LEF1 (data not shown). Even using increasing amounts of β-catenin did not result in any effect on TESCO activation by SF1 or SF1 + SOX9 (Figure 3.6c).

Next, the possible enhancement of forkhead transcriptional activity by interaction with β-catenin was analysed. As FOXL2 represses TESCO activation, the presence of β-catenin could result in a stronger repression. However, co-transfection assays showed that addition of β-catenin did not have any effect on the FOXL2-mediated repression of TESCO activity (Figure 3.7).
**Figure 3.6:** Effect of β-catenin and LEF/TCFs on TESCO activation *in vitro*. β-catenin did not activate TESCO on its own or with TCF3. Activation of TESCO by SF1 + SOX9 was not affected by addition of β-catenin, TCF3 or both [a]. Increasing amounts of β-catenin did not result in any effect on either SF1 or SF1 + SOX9 mediated activation of TESCO [b]. Assays were performed in triplicate and bars indicate the standard deviation of three biological replicates.
Figure 3.7: Effect of β-catenin on FOXL2 mediated repression of TESCO in vitro.

β-catenin did not have any significant effect on FOXL2 mediated repression of TESCO. Assays were performed in triplicate, bars indicate the standard deviation of three biological replicates, asterisks show significance with *** $p < 0.001$, ns = not significant.
3.3 Conclusions

In the early XX gonad, Sox9 expression is down-regulated in the supporting cell lineage at 11.5 dpc and this repression is maintained throughout life. Although a number of genes have been proposed as ovarian promoting or anti-testis genes, such as Wnt4, β-catenin, Dax1, Sox4, Foxl2 and ERα, it is not known if any of these genes has a primary role in the repression of Sox9. In the XY gonad, Sox9 expression is regulated via the TESCO enhancer element (Sekido and Lovell-Badge, 2008), it is possible that the down-regulation in the XX gonad is mediated via this element as well. To determine whether the proposed ovarian genes have the potential to repress Sox9 expression by modulating TESCO activity, in vitro co-transfection assays were performed.

In the WNT signalling pathway, LEF/TCFs are the executive factors in Wnt canonical pathways, which lead, in association with β-catenin, to the activation of downstream target genes. On the other hand, it has been shown that LEF/TCF proteins can physically interact with co-repressors, such as Groucho and CtBP and thereby lead to the repression of their target genes (Rooste et al., 1998). Moreover, TCF3 has been demonstrated to act as a repressor in the absence of stabilised β-catenin (Merrill et al., 2001). Thus, there are several different possibilities as to how β-catenin and LEF/TCF factors could be involved in Sox9 regulation: β-catenin in combination with any LEF/TCF factor could act as an activator of Sox9 expression via the TESCO element or TCF3 could act as a repressor in the absence of β-catenin. However, the co-transfection assays showed that β-catenin does not affect TESCO on its own or in combination
with any LEF/TCF factor, indicating that these effectors are not contributing
to the activation of the TESCO element. Moreover, SF1 + SOX9 mediated
activation of TESCO was not affected by TCF3, arguing against the hypothesis
that TCF3 might act as a repressor of Sox9 in the absence of β-catenin. It has been
reported that forkhead factors can compete with TCFs for the interaction with
β-catenin. The formation of a forkhead/β-catenin complex has been shown to
enhance forkhead transcriptional activity ([Hoogeboom et al., 2008]). However, the
results obtained in this thesis show that addition of β-catenin did not significantly
affect FOXL2 mediated repression of TESCO activity. Taken together, none of
the tested effectors of the WNT signalling pathway had any significant effect
on TESCO activation in the in vitro co-transfection assays. This could imply
that WNT signalling is not affecting Sox9 transcription or that it is not acting
simply through the TESCO element but possibly via other regulatory elements.
However, it is also possible that necessary co-factors involved in the signalling
process or in SOX9-β-catenin degradation (like Groucho or CtBP) are missing
in this type of in vitro assay system and therefore no effect can be seen due
to the limitations of the system. Another intriguing possibility is that WNT-
signalling could affect Sox9 expression only indirectly, e.g. via DAX1. It has
already been demonstrated that Dax1 expression is significantly decreased in
XX Wnt4-deficient mice and that Dax1 transcription is activated by β-catenin
(in cooperation with SF1), indicating that Dax1 is a downstream target gene of
Wnt4. Therefore, it is possible that Wnt4 signalling might act solely through β-
catenin mediated activation of Dax1 ([Mizusaki et al., 2003]) in sex determination.

All other candidate genes tested in the in vitro co-transfection assays in this
chapter did have an effect on TESCO expression. Dax1 was able to repress TESCO activation in a dosage-dependent manner. The repression effect of Dax1 was the strongest of all genes analysed. It is already known that DAX1 can form heterodimers with SF1, which results in a decrease of SF1 activity ([Ito et al., 1997; Crawford et al., 1998]). Since SF1 is required for TESCO activation, a reduction in SF1 activity in the presence of DAX1 could cause the observed repression of TESCO. However, the detailed mechanism as to how DAX1 represses TESCO activation, whether by direct binding to the TESCO sequence or by interaction with SF1, remains to be investigated.

SOX4 also repressed TESCO activation, though it had the weakest repressor effect of all genes analysed. Interestingly, SOX4 did not have a repressive effect on TESCO activation mediated by SF1 alone, but was able to repress both SF1 + SOX9 and SF1 + SRY mediated TESCO activation. The TESCO sequence contains multiple SOX binding sites, which are crucial for its activation by SRY and SOX9. SOX4 contains the same, highly conserved, DNA binding domain (HMG-box) as other SOX proteins and probably binds to the same sites in the DNA. Thus, it is possible that SOX4 competes with SOX9 and SRY for binding to the TESCO enhancer sequence. It could be speculated that SOX4 and SOX9, as members of different groups of SOX proteins, exhibit different effects on the TESCO element: SOX9 (belonging to the SOXE group) activates TESCO while SOX4 (belonging to the SOXC group) might act as a repressor or just reduces the amount of SOX9 bound to TESCO.

FOXL2 was able to repress TESCO activity in a dosage-dependent manner,
whereas ERα had no effect on TESCO activation. Interestingly, the repressive effect of FOXL2 was even more severe in the presence of ERα, suggesting a synergistic effect of the two proteins on TESCO activation. However, this synergistic repressive effect seems to be independent of the amount of ERα available.

In summary, the proposed ovarian promoting genes Dax1, Sox4 and Foxl2 (on its own or in combination with ERα) are able to repress the activation of the TESCO element in vitro. This suggests that they might indeed be involved in the regulation of Sox9 expression in vivo via the TESCO element. Moreover, the result that FOXL2 can repress TESCO activity provides the first direct link between FOXL2 and Sox9 regulation. To gain more insight into FOXL2 mediated repression of TESCO, possible mechanisms of this regulatory effect were analysed in vitro.
Chapter 4

Possible mechanisms of TESCO modulation by FOXL2

4.1 Introduction

FOXL2 is able to repress Sox9 expression via the TESCO enhancer element in vitro. However, it is important to further determine how FOXL2 interacts with TESCO and how exactly the repression is mediated.

FOXL2 is a member of the forkhead gene family of transcription factors (Crisponi et al., 2001) and members of this family are characterised by a DNA binding domain consisting of a helix-turn-helix core element of three α-helices flanked by two loops or wings (Figure 4.1). The recognition Helix 3 interacts with the major groove of DNA and Wing 2 with the minor groove. The sequence of this forkhead domain (FHD) is highly conserved, indicating a similar 3D structure and DNA recognition within the forkhead factor family, whereas regions outside the forkhead domain are poorly conserved between family members (Carlsson and Mahlapuu, 2002). Forkhead proteins bind DNA as monomers to the consensus...
binding site \((A/G)(C/T)(A/C)AA(A/T)A\) \((\text{Kaulmann et al., 1995})\) and in doing so bend the DNA up to an angle of 80-90° \((\text{Pierrou et al., 1994})\). This property is unusual as most transcription factors do not induce such a distortion in DNA. However, the HMG boxes of SOX proteins are also known to bend the DNA up to 90° by binding to the minor groove \((\text{Sekido and Lovell-Badge, 2008})\). The flanking regions on both sides of the consensus sequence are important for the protein binding affinity to the DNA. Forkhead factors have been shown to act as both transcriptional activators and repressors \((\text{Carlsson and Mahlapuu, 2002})\) and can be found in a variety of vertebrate and non-vertebrate species indicating a conserved mechanism of action \((\text{Mazet et al., 2003})\).

FOXL2 is a nuclear protein and contains the arginine/lysine-rich sequence of the classical nuclear localisation signal (NLS) at the C-terminus of the forkhead domain \((\text{Figure 4.1})\). In addition, FOXL2 also comprises a non-conventional NLS (KGNYWTLDPCEDMFEEKNY) just prior to the classical motif \((\text{Moumne et al., 2008})\). The C-terminal part of the FOXL2 protein contains two polyA and one polyP stretch \((\text{Figure 4.1})\). About 30% of all reported human FOXL2 mutations are attributed to expansion of the polyA tract from the normal 14 alanines to up to 24 \((\text{Moumne et al., 2008})\). In vitro analyses have shown that expansion of the polyA stretch results in mislocalisation and cytoplasmic aggregation of the protein. FOXL2 is normally exclusively localised in the nucleus and therefore cytoplasmic aggregation results in loss of its activity. On the other hand, a patient with primary amenorrhea carrying a 30 bp deletion in the FOXL2 ORF resulting in a reduction of the polyA tract down to 4 alanines, was able to naturally deliver two healthy babies \((\text{Harris et al., 2002; Gersak et al., 2004})\).
Figure 4.1: The FOXL2 protein.
The FOXL2 protein consists of 375 amino acids, the forkhead domain (FHD) ranges from amino acid 50 - 150 and includes three helices, two β-strands and two loop (or wing) structures. FOXL2 contains a classical and an additional nuclear localisation sequence (NLS) at the C-terminal end of the FHD. Outside the FHD are two poly-A stretches and one poly-P stretch.

Only a few target genes for FOXL2 have been described so far. The first direct target to be identified was the promoter of murine gonadotrophin releasing hormone receptor (GnRHR). *In vitro* assays demonstrated that FOXL2 together with SMAD3/4 activated the GnRHR activating element (GRAS) (*Ellsworth et al.*, 2003). Another FOXL2 target gene is Cyp19 which encodes aromatase, the enzyme that converts androgens into estrogens. In the goat, FOXL2 and CYP19 co-localise in somatic cells of the XX gonad (*Pannetier et al.*, 2006a) and Cyp19 expression is drastically decreased upon FOXL2 loss in XX foetuses carrying the PIS mutation (*Pailhoux et al.*, 2001). Moreover, FOXL2 was able to bind and activate the goat Cyp19 promoter *in vitro*, suggesting that it is involved in the regulation of estrogen production in the female gonad (*Pannetier et al.*, 2006a).
FOXL2 could also directly bind to the promoter of human steroidogenic acute regulatory gene (StAR), which controls the rate-limiting step in steroidogenesis, and repress its transcription (Pisarska et al., 2004). StAR is expressed in differentiated granulosa cells and repression by FOXL2 is thought to prevent accelerated follicular development mediated by an increase of StAR activity, which would ultimately lead to premature ovarian failure (POF). FOXL2 transcripts with a truncation of the C-terminal domain resulting in the loss of the polyalanine tracts, failed to repress StAR activity in vitro (Pisarska et al., 2004). Recently it has been shown that the activity of FOXL2 as a transcriptional repressor of the StAR gene depends on sumoylation. Indeed, FOXL2 and the E2-conjugating enzyme Ubc9 are co-expressed in granulosa cells of small and medium follicles of the developing ovary and FOXL2 is sumoylated at lysine-25 via Ubc9-mediated sumoylation (Kuo et al., 2009). FOXL2 was also able to repress the rat Dmrt1 promoter in in vitro co-transfection assays. The Dmrt1 promoter contains several forkhead factor binding sites and removal of these sites, by shortening of the promoter sequence, resulted in the loss of repression mediated by FOXL2 (Lei et al., 2009). A bioinformatic screen using the human steroidogenic granulosa-like cell line KGN, identified new potential FOXL2 target genes, which suggest the involvement of FOXL2 the regulation of a number of different genes, including immunomodulators (e.g. IFNB1, IL12A), transcription factors (e.g. NFATC2, NR5a2) and regulators of apoptosis (e.g. BCL2A1, IER3) (Batista et al., 2007).

Forkhead factors have been described to act as co-repressors on their target genes by directly interacting with other proteins: for example, FOXG1 interacts with androgen receptor (AR) and thereby acts as an AR co-repressor (Obendorf et al., 2006b).
and FOXP1 interacts with SMRT to repress the differentiation gene c-fms in monocytes (Jepsen et al., 2008). FOXL2 has been found to directly interact with few proteins so far. It has been shown to interact with the dead box protein DP103 resulting in an increase in apoptosis in vitro (Lee et al., 2005). Moreover, FOXL2 interacts with SMAD3 to regulate the transcription of Follistatin in vitro (Blount et al., 2009) and in tilapia, FOXL2 and SF1 interact to activate Cyp19 gene expression (Wang et al., 2007). Direct interaction has also been demonstrated between FOXL2 and ERα in co-immunoprecipitation experiments in vitro (Uhlenhaut et al., 2009; Kim et al., 2009).

The results presented so far, have shown that FOXL2 can repress TESCO activity in vitro. The TESCO element contains forkhead factor binding sites suggesting that FOXL2 could directly bind to the TESCO element. Indeed, chromatin immunoprecipitation assays have demonstrated that FOXL2 can bind to the TESCO enhancer element of Sox9 (Uhlenhaut et al., 2009). In this chapter, possible mechanisms of how FOXL2 is mediating its repressive effect on TESCO was analysed in more detail in vitro.
4.2 Results

4.2.1 Analyses of mutations in TESCO binding sites

The TESCO element contains two highly conserved consensus forkhead factor binding (FOX) sites (Figure 3.1) through which the repression effect of FOXL2 could be mediated. Each of these binding sites was mutated individually as well as in combination, by replacing the FOX sites with SacI restriction sites, to determine their importance for FOXL2 binding. The mutated TESCO sequences were then cloned into the luciferase vector and their activities were analysed in co-transfection assays with SF1 + SOX9 and SF1 + SOX9 + FOXL2 using COS7 cells, respectively. Neither individual nor combined mutations of those sites resulted in a statistically significant loss of FOXL2 mediated repression of TESCO activity (Figure 4.2).

Figure 4.2: Effect of FOXL2 on TESCO with 2 mutated FOX sites. TESCO activation by SF1 + SOX9 could be repressed by addition of FOXL2, even after mutation of the highly conserved forkhead factor binding sites (#1: bp 528-534 and #2: bp 974-980) either individually or simultaneously. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.
It was possible that FOXL2 could still repress TESCO activity after mutation of these two consensus binding sites because the TESCO element might contain other forkhead factor binding sites which had not been picked up by the initial search with the MatInspector program. Thus, the TESCO sequence was searched manually for the core sequence of forkhead factor binding sites (C/A)AA(C/T)A. The search revealed 10 possible binding sites, including the two sites found in the original search, evenly distributed over the TESCO sequence (Figure 4.3).

To determine the importance of these sites, several mutation constructs of the TESCO sequence were analysed in co-transfection assays. These constructs contained 200 - 250 bp deletions in different parts of the TESCO sequence: deletion of bp 72 to 335 including two possible FOX sites (∆C4), deletion of bp 354 to 548 including three possible FOX sites (∆C5), deletion of bp 564 to 829 including one possible FOX site (∆C6), deletion of bp 847 to 1054 including one possible FOX site (∆C7). Another construct contained a shorter version of TESCO, lacking 200bp at the 3’-end which included one possible FOX site (1.0 kb). For a detailed sequence map see Appendix Figure 8.4. All of the constructs (except ∆C6) also contained established binding sites for SF1 and/or SOX9. Due to the reduced number of activation sites, the overall activation potential of these deletion constructs was diminished when co-transfected with SF1 + SOX9. However, repression of TESCO activity upon addition of FOXL2 was found in all these constructs. A slight reduction in repression ability of FOXL2 could be seen in the assays with the ∆C6 and ∆C7 constructs, but these differences were not statistically significant (Figure 4.4).
1 CATATGTCAC ATACCTAAGG TGAAAATATA CGAGCCCTGT CTAAATCGGA ACTCCAACCA
61 TGTACCATTT TCCTTAAGGC CCCACAGGAA GAAAAAGGGA AAAAGAGAGA GAGACCGACT
121 ATATTGTGAC ATTACTGTTA GCAGAACTCA GCTGTAATAC AGAACCATTT GAAAGGAATG
181 CTGCAGGAGT TCCACGGGTC AAACACAAAG TCCATGGGC TCAGGCTGAG AGATTAGACA
241 AGGAAGGGCCC TTGTTCCCAAG GAACTGAAAA CCACACACCC GACACCCTCT TTGGGATACA
301 GAAGAAGTCC AAAGATCTCTG AAGAATGCTTT CTGCTCCTTG CACCCGCTCG GCCCTTTGTC
361 CTGCAGGAGT TCCACGGGTC AAACACAAAG TCCATGGGC TCAGGCTGAG AGATTAGACA
421 AGGAAGGGCCC TTGTTCCCAAG GAACTGAAAA CCACACACCC GACACCCTCT TTGGGATACA
481 GAAGAAGTCC AAAGATCTCTG AAGAATGCTTT CTGCTCCTTG CACCCGCTCG GCCCTTTGTC
541 GGCTATCTCT TACAGCTGAG ACTTTCAAGA AGTCTGGATG AGTGGGCTTG
601 GTGGCTGAGC TCTTCTCTCT TATACCTTTTT ATCTAACAGTT TCCCAACACCC GAAGGCTCATG
661 AGAGATATCC TGGAATACAGG TATAGAACCA AGATGCAGAA MARKAGGTTGGA AGATTAGACA
721 AGCAAGCTCCC AAGATCACCC AAGAGCATCA AGTCCCGGTT GCATGAAATG GTGACCTTTCT
781 TTGTTCTCTAA TGTTCCCGATT GTGTCGCTATT TTGGCTGAAA GACCCACAGGGACGATACCC
841 TATCTTTTAAAA GGCTTGTAGCT ATGTAGTTGA TGAACCTGAA ATGGGTTGTTG AGATTAGACA
901 GTGAATTGAGG TCGTCTTCAT AAGAAGGCTG GACCTGAGAC TCCAGAATG GACGTGCTTCT
961 TCGTGTAGAG CCGCTTCTTC TCTCATGACT AAGAAGGCTG GACCTGAGAC TCCAGAATG GACGTGCTTCT
1021 TGGGCACCTT CTCATCTTCTC GCTCCTCGAG TGGGCTGAAA GACCCACAGGGACGATACCC
1081 TGGGCACCTT CTCATCTTCTC GCTCCTCGAG TGGGCTGAAA GACCCACAGGGACGATACCC
1141 TCAGTACCAT ATTTGTTC TGGCAATTTA AACAGGCTCATG GTGGTGGCGAC AGAATACATG
1201 AAAATGCCAG GAGACACCCCA GTCTCATAAT AAACAACGATG GTGGTGGCGAC AGAATACATG
1261 ATAAATTCCC AAGAAGGCTG GACCTGAGAC TCCAGAATG GACGTGCTTCT

| XXX | SF1 binding sites | T/C A A G G T/C C G/A |
| XXX | SOX binding sites | A G A A C A A T G G |
| XXX | FOXL2 core binding site | G/A T/C C/A A A C/T A |

Figure 4.3: Additional consensus FOX sites in the TESCO sequence.

The TESCO sequence was manually searched for the core sequence of forkhead factor binding sites (C/A)AA(C/T)A, which revealed 10 possible FOX sites evenly distributed over the TESCO sequence.
Figure 4.4: Repression effect of FOXL2 on TESCO deletion constructs. Five constructs with deletions of different parts of TESCO were analysed in co-transfection assays. Addition of FOXL2 after activation by SF1 + SOX9 resulted in a repression similar to the wild-type TESCO construct. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

Next, the all ten putative FOX sites in the TESCO element were mutated simultaneously by replacement of the essential central AA motif with a GC motif. The mutated TESCO sequence was then cloned into the luciferase vector and analysed \textit{in vitro} as described before. The construct was activated by SF1 + SOX9 in co-transfection assays and addition of FOXL2 still resulted in repression of the TESCO activity (Figure 4.5). The combined mutation of all FOX sites and all estrogen response elements (EREs) still resulted in repression of TESCO activity by FOXL2, but interestingly the previously observed synergistic repression effect of ERα together with FOXL2 was abolished (Figure 4.6).
Figure 4.5: Repression effect of FOXL2 on TESCO with mutations in all consensus FOX sites.
The ten possible consensus FOX sites found in the TESCO sequence were mutated and the construct used in co-transfection assays. Addition of FOXL2 after activation by SF1 + SOX9 resulted in a repression of the mutant TESCO similar to the wild-type TESCO element. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

Figure 4.6: Repression effect of FOXL2 + ERα on TESCO with all FOX/ERE sites mutated.
All FOX and ERE sites found in the TESCO element were mutated and the construct used in co-transfection assays. Addition of FOXL2 after activation by SF1 + SOX9 resulted in a repression of the mutant TESCO similar to the wild-type TESCO element. However, the synergistical effect of ERα in enhancing FOXL2 mediated repression was lost. Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.
In all the mutation constructs analysed in the above co-transfection assays, addition of FOXL2 resulted in a similar level of repression of TESCO activity. Thus, it could be possible that the repression effect seen by FOXL2 might be due to a non-specific effect of FOXL2 on the luciferase vector backbone. To rule out this possibility, increasing amounts of the original luciferase vector (without the TESCO sequence) were added in co-transfection assays to the wild-type TESCO-luciferase construct which was activated by SF1 + SOX9 and repressed by addition of FOXL2, as described before. If FOXL2 was indeed binding to cryptic binding sites in the vector backbone, excess amounts of Luciferase vector would deplete the available FOXL2, thereby reducing the amount of FOXL2 accessible to repress TESCO activity. However, even with increasing amounts of Luciferase vector, no significant decrease in the FOXL2-mediated repression of TESCO activity was found (Figure 4.7). It might be worth noting that, due to the large excess of the original luciferase vector used in this assays, the cumulative amount of DNA was more than double the amount used in the assays before, which might interfere with the transfection efficiency.

Taken together, all these results suggest that the identified FOX binding sites within the TESCO enhancer are not crucial for FOXL2 to repress TESCO activity. It is possible that the TESCO element contains additional forkhead factor binding sites, through which the repression effect of FOXL2 is mediated and which might differ from the known consensus binding site. Another possible explanation is that the repression effect of FOXL2 is not mediated through binding to the TESCO sequence but through a different mechanism, e.g. by acting as a co-repressor in association with another protein.
4.2.2 Analyses of interactions between FOXL2 and SF1

Experiments in tilapia suggest that FOXL2 and SF1 can physically interact \cite{Wang2007}. A similar interaction could be occurring in the developing mouse gonad, resulting in a repression of SF1-mediated transcriptional activation of TESCO. To test this hypothesis, a synthetic sequence element containing only SF1 binding sites and no FOX sites, was cloned into the luciferase vector (Figure 4.8a). This luciferase reporter construct was indeed activated in co-transfection assays by SF1 and addition of FOXL2 resulted in a dosage dependent repression (Figure 4.8a). This result was similar to the FOXL2-mediated repression of TESCO (Figure 3.4b). As no forkhead binding sites are present in this syn-
thetic element, the repression cannot be due to FOXL2 binding to FOX sites but
must be mediated through a different mechanism, e.g. direct interaction between
FOXL2 and SF1.

To analyse this hypothesis, a GST-FOXL2 fusion protein and a $^{35}\text{S}$-labelled SF1
protein were generated. Co-immunoprecipitation assays with the two proteins
were performed, showing that FOXL2 and SF1 can indeed directly interact (Figure 4.9).
No interaction could be detected in the controls with $^{35}\text{S}$-SF1 on its
own or $^{35}\text{S}$-SF1 together with the GST control.

Next, the effect of FOXL2 on the DNA-binding ability of SF1 was analysed. The
synthetic SF1-binding sequence, which contained SF1 but no FOX binding sites,
was radioactively labelled. This probe was then incubated with SF1 and increas-
ing amounts of FOXL2 (Figure 4.10). As expected, binding of SF1 to the DNA
was clearly visible. Interestingly, a weak interaction could also be detected in
the sample where only FOXL2 (and not SF1) was added to the DNA, indicating
that FOXL2 might be able to bind to DNA containing SF1 binding sites. In-
creasing amounts of FOXL2 resulted in an attenuation of the band representing
SF1 bound to DNA. This suggests that FOXL2 competes with SF1 for binding
to SF1 binding sites. As the DNA contains more than one SF1 binding site, it is
possible that several SF1 and FOXL2 proteins could simultaneously bind to the
sequence. The formation of such multimers could result in an even higher
bandshift and possibly explain the upper bands seen in this assay.
Figure 4.8: **Effect of FOXL2 on SF1-mediated activation.** A synthetic sequence containing only SF1 binding sites was cloned into the Luciferase vector (a). In co-transfection assays, this construct was activated by SF1. Addition of increasing amounts of FOXL2 resulted in a repression in a dosage-dependent manner (b). Assays were performed in triplicates, bars indicate the standard deviation of three biological replicates.

Figure 4.9: **Direct physical interaction between FOXL2 and SF1.** Co-Immunoprecipitation of GST-FOXL2 and $^{35}$S-SF1 showing an interaction between the two proteins.
Figure 4.10: **FOXL2 interferes with SF1 binding to DNA.**
A radioactively labelled probe containing only SF1 binding sites was incubated with SF1 or FOXL2 or both proteins. SF1 was able to bind to its binding sites as indicated by the band shift. When FOXL2 was added on its own a weak band appears indicating the possibility of FOXL2 binding to the SF1 site. Increasing amounts of FOXL2 resulted in a decrease of SF1 bound to DNA and an increase of FOXL2 bound to DNA, suggesting a competition for binding between the two proteins.
4.3 Conclusions

It has been shown so far that FOXL2 represses TESCO activity in vitro. In this chapter, it was assessed how FOXL2 mediates this repression effect. As FOXL2 is a transcription factor which can activate or repress its target genes by binding to its consensus DNA binding sites, the analysis first asked whether FOXL2 represses TESCO activity by binding to possible binding sites in the TESCO sequence. Mutation of the ten putative forkhead factor binding sites in the TESCO sequence did not abolish the repression mediated by FOXL2 in co-transfection assays. However, it is possible that the TESCO element contains additional forkhead factor binding sites which differ from the consensus forkhead binding site. It is known that some groups of forkhead factors indeed bind to sequences which only partially match the consensus site (Carlsson and Mahlapuu, 2002). Thus, FOXL2 could still be repressing TESCO activity via potential non-consensus binding sites in the in vitro assays.

FOXL2 and ERα were found to physically interact with each other in co-immunoprecipitation assays (Uhlenhaut et al., 2009; Kim et al., 2009). While the combined mutation of all FOX sites and EREs found in the TESCO element still resulted in repression of TESCO activity by FOXL2, it abolished the synergistic repression effect of ERα together with FOXL2 in vitro. Similarly, simultaneous mutation of all FOX and ERE sites in the TESCO:CFP reporter construct, resulted in in a de-repression of TESCO activity in adult ovaries in vivo (Uhlenhaut et al., 2009). These results indicate an involvement of both factors in the repression of TESCO and the importance of forkhead factors for estrogen
receptor function. However, the exact mechanism of how FOXL2 and ERα repress TESCO remains to be investigated. Furthermore, it is possible that the mutated TESCO sequence still contains some minor interaction sites for FOXL2, which do not play an important role in the normal physiological environment in vivo. However, due to the artificial system of the co-transfection assays in vitro, the excessive amount of FOXL2 available might still repress the TESCO element via these sites. Thus, the repression of the mutated TESCO element in vitro in contrast to the de-repression seen in vivo might be due to a dosage effect of FOXL2.

Interestingly, deletion of different parts of the TESCO element resulted in the same level of repression by FOXL2 as the wild-type TESCO sequence. These results suggest that FOXL2 could be additionally repressing TESCO activation via a different mechanism. Co-immunoprecipitation analysis revealed that FOXL2 and SF1 are able to physically interact with each other. Thus, it is possible that FOXL2 could mediate its repression effect via SF1, by repressing the SF1-mediated transcriptional activation of TESCO. Recently, a paper was published which identified a new FOXL2-specific DNA binding site GTCAAGG(T/C)CA \citep{Benayoun2008} which shows close similarity to the SF1-binding site (T/C)(T/C)AAGG(T/C)C(G/A). This suggests the possibility that FOXL2 might be able to bind to the SF1 DNA site. Indeed, gel mobility shift assays showed that FOXL2 can bind to a DNA sequence containing only SF1-binding sites and no FOX sites. Moreover, increasing amounts of FOXL2 resulted in a decrease of SF1 bound to its DNA binding site. Likewise, increasing amounts of FOXL2 led to a dosage-dependent repression of an luciferase reporter construct contain-
ing the same DNA sequence in co-transfection assays. These results suggest a mechanism involving competition between FOXL2 and SF1 for binding to the SF1 site. In this hypothesis, binding of SF1 could result in TESCO activation whereas binding of FOXL2 might result in TESCO repression.

In summary, these results suggest a cooperation of FOXL2 and ERα in repressing TESCO activity. It is possible that the two proteins interact as a functional unit which can either bind to FOX sites or EREs to repress the TESCO element. However, the data obtained here also suggest the possibility of a second mechanism of FOXL2-mediated repression involving SF1. There could be several hypotheses: (i) FOXL2 might physically interact with SF1 and thereby reduce the amount of SF1 available to activate TESCO, (ii) FOXL2 might interact with SF1 which is bound to its DNA binding site in the TESCO element, thereby inhibiting its activation capability and (iii) FOXL2 might be competing with SF1 for binding at the TESCO element, resulting in a repression of TESCO rather than is activation.

The results obtained in this thesis so far have provided evidence that FOXL2 can repress TESCO activity in vitro and might therefore be involved in the regulation of Sox9 in the XX gonad in vivo. To investigate this further, the repressing effect of FOXL2 on TESCO activity was analysed next in vivo.
Chapter 5

The effect of Foxl2 on TESCO activity in vivo

5.1 Introduction

The female-specific expression pattern of Foxl2 in the gonad is highly conserved amongst vertebrates, and also in species with very distinct mechanisms of sex determination. For example, in the red-eared slider turtle (*Trachemys scripta*), in which the sex of the offspring is dependant on the egg incubation temperature, the Foxl2 transcript levels are higher at female promoting temperatures than at male promoting temperatures ([Loffler et al., 2003](#)). In the tilapia fish (*Oreochromis niloticus*), Foxl2 is highly expressed in XX individuals at the critical period of sex determination (between 5 to 6 days after hatching) whereas it is barely detectable in XY individuals ([Ijiri et al., 2008](#)). In rainbow trout (*Oncorhynchus mykiss*), XY fish treated with estrogens up-regulate Foxl2 expression and become feminised, whereas XX fish treated with androgens show signs of masculinisation and down-regulate Foxl2 expression ([Baron et al., 2004](#)). In frog
(Xenopus laevis), Foxl2 expression is higher in the female ZW gonad than in the male ZZ gonad (Okada et al., 2009). A similar sexual dimorphic pattern is also observed in the chick, where Foxl2 is specifically expressed in the ZW gonad from the beginning of sex determination (Loffler et al., 2003). In the mouse, Foxl2 is expressed in the somatic cells of the XX gonad from 12.5 dpc onwards, whereas it is not detected in XY gonads (Uda et al., 2004). The conserved female-specific expression pattern of Foxl2 in the developing gonad suggests the potential of this gene to act as a conserved factor in ovarian development. However, the molecular mechanisms explaining how the transcription factor FOXL2 regulates ovarian development and differentiation are not yet fully understood.

Mice heterozygous for a null mutation of Foxl2 display no phenotype, whereas homozygotes mutant mice (Foxl2−/−) are born with open necrotising eyes, eyelid hypoplasia, craniofacial defects and smaller body size, which are features comparable to human BPES (Uda et al., 2004). In addition, XX Foxl2−/− mice show hypoplastic uterine tubes and are infertile, whilst XY Foxl2−/− mice remain fully fertile and testis development is unaffected. The same phenotype was found in mice carrying a LacZ gene insertion into the Foxl2 locus (Foxl2LacZ) resulting in a null allele (Schmidt et al., 2004). Directly after birth the numbers of primordial follicles and oocytes are similar between wild-type and Foxl2−/− mutant ovaries, but 2 weeks postnatally, when secondary follicles are normally surrounded by two cuboidal granulosa cell layers in the wild-type ovary, the mutant follicles contain only one layer of granulosa cells. Moreover, these cells have not yet completed the transition from squamous to cuboidal type. At 8 weeks, mutant ovaries are significantly smaller than wild-type and mutant follicles contain significantly fewer

110
granulosa cells due to decreased proliferation. In addition, no quiescent primordial follicles are found indicating a depletion of the primordial follicle pool (Uda et al., 2003). The expression of Sox9 and some other markers of testis differentiation such as Fgf9, Fgfr2, Dhh, Wt1, Sfi, Gata4 is up-regulated in the mutant ovary 1 week after birth (Ottolenghi et al., 2005).

Recently, our collaborators Henriette Uhlenhaut and Mathias Treier, have generated a mouse line carrying a conditional Foxl2 allele (Foxl2^{flox/flox}). To determine Foxl2 function in the adult ovary, an ubiquitously expressed inducible Cre recombinase allele (Rosa26:CreERT2) was bred into this line to allow the deletion of Foxl2 upon administration of tamoxifen (thus giving Foxl2^{Δ/Δ}). Analysis 3 weeks after the tamoxifen administration to 8 week old mice, revealed XX gonadal sex reversal (Uhlenhaut et al., 2009). The gonads of these mice contained structures resembling seminiferous tubules with Sertoli-like cells as indicated by tripartite nucleoli and veil-like cytoplasmic extensions pointing towards the lumen. The tubule-like structures were also surrounded by a prominent basal lamina. One week after tamoxifen administration, the morphology of the follicles and the oocytes in Foxl2^{Δ/Δ} gonads were still intact, but the granulosa cells surrounding the oocytes were expressing Sox9. These cells started to transdifferentiate into Sertoli-like cells despite the presence of an oocyte, suggesting that granulosa cells undergo cell autonomous reprogramming into Sertoli-like cells upon loss of Foxl2. A number of other Sertoli cell markers were also up-regulated in these cells, including Gata1, Tif2, Dmrt1 and Dhh. Leydig cell markers, such as Hsd17b3 (the rate-limiting enzyme in testosterone production in the testis) were also up-regulated in the XX Foxl2^{Δ/Δ} gonads. Moreover, Hsd17b3 was found
to be expressed in a similar pattern to adult testis, suggesting the presence of Leydig-like cells. Furthermore, similar levels of testosterone were detected in the blood of XX Foxl2^{∆/∆} mice compared with XY wild-type littermates. Looking at events immediately after tamoxifen administration revealed that FOXL2 protein was still present 2 days after whereas SOX9 was not yet expressed. However, SOX9 was detected already at high levels 4 days after tamoxifen administration, by which time FOXL2 expression had disappeared. No evidence could be found of any overlap, suggesting that expression of FOXL2 and SOX9 are mutually exclusive.

All the evidence suggested that FOXL2 is required in granulosa cells of the postnatal ovary to antagonise Sox9 expression and actively prevent Sertoli cell differentiation and subsequent masculinisation of the gonad. The data presented in the previous chapters of this thesis showed that FOXL2 can repress Sox9 expression \textit{in vitro} via the TESCO enhancer element (see Chapter 3).

To test whether the same mechanism occurs \textit{in vivo} Foxl2^{LacZ} mice, which were generated by the replacement of most of the Foxl2 coding region by β-galactosidase (Schmidt et al., 2004), were crossed to a transgenic mouse line expressing a CFP reporter under the control of the TESCO enhancer element (TESCO:CFP) (Sekido and Lovell-Badge, 2008). The expression of CFP in the TESCO:CFP line correlates with the expression of endogenous SOX9: in XY gonads CFP is expressed in Sertoli cells from 11.5 dpc until adult stages, whereas in XX gonads CFP is not expressed at all in granulosa cells (Figure 5.1). If FOXL2 represses Sox9 expression via the TESCO element \textit{in vivo}, loss of Foxl2 in the XX go-
nad should lead to a de-repression of TESCO and therefore activation of CFP expression in the XX TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ} ovary.

Figure 5.1: **CFP expression in the TESCO:CFP reporter line in vivo.** Immunohistochemistry showing CFP expression in XY, but not XX gonads, of the TESCO:CFP reporter line at 13.5 dpc, equates to the expression pattern of Sox9. Scale bar = 100\,\mu m; insert shows 2.5x higher magnification.
5.2 Results

5.2.1 De-repression of TESCO in Foxl2 mutant mice

In the original paper describing the Foxl2\textsuperscript{LacZ/LacZ} phenotype, it was reported that homozygotes are born at the expected Mendelian ratio, although about 95% of the mutant animals die before weaning (Schmidt et al., 2004). However, after being bred onto a C57BL/6 background for several generations, it was not possible to find any homozygous mutant mice amongst the litters born or even amongst the embryos harvested at 12.5 dpc (a total of 207 mice and 114 embryos were generated from heterozygous Foxl2 matings on the C57BL/6 background). As the original mutation was maintained on a mixed background (129/BlackSwiss/CD1), this result suggests a variable penetrance of the Foxl2 mutation in different mouse strains and a high sensitivity of the C57BL/6 background for the Foxl2 mutation.

To circumvent this problem, the Foxl2 mutant mice were first bred onto an MF1 hybrid background, which resulted in the appearance of live born mutant mice after four generations. These Foxl2[MF1] homozygous mutant mice were smaller than wild-type and heterozygous littermates and they were born with open eyes and craniofacial defects as described in Schmidt et al. (2004). The Foxl2[MF1] mice were then bred with TESCO:CFP reporter mice to generate the desired TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ} genotype.

To determine the possible de-repression of TESCO activity, XX TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ} gonads were collected and compared to control gonads from XY TESCO:CFP; Foxl2\textsuperscript{LacZ/wt} and XX TESCO:CFP; Foxl2\textsuperscript{LacZ/wt} mice at different time points during embryogenesis (12.5 dpc to 18.5 dpc) and after birth (P0 to
8 weeks). The gonads were then sectioned and analysed for CFP expression. Ovaries of XX heterozygous and homozygous Foxl2 mutant mice are indistinguishable directly after birth (P0). However, from 7 dpp onwards morphological differences could be found between the two genotypes and the ovarian dysgenic phenotype of XX homozygous mutants became more prominent with age. From 4 weeks onwards, it was clearly evident that oocytes (asterisks) in $\text{Foxl2}^{\text{LacZ}/\text{LacZ}}$ ovaries are surrounded by fewer layers of granulosa cells (arrows), compared to several layers of granulosa cells in the $\text{Foxl2}^{\text{LacZ}/\text{wt}}$ ovaries (Figure 5.2), as described in the original paper (Schmidt et al., 2004).

As expected, during embryonic development, CFP was expressed in the Sertoli cells within the testis cords in XY TESCO:CFP; Foxl2$^{\text{LacZ}/\text{wt}}$ gonads, whereas it was absent in XX TESCO:CFP; Foxl2$^{\text{LacZ}/\text{wt}}$ gonads between 12.5 dpc and 18.5 dpc. No CFP expression could be detected in the gonads of XX TESCO:CFP; Foxl2$^{\text{LacZ}/\text{LacZ}}$ mice at any stage during embryogenesis. However, just after birth (P0) a very few CFP-positive cells were found (Figure 5.3).
Figure 5.2: **Morphology of XX Foxl2<sup>LacZ/LacZ</sup> gonads.**

H+E staining of XX Foxl2<sup>LacZ/wt</sup> and Foxl2<sup>LacZ/LacZ</sup> gonads at different time points after birth and during adulthood. No morphological difference could be seen at P0. From 4 weeks of age it was evident that fewer layers of granulosa cells (arrows) surround the oocytes (asterisks) in Foxl2<sup>LacZ/LacZ</sup> ovaries. Scale bar = 50μm.
Figure 5.3: TESCO activity in TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ} gonads during embryogenesis.

Cryosections of XY and XX gonads of TESCO:CFP mice, with heterozygous and homozygous loss of Foxl2, at different time points during embryogenesis and just after birth (P0). Immunostaining shows DAPI in blue and CFP in green. TESCO:CFP expression could be detected in Sertoli cells in the XY gonads (left panel), whereas it was absent in XX TESCO:CFP; Foxl2\textsuperscript{LacZ/wt} gonads (middle panel). No de-repression of TESCO activity was found in XX TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ} gonads during embryogenesis, but a few CFP-positive cells could be detected just after birth (right panel). Scale bar = 50\textmu m; insert shows 2.5x higher magnification of CFP-positive cells in ovary with homozygous loss of Foxl2.
At postnatal stages, CFP expression was detected in the Sertoli cells of all XY TESCO:CFP; Foxl2LacZ/wt gonads from 7 dpp until 8 weeks of age. During this time frame the normal reorganisation of the testis cords could be observed, including the migration of Sertoli cells towards the centre of the cords just after birth, the migration of the Sertoli cell nuclei back to the basal area of the seminiferous tubules after puberty and the finger-like cytoplasmic extension of adult Sertoli cells from the basal to the adluminal compartment of the seminiferous tubules (Figure 5.4). In XX TESCO:CFP; Foxl2LacZ/wt gonads, no CFP expression was seen before 4 weeks of age, except for a non-specific staining in oocytes. However, at 4 and 8 weeks of age, CFP expression was detected in some theca cells surrounding the follicles. It has been reported that Sox9 is expressed in the inner layer of theca cells in the adult ovary but does not coincide with the expression of the steroidogenic markers P450 and 3/βHSD, suggesting that the SOX9-positive cells are a subpopulation of non-steroidogenic theca cells (Notar nicola et al., 2006). In the XX TESCO:CFP; Foxl2LacZ/LacZ mice, CFP expression was detected in all gonads from 7 dpp to 8 weeks of age (Figure 5.4). The CFP-positive cells in the homozygous Foxl2 mutant gonads were mostly found in the remaining layer surrounding the oocytes.
Figure 5.4: TESCO activity in postnatal TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup> gonads.

Cryosections of XY and XX gonads of TESCO:CFP mice, with heterozygous and homozygous loss of Foxl2, at different time points postnatally. Immunostaining shows DAPI in blue and CFP in green. CFP expression was detected in Sertoli cells in XY gonads (left panel) and in a potential subpopulation of theca cells in XX TESCO:CFP; Foxl2<sup>LacZ/wt</sup> gonads from 4 weeks onwards (white arrows, middle panel). De-repression of TESCO could be seen in XX TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup> gonads in cells surrounding the oocytes at all stages analysed (right panel). Scale bar = 50 μm; inserts show 2.5x higher magnifications.
5.2.2 De-repression of SOX9 in Foxl2 mutant mice

To determine whether de-repression of TESCO activity coincided with de-repression of endogenous Sox9, the expression of Sox9 was analysed in Foxl2 mutant gonads during embryonic development and after birth.

While Sox9 was expressed normally in the Sertoli cells of embryonic XY gonads, no Sox9 expression could be detected in either XX TESCO:CFP; Foxl2LacZ/wt or XX TESCO:CFP; Foxl2LacZ/LacZ gonads between 12.5 dpc and 18.5 dpc (data not shown). After birth, Sox9 expression was found in the Sertoli cells of XY gonads from P0 to 8 weeks of age and in a few cells in the ovaries of 4 and 8 week old XX TESCO:CFP; Foxl2LacZ/wt mice, which might represent the sub-population of theca cells as noted above (Notarnicola et al., 2006). However, endogenous SOX9 was detected in the ovaries of XX TESCO:CFP; Foxl2LacZ/LacZ mice from 7 dpp to 8 weeks of age, in most of the residual cells surrounding the oocytes (Figure 5.5). Co-staining for nuclear SOX9 and cytoplasmic CFP revealed the expression of both proteins in the same cells. Interestingly, whereas CFP expression was found in ovaries of XX TESCO:CFP; Foxl2LacZ/LacZ mice from P0 (Figure 5.3), SOX9 was not detected until later (Figure 5.3).
Figure 5.5: Endogenous Sox9 expression in postnatal TESCO:CFP; Foxl2<sup>lacZ/wt</sup>.
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of Foxl2 at different time points postnatally. Immunostaining shows DAPI in blue, CFP in green and SOX9 in red. Inserts show higher magnifications. CFP and SOX9 co-localised in Sertoli cells in the XY gonads (left panel), in a possible subpopulation of theca cells in the XX TESCO:CFP; Foxl2<sup>lacZ/wt</sup> gonads (middle panel) and in cells surrounding the oocytes in XX TESCO:CFP; Foxl2<sup>lacZ/lacZ</sup> gonads (right panel). Scale bar = 50µm; inserts show 2.5x higher magnifications.
As mentioned above, expression from both TESCO and Sox9 could be seen in the cells which surround the oocytes in a typical granulosa cell manner. To determine whether indeed the remaining granulosa-like cells are the cells up-regulating Sox9, the Foxl2 mutant gonads were analysed for the expression of LacZ, a reporter gene inserted into the Foxl2 locus and therefore expressed under the control of the endogenous Foxl2 promoter. In double immunostainings, the gonadal expression of SOX9 and β-galactosidase was analysed in 4 week old mice (Figure 5.6). XY TESCO:CFP; Foxl2LacZ/WT gonads showed Sox9 expression in the Sertoli cells, but no expression of β-galactosidase. On the other hand, in XX TESCO:CFP; Foxl2LacZ/WT gonads, β-galactosidase could be found predominantly in the granulosa cells, but also in some interstitial cells (large white arrow). Although it has been shown that Foxl2 is also expressed in a subpopulation of theca cells (Uhlenhaut et al., 2009), in this analysis no β-galactosidase activity could be detected in any theca cells. As described before, Sox9 expression could be detected in some cells of the XX TESCO:CFP; Foxl2LacZ/WT gonads that might represent a subpopulation of theca cells (small white arrow). In the XX TESCO:CFP; Foxl2LacZ/LacZ gonads, the cells surrounding the oocytes expressed both Sox9 and β-galactosidase. However, some cells in the XX homozygous mutant gonad were positive for β-galactosidase but not for SOX9 and might correspond to the interstitial cells which were also found in the XX Foxl2 heterozygous mutant gonads.
5.2.3 The impact of oocyte depletion on the supporting cell lineage

It has been suggested that oocytes are required for the maintenance of granulosa cells and that the ovarian pathway is actually under the control of the germ cells (Burgoyne, 1988; McLaren, 1991). If these cells are lost at the time of folliculogenesis, testis-like structures form in cultured fetal rat ovaries (Prépin and Hida, 1989). The same effect can be seen naturally in freemartin cattle, where female embryos have a vascular connection to their male twins. Here, the oocytes gradually degenerate and testicular cords form in the ovary, including Sertoli cells which produce AMH and Leydig cells which produce testosterone (McLaren, 1991). Other studies have shown that oocytes can interfere with the organisation of testis cords and thus might be involved in antagonising testis...
differentiation (Yao et al., 2003). Based on this evidence it has been proposed that the loss of oocytes could by itself cause the transdifferentiation of granulosa to Sertoli-like cells.

To investigate this possibility, oocytes were specifically depleted in neonatal mice and Sox9 expression was analysed to determine whether the loss of oocytes could result in a de-repression of Sox9. For this, mice carrying a conditionally expressed gene for diphtheria toxin fragment A (DTA) in the ROSA26 locus were used (R26DTA). These express the active DTA only upon Cre-mediated recombination (Ivanova et al., 2005). Diphtheria toxin is secreted by the parasite Corynebacterium diphtheriae and consists of subunit A and B. Subunit B mediates the internalisation of the toxin by binding to its receptor, whereas subunit A terminates protein synthesis and eventually leads to apoptosis of the cell (Maxwell et al., 1986). The R26DTA mice were bred to mice carrying Cre-recombinase under the control of the germ-line specific promoter of the zona pellucida 3 gene (Zp3:Cre), which is specifically expressed in oocytes (De Vries et al., 2000). Zp3:Cre is active in ovaries from 5 dpp onwards, in primary, secondary, and later follicular stages, but not in adjacent somatic cells (Lan et al., 2013). Therefore, upon Zp3:Cre mediated recombination, the DTA will be active exclusively in oocytes from the primary follicular stage onwards, resulting in a global loss of oocytes.

To investigate the possible up-regulation of Sox9 expression upon oocyte loss, gonads of XY Zp3:Cre; R26DTA, XX R26DTA and XX Zp3:Cre; R26DTA mice were collected at different time points after birth (P0 to 8 weeks). The gonads were then sectioned and analysed for Sox9 expression. As expected, Sox9 expres-
sion could be detected in Sertoli cells of XY Zp3:Cre; R26DTA gonads at all ages between P0 and 8 weeks of age and in the putative subpopulation of theca cells of four and eight week old XX Zp3:Cre-negative R26DTA gonads (white arrows). As Zp3:Cre is not active at P0, ovaries of XX Zp3:Cre; R26DTA mice just after birth were similar to ovaries of XX R26DTA mice, indicating that indeed the DTA had no effect in those ovaries yet. The first morphological differences could be found at 7 dpp and became more evident with age, resulting eventually in a complete oocyte-depleted ovary at 4 and at 8 weeks of age. Adult XX Zp3:Cre; R26DTA ovaries were drastically smaller compared to those of XX Zp3:Cre-negative littermates. Despite the severely altered morphology, up-regulation of Sox9 expression was not detected at any stage between P0 and 8 weeks of age in these XX Zp3:Cre; R26DTA ovaries (Figure 5.7).
Figure 5.7: Sox9 expression in gonads of Zp3:Cre; R26DTA mice. Cryosections of XY and XX gonads of R26DTA mice with or without Zp3:Cre at different time points postnatally. Immunostaining shows DAPI in blue and SOX9 in green. SOX9 could be detected in Sertoli cells of XY Zp3:Cre; R26DTA gonads (left panel) and in a few cells in XX R26DTA gonads (white arrows, middle panel). SOX9 was not detected in XX Zp3:Cre; R26DTA gonads (right panel). Scale bar = 50 μm.
5.3 Conclusions

In this chapter the effect of Foxl2 on TESCO activity was analysed in vivo using Foxl2<sup>LacZ</sup> mice crossed to TESCO:CFP reporter mice. Homozygous loss of Foxl2 did not have any effect on either TESCO activity or endogenous Sox9 expression during embryogenesis. However, after birth homozygous loss of Foxl2 in XX gonads resulted in the de-repression of TESCO activity and thus activation of CFP expression from P0 onwards. Just after birth, very few CFP-positive cells could be detected in the gonads of these XX TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup> mice and their number increased with age. From 4 weeks onwards, it was clear that the cells which show de-repression of TESCO were mainly the cells surrounding the oocytes. In addition, endogenous Sox9 expression was detected in ovaries of Foxl2 homozygous, but not heterozygous, mutant mice. Sox9 expression coincided with CFP expression in the cells surrounding the oocytes. These cells also expressed β-galactosidase, which is largely specific to granulosa cells as it is expressed under the endogenous Foxl2 promoter. All this evidence indicates that indeed the remaining supporting cells which are surrounding the oocytes in the XX Foxl2<sup>LacZ/LacZ</sup> gonads up-regulate Sox9 expression upon loss of Foxl2 and thus switch their cell fate from the female supporting lineage to male Sertoli-like cells.

Interestingly, TESCO and endogenous Sox9 became de-repressed at different time points in the XX Foxl2<sup>LacZ/LacZ</sup> gonads. While a few CFP-positive cells could already be detected at P0, Sox9 was still repressed. There are several possible explanations for these results: (i) endogenous Sox9 expression might be repressed
via different mechanisms compared to the TESCO element in XX gonads at the time of birth, (ii) the fact that only very few CFP-positive cell could be found at this time point suggests that other genes might be involved in the repression of TESCO activity (and therefore Sox9 expression) around birth, (iii) TESCO:CFP is a transgene and might be inserted at a more accessible position in the genome than the endogenous Sox9 gene. Thus, it is possible that the differential de-repression profile of TESCO:CFP transgene and endogenous Sox9 might be due to epigenetic differences.

Both TESCO and Sox9 became clearly de-repressed with increasing age in the XX Foxl2LacZ=LacZ gonads, indicating that Foxl2 might be the important gene in repressing Sox9 in the adult. These results match with the findings of our collaborators in adult XX Foxl2^{Δ/Δ} mice, where endogenous Sox9 expression could be detected after the complete loss of FOXL2. Moreover, when bred on the TESCO:CFP background, CFP expression was also up-regulated in a typical granulosa cell location of the adult ovary after Foxl2 deletion (Uhlenhaut et al., 2009).

For a long time it had been proposed that oocytes are essential to maintain granulosa cell fate. In different models the degeneration of oocytes was associated with the appearance of testis cord like structure in XX gonads and the transdifferentiation of granulosa cells into Sertoli-like cells (McLaren, 1991). When 13.5 dpc XX gonads were grafted to the kidney capsule of adult mice, they developed cord like structures and started to express Sox9 (Morais da Silva et al., 1996). However, the signals responsible for this testis development of the grafted XX
gonads are not known. To determine whether the loss of oocytes could result in a de-repression of Sox9, oocytes were specifically depleted by activating the DTA upon Zp3:Cre mediated recombination. In the Zp3:Cre; R26DTA mice, the active DTA caused apoptosis in oocytes from the primary follicular stage, resulting in a much smaller, completely oocyte-depleted ovaries from 7 dpp onwards. Although follicular growth was significantly impaired, no Sox9 expression was detected in these ovaries, indicating that the supporting cells in these oocyte-depleted ovaries did not switch to Sertoli-like cells. These results are consistent with the data of our collaborators, who generated Gdf9:Cre; R26DTA mice to analyse oocyte depletion in the ovary. Gdf9 is expressed in oocytes from the primordial follicle stage onwards at 3 dpp, whereas ZP3 expression starts at 5 dpp (Lan et al., 2004). Analyses of these Gdf9:Cre; R26DTA mice showed similar results: no Sox9 expression could be detected in the ovaries upon oocyte loss (Uhlenhaut et al., 2009). These results differ from the ones obtained in the analyses of the Foxl2LacZ/LacZ mice which showed a postnatal de-repression of Sox9. Moreover, when oocytes were depleted in adult female mice using an inducible form of the human Diphtheria toxin receptor (hDTR), which only induces oocyte-specific cell ablation after administration of diphtheria toxin (Gdf9:Cre; R26iDTR), Sox9 expression was also not detected (Uhlenhaut et al., 2009). Again these results differ from the ones obtained with the conditional adult XX Foxl2Δ/Δ mice, which showed an up-regulation of Sox9 expression at a comparable time after FOXL2 depletion.

Taken together, Sox9 de-repression could not be detected in XX gonads upon oocyte depletion - it occurs only upon loss of Foxl2. Moreover, oocytes could
still be found in the ovaries of both XX $\text{Foxl2}^{LacZ/LacZ}$ and $\text{Foxl2}^{\Delta/\Delta}$ mice. These results indicate that the de-repression of Sox9 in XX gonads is a consequence of loss of Foxl2 expression, which results in a transdifferentiation of granulosa cells into Sertoli-like cells and that oocytes are not directly required to maintain the granulosa cell fate at least during postnatal ovarian development. The loss of oocytes in Foxl2 mutants might be a consequence of the granulosa cell transdifferentiation to Sertoli-like cells rather than a cause. It is not surprising that oocytes fail to survive in a testicular environment.

In summary, Foxl2 seems to be the only critical factor ensuring the maintenance of the granulosa cell lineage in the adult ovary. It does so by repressing Sox9 expression via the TESCO element. However, these data also indicate that FOXL2 is not the primary factor to repress Sox9 expression during embryonic development. Thus, other factors must be involved to ensure both the establishment and maintenance of the female supporting precursor cell during embryogenesis.
Chapter 6

The effect of Wnt4 on TESCO activity in vivo

6.1 Introduction

Loss of Foxl2 expression results in the de-repression of TESCO activity and endogenous Sox9 expression after birth, indicating that FOXL2 is a critical factor for maintaining granulosa cell fate postnatally (Chapter 5 and Uhlenhaut et al. (2009)). However, Foxl2 does not seem to be the important factor to repress both TESCO and Sox9 during embryogenesis. This raises the question which factor(s) are responsible for the initial down-regulation of Sox9 expression in the XX gonad at 11.5 dpc and maintenance of this repression throughout embryonic development.

Wnt4 is a candidate to fulfil this role. In the mouse, Wnt4 is expressed in the indifferent gonad from 10.5 dpc onwards in both sexes and becomes female-specific due to its down-regulation in XY gonads at 11.5 dpc (Vainio et al., 1999). XX
mice carrying a targeted null mutation of Wnt4 (Wnt4−/−) show partial masculinisation of the gonad (which takes on a rounder shape, is unencapsulated and develops closely associated with a fat body) and the developing gonadal duct has a region resembling the epididymal region of the male Wolffian duct. Leydig-like cells, expressing the two male steroidogenic genes 3βHSD and P450c17 are present in the mutant XX gonad and produce testosterone (Vainio et al., 1999). Microarray studies revealed that several genes involved in testosterone synthesis are up-regulated in XX Wnt4−/− gonads between 12.5 dpc and 14.5 dpc (Heikkila et al., 2005). In these mice, steroidogenic adrenal precursors, derived from the mesonephric region where the adrenal is forming, migrate into the gonad and cluster in the area closest to the developing adrenal. This lead to the hypothesis that during early gonadal development in XX mice, WNT4 represses the migration of steroidogenic cells from the mesonephros into the gonad (Jeays-Ward et al., 2003). Moreover, mesonephric cells, which in XY gonads contribute to the formation of the male-specific blood vessel, migrate into XX Wnt4−/− gonads and an ectopic coelomic vessel develops. This suggests that Wnt4 is necessary to prevent both migration of endothelial cells into the XX gonad as well as formation of the vessel (Jeays-Ward et al., 2003). However, neither testis cord formation nor masculinisation of the external genitalia could be observed in XX Wnt4−/− mice. Moreover, while Sox9 expression could be detected transiently in these mutant gonads at 11.5 dpc, it was already down-regulated by 12.5 dpc (Kim et al., 2006b). Lack of Wnt4 expression also results in loss of germ cells by the time of birth. While the early migration and proliferation of the germ cells seem to be normal in XX Wnt4−/− mice, almost 90% of the germ cells undergo apopto-
sis after 16.5 dpc (Yao et al., 2004), indicating a role for Wnt4 in maintaining oocyte health. Interestingly, XY Wnt4−/− mice display defects in early testis development, including reduced expression of Sox9, Dhh and Amh at 11.5 dpc (Jeays-Ward et al., 2004). As Sry expression is unaffected, this data suggests that Wnt4 is involved in Sertoli cell differentiation downstream of Sry but either up-stream of or in parallel to Sox9. WNT4 has a mutually antagonistic relationship with FGF9 (Kim et al., 2006b) and it is possible that precocious activity of the latter leads to precocious development of testis cords with too few Sertoli-cells dividing more slowly than pre-Sertoli cells. XY Wnt4−/− gonads also exhibit an increase in the number of steroidogenic cells indicating that Wnt4 might play a regulatory role in steroidogenesis in both XX and XY gonads (Jeays-Ward et al., 2004).

XX mice carrying double homozygous mutations in Wnt4 and Foxl2 (Foxl2−/−, Wnt4−/−) develop some seminiferous tubule-like structures at P0, which contain oocytes as well as differentiated type A spermatogonia (Ottolenghi et al., 2007). Several male-specific marker genes such as Sox9, Dmrt1, Amh, Ptgds, Hsd17b3 and Cyp26b1 are up-regulated in XX Foxl2−/−, Wnt4−/− gonads just after birth (Ottolenghi et al., 2007).

In contrast to the data from WNT4 duplications in human patients, misexpression of either the human WNT4 gene with its endogenous regulatory sequences or an Sf1:Wnt4 construct in XY transgenic mice does not cause male-to-female sex reversal. In the XY Wnt4 transgenic gonads, the coelomic blood vessel develops, although it is disorganised with multiple branches and fails to form one
main vessel. This suggests that WNT4 is not sufficient to prevent the formation of the coelomic blood vessel in XY gonads, although the level and timing of Wnt4 expression may not have been sufficient (Jeays-Ward et al., 2003; Jordan et al., 2003). These data not only imply that additional Wnt4-independent pathways must exist in XX gonads to prevent the initiation of vessel formation but also that Wnt4 needs to be adequately down-regulated in XY gonads in order to form normal vessels. While misexpression Sf1::Wnt4 in XY gonads does not affect Leydig cell differentiation (Jeays-Ward et al., 2003), the presence of human WNT4 results in reduced levels of testosterone due to a decreased expression of StAR, which is involved in the rate-limiting step of steroidogenesis (Jordan et al., 2003).

In XY gonads, Fgf9 has been shown to be involved in the maintenance of Sox9 expression via the establishment of a feed-back loop between SOX9 and FGF9 (Kim et al., 2006b). In XY Fgf9−/− gonads, Wnt4 expression is not down-regulated at 11.5 dpc as it normally occurs. However, both Sry and Sox9 are initially expressed. Furthermore, addition of FGF9 to XX gonad cultures ex vivo resulted in the down-regulation of Wnt4 expression (Kim et al., 2006b), while Fgf9, which is normally expressed only in XY gonads after 11.5 dpc, was detected in XX Wnt4−/− gonads at 12.5 dpc (Kim et al., 2006b). These results suggest an antagonistic relationship between FGF9 and WNT4 signalling in the developing gonad: the establishment of active FGF9 signalling in the XY gonad represses Wnt4 expression and thereby ensures normal testis development, whereas the establishment of WNT4 signalling represses Fgf9 activity in XX gonads to secure normal ovarian development. In XX gonads with ectopic Sry expression induced after the critical time window of sex determination, Sox9 expression could not
be maintained. This was attributed to the fact that delayed \textit{Sry} expression can not result in sufficiently high levels of \textit{Sox9} expression to enable the XX gonad to activate FGF9 signalling. As the XX gonads did not switch from the female WNT4 signalling to the male FGF9 signalling program, \textit{Sox9} expression could not be maintained and therefore no female-to-male sex reversal occurred (Hiramatsu \textit{et al.}, 2009). Taken together, the establishment of WNT4 signalling seems to be crucial in tipping the balance of sex determination towards female development.

So far, a number of genes have been described as possible downstream targets for WNT4 signalling. For example \textit{Dax1} expression is significantly reduced in \textit{Wnt4}−/− mice (Mizusaki \textit{et al.}, 2003). Moreover, addition of WNT4 to cultured mouse Leydig cells or Sertoli cells resulted in an up-regulation of endogenous \textit{Dax1} expression (Jordan \textit{et al.}, 2001). Furthermore, both \textit{Fst} and \textit{Bmp2} have been suggested as downstream effectors of WNT4 signalling. \textit{Fst} encodes an activin binding protein, which can antagonise members of the TGF\textbeta superfamily. It was originally identified as a potent inhibitor of pituitary FSH (Ueno \textit{et al.}, 1987) and is involved in regulation of the hypothalamic-pituitary-gonadal axis (Phillips and de Kretser, 1998). \textit{Fst} is expressed exclusively in XX gonads from 11.5 dpc and decreases after 14.5 dpc (Yao \textit{et al.}, 2004). In XX \textit{Wnt4}−/− mice, no expression of \textit{Fst} can be detected, whereas \textit{Wnt4} is expressed normally in XX \textit{Fst}−/− mice. This suggests that \textit{Fst} is a downstream effector of Wnt4 signalling (Yao \textit{et al.}, 2004). Moreover, XX \textit{Fst}−/− mice form testis-specific coelomic blood vessels and suffer germ cell depletion similar to the phenotypes observed in \textit{Wnt4}−/− mice (Yao \textit{et al.}, 2004). Testis differentiation was found to be normal in XY \textit{Fst}−/− mice. BMP2 belongs to the family of bone morphogenic proteins.
(Bmp), which are multifunctional regulators of cell growth and differentiation and were originally identified in cartilage formation ([Wozney et al., 1988]. Bmp2 is expressed in the gonads of both sexes at 10.5 dpc and becomes female-specific around 11.5 dpc, before its expression decreases after 14.5 dpc ([Yao et al., 2004]). The expression is restricted to cells just under the coelomic epithelium. The gonadal phenotype of Bmp2−/− mice could not be analysed as the mice die before 10.5 dpc and thus prior to the time of sex determination ([Zhang and Bradley, 1996]). However, Bmp2 expression is absent in Wnt4−/− mice suggesting that it is a downstream effector of Wnt4 signalling ([Yao et al., 2004]).

In summary, WNT4 is an important anti-testis factor, implicated in opposing the male pathway by repressing SOX9 and FGF9. This raises the question whether Wnt4 could be involved in the regulation of Sox9 expression in vivo via the TESCO enhancer element. To investigate this possibility, the effect of Wnt4 deletion on TESCO activity was analysed in XX gonads by crossing Wnt4 mutant mice to the TESCO:CFP reporter line. If WNT4 represses Sox9 expression via the TESCO enhancer element in vivo, loss of Wnt4 in the XX gonad should result in a de-repression of TESCO activity and therefore activation of CFP expression in XX TESCO:CFP; Wnt4−/− ovaries.
6.2 Results

6.2.1 The de-repression of TESCO in *Wnt4* mutant mice

To test whether TESCO became de-repressed in the developing gonad upon loss of *Wnt4* expression, the TESCO:CFP reporter mice were bred to *Wnt4* mutant mice. The targeted deletion of *Wnt4* was established by replacing the third and fourth exon of *Wnt4* with a selection cassette containing neomycin phosphotransferase resulting in a functional null allele ([Stark et al., 1994](#)).

*Wnt4*−/− mice die within 24 hours after birth due to kidney failure and therefore no analyses were possible after P0 ([Vainio et al., 1999](#)). XX TESCO:CFP; *Wnt4*−/− gonads, as well as XY TESCO:CFP; *Wnt4*+/− and XX TESCO:CFP; *Wnt4*+/− control gonads, were collected at different time points during embryonic development (12.5 dpc to 18.5 dpc) and at birth (P0). Gonads were then sectioned and analysed for CFP expression. CFP expression was detected in XY TESCO:CFP; *Wnt4*+/− gonads in the Sertoli cells. In XX TESCO:CFP; *Wnt4*+/− gonads, occasionally a few CFP-positive cells could be detected from 14.5 dpc onwards. In XX TESCO:CFP; *Wnt4*−/− gonads, few CFP expressing cells were detected at 14.5 dpc, while more CFP-positive cells could be found between 16.5 dpc and P0 (Figure 6.1).
Figure 6.1: TESCO activity in $\text{TESCO}:\text{CFP}; Wnt4^{-/-}$ gonads during embryogenesis.
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of $Wnt4$ at different time points during embryogenesis and just after birth (P0). Immunostaining shows DAPI in blue and CFP in green. TESCO expression could be detected in the Sertoli cells in XY gonads (left panel). Very few CFP-positive cells could be found in XX $\text{TESCO}:\text{CFP}; Wnt4^{+/-}$ gonads (middle panel). De-repression of TESCO activity was found in XX $\text{TESCO}:\text{CFP}; Wnt4^{-/-}$ from around 14.5 dpc to P0 (right panel). Scale bar = 50$\mu$m.
To determine whether the TESCO element was de-repressed in the granulosa cells of XX TESCO:CFP; Wnt4\(^{-/-}\) gonads, co-immunostainings of CFP and FOXL2 were analysed in 18.5 dpc gonads. As expected, XY TESCO:CFP; Wnt4\(^{+/−}\) gonads expressed CFP, as shown before, but no FOXL2. On the other hand, XX TESCO:CFP; Wnt4\(^{+/−}\) gonads showed expression of FOXL2 and occasionally very few CFP-positive cells. Expression of both proteins was detected in XX TESCO:CFP; Wnt4\(^{-/-}\) gonads. Although both proteins were expressed, strong signals for FOXL2 and CFP did not co-localise in the mutant XX gonads (Figure 6.2). Only occasionally a very weak cytoplasmic CFP signal could be found with a strong nuclear FOXL2 signal and vice versa.

**Figure 6.2:** FOXL2 and CFP do not co-localise in TESCO:CFP; Wnt4\(^{-/-}\) gonads.
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of Wnt4 at 18.5 dpc. Immunostaining showing DAPI in blue, CFP in green and FOXL2 in red. CFP was detected in the Sertoli cells in the XY gonads and occasionally in scattered cells in the XX TESCO:CFP; Wnt4\(^{+/−}\) gonads, whereas FOXL2 was detected in XX TESCO:CFP; Wnt4\(^{+/−}\) but not XY gonads. XX TESCO:CFP; Wnt4\(^{-/-}\) gonads showed cells which express either CFP or FOXL2. Scale bar = 50μm; inserts show 2.5x higher magnifications.
6.2.2 SOXE expression in Wnt4 mutant mice

Next, the expression pattern of endogenous Sox9 was analysed between 12.5 dpc and P0 to determine if it coincided with the CFP expression. As expected, Sox9 expression was detected in Sertoli cells of XY TESCO:CFP; Wnt4+/− gonads, while it could not be detected in control XX TESCO:CFP; Wnt4+/− gonads. However, no Sox9 expression was found in XX TESCO:CFP; Wnt4−/− gonads between 12.5 dpc and P0 (Figure 6.3). Thus, the cells which express TESCO:CFP did not show a synchronous de-repression of endogenous Sox9 expression.

It has been described before that other members of the SoxE family also play an important role in early testis development. Sox8 is expressed in Sertoli cells in the XY gonad from 12.0 dpc onwards and has not been detected in XX gonads (Schepers et al., 2003). Recently, it has also been reported that Sox10 is expressed exclusively in the XY gonad at the time of sex determination (Polanco et al., 2010). Both, Sox8 and Sox10 share a high sequence identity with Sox9 and it has been proposed that at least SOX8 and SOX9 might act redundantly with respect to each others function. To determine whether any SOXE signal could be detected in XX TESCO:CFP; Wnt4−/− gonads, an antibody recognising all three members of the SOXE proteins (SOX8, SOX9, SOX10) was used.

Since SOX9 expression is absent in XX Wnt4−/− gonads (Figure 6.3) any SOXE signal identified would correspond to SOX8 or SOX10. As expected, SOXE expression was found in the Sertoli cells in XY TESCO:CFP; Wnt4+/− gonads at all time points analysed, presumably representing the expression patterns of both Sox8 and Sox9 (Figure 6.4). No SOXE signal was detected in control XX
TESCO:CFP; Wnt4+/− gonads. In XX TESCO:CFP; Wnt4−/− gonads, some occasional SOXE-positive cells could be found at 12.5 dpc, a few more cells at 14.5 dpc and more cells expressing SOXE were detected between 16.5 dpc and P0 (Figure 6.4), similar to the pattern of TESCO de-repression in XX TESCO:CFP; Wnt4−/− gonads (Figure 6.1).
Figure 6.3: Endogenous Sox9 expression in TESCO:CFP; Wnt4−/− gonads during embryogenesis.
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of Wnt4 at different time points during embryogenesis and just after birth (P0). Immunostaining shows DAPI in blue and SOX9 in green. SOX9 could be detected in Sertoli cells in the XY TESCO:CFP; Wnt4+/− gonads (left panel) but not in XX TESCO:CFP; Wnt4+/− or XX TESCO:CFP; Wnt4−/− at any time during embryogenesis (middle and right column, respectively). Scale bar = 50μm.
Figure 6.4: Endogenous SoxE expression in TESCO:CFP; Wnt4−/− gonads during embryogenesis.
Cryosections of XY and XX gonads of TESCO:CFP mice with heterozygous and homozygous loss of Wnt4 at different time points during embryogenesis and at P0. Immunostaining shows DAPI in blue and SOXE in green. SOXE signals could be found in Sertoli cells in the XY TESCO:CFP; Wnt4+/− gonads (left panel) but not in XX TESCO:CFP; Wnt4+/− gonads (middle panel). De-repression of SOXE, presumably SOX8 and/or SOX10, was found in XX TESCO:CFP; Wnt4−/− from around 14.5 dpc until birth (right panel). Scale bar = 50μm.
6.2.3 De-repression of TESCO and endogenous Sox9 expression in Foxl2/Wnt4 double mutant mice

Next, the effect of the combined loss of both Foxl2 and Wnt4 on TESCO activity was analysed in gonads of XX Foxl2/Wnt4 double mutant mice (Foxl2LacZ/LacZ; Wnt4−/−) at birth (Figure 6.5). CFP expression could be detected normally in Sertoli cells in XY Foxl2LacZ/LacZ; Wnt4−/− gonads, indicating that both Foxl2 and Wnt4 are not critical for Sertoli cell differentiation or testis cord formation. Analyses of XX TESCO:CFP; Foxl2LacZ/LacZ; Wnt4+/− gonads showed similar results to those of heterozygous XX TESCO:CFP; Wnt4+/− single mutants with a few CFP-positive cells. However, XX TESCO:CFP; Foxl2LacZ/LacZ; Wnt4−/− gonads showed an increase in the number of CFP-positive cells compared to each single mutant, suggesting a stronger de-repression of TESCO activity at P0 in the Foxl2LacZ/LacZ; Wnt4−/− double mutants. The CFP-positive cells in these gonads are forming seminiferous tubule-like structures which resemble the ones seen in XY gonads. Endogenous SOX9 expression could be found in the Sertoli cells of XY Foxl2LacZ/LacZ; Wnt4−/− gonads, but not in the analysed XX TESCO:CFP; Foxl2LacZ/LacZ; Wnt4+/− gonads. XX TESCO:CFP; Foxl2LacZ/LacZ; Wnt4−/− gonads showed endogenous Sox9 expression in a pattern similar to the TESCO:CFP reporter. This was in contrast to each single mutant, where SOX9 was not de-repressed at P0 (Figure 6.3 and Figure 5.5).
Figure 6.5: TESCO activity in TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup>; Wnt4<sup>−/−</sup> double mutant gonads at P0.

Cryosections of XY and XX gonads of TESCO:CFP mice with homozygous loss of Foxl2 and heterozygous or homozygous loss of Wnt4 at P0. Immunostaining shows DAPI in blue and CFP in green. TESCO expression could be detected in the Sertoli cells in the XY TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup>; Wnt4<sup>−/−</sup> gonads. Very few CFP-positive cells could be found in XX TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup>; Wnt4<sup>+/−</sup> gonads. More CFP-positive cells were found in XX TESCO:CFP; Foxl2<sup>LacZ/LacZ</sup>; Wnt4<sup>−/−</sup> gonads than in the single mutants. Scale bar = 50 μm.
6.3 Conclusions

According to published data, Wnt4 is a candidate for an anti-testis or ovarian-determining gene which could be involved in the repression of Sox9 in XX gonads during embryonic development. As Wnt4 expression becomes female-specific coincidentally with the down-regulation of Sox9 at 11.5 dpc, it is possible that it is the crucial gene to mediate the down-regulation of Sox9 expression in embryonic XX gonads. In this chapter it was analysed whether Wnt4 can repress Sox9 expression in vivo and whether this repression is mediated via the TESCO enhancer element. As homozygous Wnt4 mutant mice die within 24 h after birth due to kidney failure, the gonadal phenotype of these mice could only be analysed up until P0.

Homozygous loss of Wnt4 resulted in some de-repression of TESCO activity in the XX gonad around 14.5 dpc. This result suggests that WNT4 signalling is involved in the repression of TESCO activity from 14.5 dpc until after birth, but not prior to 14.5 dpc. Interestingly, no Sox9 expression could be detected in XX Wnt4−/− gonads at the time points analysed, either during embryogenesis or at P0. This is consistent with results from Kim et al. (2006b), where transient expression of Sox9 in XX Wnt4−/− gonads was detected only at 11.5 dpc and then found to be down-regulated by 12.5 dpc. The time course of Sox9 expression in XX TESCO:CFP; Wnt4−/− gonads shown here, demonstrates that the down-regulation of Sox9 is maintained after 12.5 dpc until birth, although some cells express the TESCO:CFP reporter from 14.5 dpc onwards. The fact that the TESCO element became de-repressed upon loss of Wnt4 while Sox9 expression is
not up-regulated suggests that the endogenous Sox9 gene is controlled in a more complex manner, independently of the TESCO element in XX gonads during embryonic development. The TESCO element is the critical 1.3 kb core region of the endogenous 3.2 kb Sox9 enhancer TES and thus it might be possible that the complete TES element contains relevant repressor binding sites outside the TESCO region. In fact, when the larger TES element was cloned into the luciferase vector and tested in co-transfection assays, the element could not be activated by increasing amounts of SF1 and SOX9 (data not shown), suggesting that TES might contain a strong repressor element outside TESCO. However, as mentioned before, TESCO:CFP is a transgene and it is possible that the differential de-repression profile of TESCO:CFP transgene and endogenous Sox9 might be at least partially due to epigenetic differences and integration site.

Coinciding with the de-repression of TESCO in XX Wnt4−/− gonads, was the activation of SOXE expression, that is SOX8 and/or SOX10, from 14.5 dpc until P0. This result suggests that WNT4 represses Sox8 and/or Sox10 expression, in addition to Sox9, in XX gonads during this time of embryonic development. Both, Sox8 and Sox10 belong to the same group of SOXE proteins as Sox9 and the two proteins share a high sequence homology. Studies in mice with double mutation of both Sox8 and Sox9 have suggested that the two genes have redundant functions during embryonic testis development (Chaboissier et al., 2004; Barrionuevo et al., 2009).

Interestingly, the cells which showed a de-repression of TESCO activity in XX Wnt4−/− gonads were not expressing FOXL2. This suggests that TESCO ac-
tivity becomes de-repressed in a subset of granulosa cells which have lost Foxl2 expression, or in some undifferentiated granulosa precursor cells in the gonads of these mice. The result that expression of TESCO and FOXL2 are mutually exclusive, once more indicates that FOXL2 antagonises TESCO activity. This is consistent with the results in adult XX Foxl2Δ/Δ mice, were the expression of FOXL2 and SOX9 were found to be mutually exclusive (Uhlenhaut et al., 2003). FOXL2 seems to be able to keep TESCO activity repressed in the granulosa cell precursor cells in XX Wnt4−/− gonads during embryonic development. As TESCO does not become up-regulated in XX Foxl2LacZ/LacZ gonads before birth, it suggests that Wnt4 is the more important gene to repress TESCO during embryogenesis, but in the absence of Wnt4, FOXL2 can still repress TESCO activity. To determine whether Foxl2 and Wnt4 might be acting in redundant pathways to secure repression of TESCO activity and endogenous Sox9 expression in the developing XX gonad, mice carrying mutations of both genes were analysed. XX Foxl2LacZ/LacZ, Wnt4+/− gonads were similar to those of Wnt4+/− mice with very few CFP-positive cells. XX Foxl2LacZ/LacZ, Wnt4−/− gonads showed more cells expressing CFP than in either of the single mutants. Moreover, cord-like structures could be seen in the double mutant gonads and Sox9 expression was de-repressed. This observation was different from that in each single mutant where neither XX Foxl2LacZ/LacZ nor XX Wnt4−/− gonads showed up-regulation of Sox9 expression at P0 (Figure 6.6). These results indicate a synergistic effect of both Foxl2 and Wnt4 in repressing TESCO and Sox9 expression at birth.

Taken together these results indicate that WNT4 signalling is at least partially involved in the repression of TESCO activity during embryonic development.
However, the expression of endogenous Sox9 seems to be repressed by additional factors independently from the TESCO element. Around the time of birth both Wnt4 and Foxl2 seem to be working together to ensure the repression of TESCO activity and Sox9 expression in the developing XX gonad.

Figure 6.6: **Summary of in vivo data.**
In gonads of XX Wnt4\(^{-/-}\) mice, TESCO:CFP and SOX8 are de-repressed from 14.5 dpc until P0, while Sox9 is still repressed (cross indicates that Wnt4\(^{-/-}\) mice die within 24h after birth). In gonads of XX Foxl2\(^{LacZ/LacZ}\) mice, TESCO:CFP becomes de-repressed at P0, whereas SOX9 first appears at 7 dpp. In gonads of XX double mutants, both TESCO:CFP and SOX9 can be detected at P0.
Chapter 7

General Discussion

The decision whether a mammalian embryo develops as male or female depends on the presence or absence of the Y chromosome. For some, the discovery of Sry as the sex determining factor on the Y almost 20 years ago, strengthened the idea that male development would be an active process whereas female development would be merely a default pathway. However, due to the discovery of a number of genes involved in both male and female sex determination, it has become evident that it is not that simple. In both humans and mice, cases of XX female-to-male sex reversal have been described that result from LoF mutations in several genes, which are now considered to be ovarian-promoting or at least anti-testis genes as they are playing important roles in the active repression of the male pathway.

Recently, it has been shown that Sox9 is the critical downstream target gene of Sry in the developing XY gonad and that its testis-specific expression is regulated via the 1.3 kb enhancer element TESCO (Sekido and Lovell-Badge, 2008). A model has been proposed in which TESCO activity (and therefore Sox9 expression) in the XY supporting cell lineage is initiated by SF1 at 10.5 dpc, up-
regulated by SRY by 11.5 dpc and maintained via a positive feedback loop including SOX9 itself and other factors, such as FGF9 and PGD2 signalling (Sekido and Lovell-Badge, 2008; Kim et al., 2006; Wilhelm et al., 2005). SOX9 directs the supporting cell lineage of the XY gonad to become Sertoli cells, which is the crucial event in testis development. In contrast, in XX gonads, although Sox9 expression is initiated by SF1, it is then actively down-regulated by 11.5 dpc and this repression is maintained in granulosa cells usually throughout life. Until now, it has not been known which genes are responsible for the initial down-regulation of Sox9 and the maintenance of Sox9 repression or whether this repression is mediated via the TESCO element in XX gonads.

7.1 The ovarian-promoting genes Dax1, Sox4 and Foxl2 repress TESCO activity in vitro

Several genes have been proposed as ovarian promoting genes due to their differential expression profiles in XX and XY gonads during the time of sex determination. These include Dax1, Sox4, Wnt4, Foxl2 and ERα. To analyse whether these candidate genes could be involved in the regulation of Sox9 expression via the TESCO element, the 1.3 kb sequence was first searched for putative binding sites. The analysis revealed that the TESCO sequence is indeed full of potential transcription factor binding sites. Amongst others, sites were found for GATA, zinc finger transcription factors, LIM homeobox factors, TATA-binding factors, SOX proteins, nuclear receptors, LEF/TCFs, forkhead factors and estrogen response elements. This suggests the possibility that the listed ovarian promoting
genes could be involved in the modulation of TESCO activity.

The potential of these genes to modulate Sox9 expression via the TESCO element was then analysed in co-transfection assays in vitro. In this thesis, it has been demonstrated that, while WNT effectors did not significantly affect TESCO activation mediated by SF1 and SOX9/SRY, DAX1, SOX4 and FOXL2 were able to repress TESCO activity in a dosage-dependent manner. Interestingly, ERα on its own did not have any effect on TESCO activation, but acted synergistically with FOXL2 resulting in an enhanced repression effect which seems to be independent from the amount of ERα, at least at the levels tested.

The implication of WNT4 signalling on TESCO expression was analysed via its downstream effectors β-catenin and LEF/TCFs. No effect on TESCO activity could be found by either β-catenin on its own, either LEF1, TCF1, TCF3 or TCF4, or by combinations of β-catenin and any of the LEF/TCFs. This could suggest that WNT4 signalling is not acting on Sox9 expression via the TESCO element or that it acts on TESCO activity via a non-canonical pathway (for review see Komiya and Habas, 2008). Another possibility is that the COS7 cell line that was used might lack important co-factors which are crucial for WNT dependent transcriptional regulation (like Groucho or CtBP). For more conclusive results the experiments should be repeated in a more suitable cell line, e.g. in primary cultures of granulosa cells. It is not known yet which LEF/TCFs are expressed in the supporting cells of the gonad and therefore could be involved in any regulation of sex determination. Thus, in future experiments, it should firstly be determined which LEF/TCFs are indeed expressed in the granulosa cells in XX gonads during
embryogenesis and postnatally, and thus could be involved in the regulation of Sox9 expression. Moreover, ChIP assays are necessary to analyse which of these factors, if any, are bound to the TESCO element in vivo and thus might indeed be involved in its regulation. It is also possible that WNT4 signalling modulates Sox9 expression not by transcriptional regulation but via a different mechanism. It has been reported in chondrocyte differentiation that SOX9 physically interacts with β-catenin which causes the degradation of both proteins via the proteosomal machinery (Akiyama et al., 2004). It might be possible that the same is happening in the gonad, thereby regulating the amount of available SOX9. Nevertheless, reduced levels of SOX9 in the presence of β-catenin should still cause a reduction of TESCO activation in the co-transfection assays, as SOX9 is needed for TESCO activation. However, the luciferase reporter assay might not be a sufficient system to analyse any potential SOX9 degradation effect. To determine the effect of β-catenin on SOX9, different amounts of β-catenin and SOX9 could be transfected into a cell line which does not endogenously express both genes and a possible reduction of the two proteins could be monitored e.g. by western blotting analysis. Finally, it is also possible that WNT4 is only involved in the regulation of Sox9 expression via activating its downstream target Dax1 (Mizusaki et al., 2003), which is unlikely to happen in COS7 cells.

In the co-transfection assays, DAX1 was able to repress TESCO activation. It had already been reported that DAX1 can form heterodimers with SF1 and decreases SF1 activity (Ho et al., 1997; Crawford et al., 1998). As SF1 is needed for TESCO activation, less SF1 activity could result in the observed repression effect on TESCO activity upon addition of DAX1. However, the precise mechanism as
to how DAX1 represses TESCO activity remains to be investigated. In future experiments it would be necessary to determine whether DAX1 directly binds to the TESCO sequence, e.g. by ChIP assay. Moreover, it would also be interesting to analyse the effect of DAX1 on TESCO activity in vivo. If DAX1 is necessary for the repression of TESCO activity, mice carrying a deletion of the Dax1 gene crossed to the TESCO:CFP reporter line might show an up-regulation of CFP expression in XX gonads. However, the role of Dax1 in sex determination is not completely understood and the published Dax1 mutant mice are still a point of controversy. Thus, it might be necessary to generate a targeted deletion of both Dax1 exons or to establish a complete knock down of Dax1 expression via other methods such as RNAi. These in vivo models might provide a more reliable model to analyse the effect of loss of Dax1 expression in general and on Sox9 expression and TESCO activation in particular.

SOX4 was also found to be able to repress TESCO activity in vitro, but only when activated by combinations including SOX9 or SRY. The SOX4 protein contains the highly conserved DNA binding domain (HMG-box) characteristic of the SOX proteins and it is likely that it can bind to the same SOX binding sites in the TESCO element as SOX9 and SRY. Thus, SOX4 could compete with SOX9 and/or SRY for binding to the TESCO element and thereby prevent the activation of TESCO. As SOX4 and SOX9 are members of different groups of SOX proteins it is also possible that SOX9 (belonging to the SOXE group) acts as a transcriptional activator on TESCO and SOX4 (belonging to the SOXC group) could act as a transcriptional repressor. Indeed, it has already been shown that a prolonged expression of Sox4 in oligodendrocytes results in a reduction of
myelin gene expression in vivo (Chew and Gallo, 2009). In future experiments, such as EMSA or ChIP assays, it should be determined whether SOX4 is indeed able to bind to the SOX binding sites in the TESCO element and whether this might result in a competition for binding with SOX9. Furthermore, the effect of SOX4 on TESCO activity and endogenous Sox9 expression should be analysed in vivo. For this, mice with a deletion of the Sox4 gene should be crossed to the TESCO:CFP reporter line. If SOX4 is repressing TESCO activation, homozygous loss of Sox4 might result in an up-regulation of CFP and possibly endogenous Sox9 expression in XX gonads. However, Sox4−/− mice die at 14.5 dpc due to heart malformation (Schilham et al., 1996) allowing only the analysis of a possible role of SOX4 in the early repression of Sox9. To further determine the involvement of SOX4 in Sox9 repression after 14.5 dpc, a conditional mutation of Sox4 would be required.

The most interesting effect seen in the co-transfection assays in vitro was the repression of TESCO by FOXL2. This effect was dosage-dependent and provides the first evidence of a direct link between Foxl2 and the regulation of Sox9, suggesting that this regulation could be mediated via the TESCO element.

7.2 FOXL2 represses TESCO activity synergistically with ERα

FOXL2 and ERα were able to synergistically repress TESCO activation in vitro. While FOXL2 belongs to the forkhead family of transcription factors which can bind to DNA at the consensus binding site (A/G)(C/T)(A/C)AA(A/T)A (Kauf-
ERα can bind to EREs (palindromes of GGTCA) (Klinge, 2001), both of which are present in the TESCO element. ChIP analysis in adult ovaries have revealed that both FOXL2 and ERα are indeed binding to the TESCO sequence and co-immunoprecipitation assays have demonstrated that FOXL2 and ERα physically interact (Uhlenhaut et al., 2009). Moreover, it has been shown in this thesis that mutation of all identified FOX and ERE sites in the TESCO sequence resulted in a loss of the synergistic repression effect mediated by ERα in vitro, though the repressive effect of FOXL2 was persisting. Analyses of the same mutations in the TESCO element in vivo showed a de-repression of TESCO:CFP in the adult ovary (Uhlenhaut et al., 2009). These data suggest that a potential FOXL2/ERα complex might bind to either FOX or ERE sites in the TESCO element and that Sox9 repression is mediated via these sites in the TESCO element in the adult ovary. A possible explanation for the differing results in vitro and in vivo could be the presence of some minor interaction sites for FOXL2 in the mutated TESCO sequence. These might not play a crucial role in vivo and TESCO activity becomes de-repressed upon loss of the major FOX and ERE sites. However, in the in vitro co-transfection assays these minor sites might still mediate the repression of TESCO activity due to the excessive amount of available FOXL2. Although the conclusions which can be drawn from the in vitro assays should be regarded carefully due to the limitations of this artificial system, the results stress the same trend seen in vivo: the importance of the synergistic repressive effect of FOXL2 and ERα on TESCO activity.

In contrast to the adult ovary, FOXL2 seems not to be the crucial factor for TESCO repression during embryogenesis as loss of Foxl2 does not result in the
up-regulation of TESCO activity or Sox9 expression before birth. In mice, no steroidogenesis takes place during XX embryonic development ([Pannetier et al., 2006a]), thus no production of estrogens occurs during this time period. It is possible that, due to the lack of estrogen signalling, ERα is not translocated into the nucleus and thus cannot interact with FOXL2 which results in the inability of FOXL2 to repress TESCO. To understand this mechanism better, it should be analysed via ChIP assays whether FOXL2 is able to bind to the TESCO element in the absence of ERα during embryogenesis. In contrast to mice, production of estrogens has been found during early ovarian development in the goat. It has been shown that FOXL2 activates Cyp19a1 expression in goat ([Pannetier et al., 2006a]), indicating that loss of Foxl2 expression in XX goats with female-to-male sex reversal (PIS mutation) might also result in the additional absence of estrogens during embryonic development. The phenotype in PIS mutant goats is evident much earlier than in Foxl2 mutant mice, suggesting that indeed estrogens play an important role in repressing the testis pathway during embryonic development in the goat. Loss of Foxl2 therefore seems to be the primary cause of the XX sex reversal in PIS animals.

In XX mice however, double mutations of both ERα and ERβ (α/βERKO) result in mostly normal pre-pubertal ovaries ([Couse et al., 1999]). In such mice, the early differentiation of the reproductive tract occurs normally and all Müllerian duct-derived structures are developed. However, the pre-pubertal ovaries possess adult-like follicles indicating a precocious maturation of the ovary. In contrast, ovaries of adult α/βERKO mice show the appearance of some seminiferous tubule like structures and Sertoli-like cells. Moreover, Sox9 expression becomes
up-regulated, resulting in an adult sex-reversed phenotype similar to the one
described in adult Foxl2Δ/Δ mice (Uhlenhaut et al., 2009), although less pro-
nounced. While the phenotype of α/βERKO mice first becomes evident after
puberty, the phenotype of Foxl2LacZ/LacZ mice is visible as early as 7 dpp. These
differences in timing indicate that FOXL2 cannot just depend on an interaction
with ERs to regulate female sex determination.

7.3 FOXL2 interferes with SF1-mediated activation of TESCO in vitro

In this thesis it has been demonstrated that FOXL2 interacts directly with SF1.
It was also shown that mutation of all putative FOX sites in the TESCO element
did not result in a loss of TESCO repression mediated by FOXL2, suggesting that
FOXL2 does not act, at least not exclusively, via these predicted binding sites.
Moreover, the data suggests that FOXL2 can bind to DNA at the SF1 binding
site and might be competing with SF1 for binding to the TESCO sequence.
Recently a paper was published which postulated a new FOXL2-specific DNA
binding site GTCAAGG(T/C)CA (Benayoun et al., 2008). This new binding site
resembles very closely the SF1 binding site (T/C)(T/C)AAGG(T/C)C(G/A).
Taken together, these data allows to hypothesise several ways by which FOXL2
could interact with SF1 to regulate TESCO activity (Figure 7.1): (i) FOXL2
(possibly together with ERα) might physically interact with SF1 and thereby
reduce the amount of SF1 available to activate TESCO, (ii) FOXL2 (possibly
together with ERα) might interact with DNA-bound SF1, thereby inhibiting its
transcriptional activation capability, (iii) FOXL2 might be competing with SF1 for binding to the TESCO element, resulting in a repression of TESCO rather than an activation. In future experiments, the exact binding sites for FOXL2 in the TESCO element should be analysed e.g. by in vivo footprinting assays, to determine if FOXL2 is indeed able to bind to SF1 binding sites or if it could possibly bind to other sites which differs from the consensus forkhead factor binding site. Further structural analyses should also determine the interaction between FOXL2/ERα and FOXL2/SF1 in more detail to find out which domains might be involved in protein-protein interactions and how they might bind to DNA as protein complexes.

Figure 7.1: Model of possible interactions between FOXL2 and SF1 to regulate Sox9 expression in XX gonads.
SF1 initiates low expression of Sox9 in the XX gonad, but this is then down-regulated and it is maintained in a repressed state in the granulosa cells throughout life. It is likely that the repression is mediated via the TESCO element, and FOXL2 (and ERα) could contribute to this in the embryo and then become solely responsible after birth, in several ways: (i) FOXL2 (+ ERα?) could interact with SF1, preventing the latter from binding DNA, (ii) FOXL2 (+ ERα?) could interact with DNA-bound SF1, interfering with its ability to activate transcription or actively recruit co-repressors (iii) FOXL2 interacts with ERα and the complex might bind to TESCO at FOX sites, EREs or possibly at SF1 binding sites and directly mediate repression.
7.4 FOXL2 represses TESCO activity and endogenous Sox9 expression postnatally

The in vivo analyses conducted in this thesis, showed that loss of Foxl2 expression resulted in an up-regulation of TESCO activity in XX TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ} gonads. This indicates that FOXL2 is repressing TESCO activation in vivo as well as in vitro. However, the de-repression of TESCO activity was only seen postnatally and not at any of the time points analysed during embryogenesis. Endogenous Sox9 expression was also up-regulated postnatally in the same cells as CFP, in cells surrounding oocytes. These cells are likely to have been granulosa cells as they also express \( \beta \)-galactosidase, a marker expressed under the control of the endogenous Foxl2 promoter. Thus, it seems that the de-repression of both TESCO activity and endogenous Sox9 expression occurs in granulosa cells which then take on Sertoli-like properties. In Foxl2 mutant mice, these cells are organised in only one or two layers around the oocyte, in contrast to several layers of granulosa cells in wild-type ovaries. This seems to be due to a failure in proliferation (Uda et al., 2004) as might be expected if they are Sertoli cells. The up-regulation of Sox9 expression in this layer of supporting cells indicates a switch from the female to a male fate. A similar result was obtained in the conditional Foxl2\textsuperscript{\Delta/\Delta} mice where, upon loss of Foxl2 in the adult ovary, both TESCO:CFP and Sox9 became expressed in the layer of granulosa cells surrounding the oocytes (Uhlenhaut et al., 2009). Taken together, these results indicate that FOXL2 is indeed necessary to repress Sox9 postnatally and that this regulation is accomplished via the TESCO element in vivo.
However, it is also possible that the loss of FOXL2 results in a de-repression of endogenous Sox9 independent of the TESCO element. The de-repressed endogenous SOX9 could then act back on the TESCO element in a positive feedback loop in the same way that SOX9 contributes to its own regulation during early testis differentiation (Sekido and Lovell-Badge, 2008). It is thought that SOX9 levels need to reach a critical threshold first before such a feedback loop can be established. Thus, if loss of Foxl2 indeed resulted in a de-repression of Sox9 expression before activation of the TESCO element, there might be a detectable delay between the onset of endogenous Sox9 expression and activation of CFP in XX TESCO:CFP; Foxl2$\text{LacZ}/\text{LacZ}$ gonads. However, CFP activation preceded the de-repression of Sox9 expression in such gonads. While a few CFP positive cells could already be found, endogenous Sox9 expression was not detectable at P0, but it could be seen at 7 dpp. This is concordant with the finding that XX Foxl2$\text{LacZ}/\text{LacZ}$ ovaries are indistinguishable from wild-type ovaries at birth but phenotypic differences were detected at 7 dpp (Schmidt et al., 2003; Uda et al., 2004). In future experiments, the timing and relationship between TESCO:CFP and SOX9 should be analysed in more detail and a closer look should be taken at the expression profile of the two proteins between P0 and 1 week of age. Also, the conditional Foxl2 mutant mice (Foxl2$^{fl/fl}$) could be used to further analyse the de-repression of both TESCO activity and Sox9 expression in the mutant ovaries. By crossing them with the TESCO:CFP reporter line it would be possible to analyse the exact timing between the loss of FOXL2 and the first appearance of CFP or SOX9. It would also be interesting to analyse the effect of loss of Foxl2 on TESCO activity in the context of a Sox9 null background, using the conditional
Sox9 null allele \((\text{Sox9}^{fl/fl})\) (Chaboissier et al., 2004). In this background, any de-repression of TESCO:CFP would have to be independent from endogenous SOX9 and instead directly due to the loss of FOXL2-mediated repression on the TESCO element. A different approach would be to first determine whether the TESCO element is the only regulatory element necessary to control Sox9 expression in the gonad by generating a targeted deletion of the TESCO enhancer \((\text{TESCO}^\Delta)\). The deletion of the TESCO element \textit{in vivo} should result in a complete loss of gonadal Sox9 expression. The TESCO:CFP reporter and the Foxl2\textit{LacZ} allele could then be bred onto the TESCO\textit{^\Delta} background, where any de-repression of TESCO:CFP would be SOX9 independent.

Both TESCO and Sox9 become robustly de-repressed with increasing age in the XX TESCO:CFP; Foxl2\textit{LacZ/LacZ} gonads, suggesting that Foxl2 becomes the only important gene to maintain Sox9 repression in the adult. These findings were substantiated in the conditional Foxl2\textit{^\Delta/\Delta} mice, where deletion of Foxl2 in 8 week old females resulted in the de-repression of Sox9 and the transdifferentiation of granulosa cells into Sertoli cells (Uhlenhaut et al., 2009). Taken together these data indicate that FOXL2 is indeed a only critical factor needed to repress Sox9 expression in the adult ovary. Interestingly, de-repression of Sox9 starts from 1 week of age in XX TESCO:CFP; Foxl2\textit{LacZ/LacZ} gonads, suggesting that FOXL2 becomes important for repression of Sox9 expression at this time. In future experiments, Foxl2 should be deleted at different time points around and after birth, using the conditional Foxl2\textit{^fl/fl} mice, to determine the exact time point from which Foxl2 acts as the only critical factor required to repress Sox9 expression postnatally. Moreover, using the conditional Foxl2\textit{^fl/fl} mice, it would be inter-
esting to analyse whether deletion of Foxl2 just before birth (i.e. Foxl2 would be expressed during most of the embryonic development) results in the same postnatal phenotype as seen in the Foxl2LacZ/LacZ mice (where Foxl2 is never expressed). It is possible that loss of Foxl2 from the early stages of embryogenesis results in the activation of redundant pathways to ensure proper ovarian development.

In future experiments, the effect of Foxl2 misexpression in XY gonads should also be addressed. It has already been shown that misexpression of transgenic Foxl2 under the control of an ubiquitous heat-shock inducible promoter in XY gonads resulted in disorganised tubules and ovotestis-like structures at 13.5 dpc (Ottolenghi et al., 2007). However, no further analyses have been carried out in the gonads of these mice. It would be interesting to analyse both endogenous Sox9 expression and TESCO:CFP reporter expression upon misexpression of Foxl2 in XY gonads. If FOXL2 is indeed repressing Sox9 via TESCO, misexpressed Foxl2 should result in the down-regulation of both TESCO:CFP and endogenous Sox9 in these gonads. To test this hypothesis, a construct for conditional misexpression of Foxl2 has already been designed (IZ/Foxl2) and injected into fertilised mouse eggs. Mice carrying this transgene will express the LacZ gene ubiquitously and, upon cre-mediated recombination, HA-tagged Foxl2 will be expressed from a CMV promoter (Figure 7.2). These mice will be crossed to the TESCO:CFP line and to mice expressing a gonad-specific cre-recombinase (e.g. Sf1:Cre, Amh:Cre) to analyse the effect of Foxl2 expression on both TESCO activity and Sox9 expression in the XY gonad. Another approach could be to analyse the effect of Foxl2 transfection into cultured Sertoli cells in vitro. Unfortunately, in the supposed Sertoli cell line TM4, SOX9 protein could not be
detected either by immunohistochemistry or by Western blot analysis (data not shown and [Beverdam et al., 2003]). Therefore these cell line could not be used to analyse the potential effect of FOXL2 on Sox9 expression in vitro. In future experiments, primary cultures of Sertoli cells from the TESCO:CFP reporter mouse line could be employed to analyse the effect of misexpression of Foxl2 on both TESCO activity and endogenous Sox9 expression in vitro.

Figure 7.2: IZ/Foxl2: A construct for conditional misexpression of Foxl2. Mice carrying this transgene will express β-galactosidase ubiquitously. Upon cre-mediated recombination the two loxP sites (pink triangles) will recombine and Foxl2, which is N-terminally tagged with a HA-epitope, will be expressed.

7.5 Sox9 expression is not up-regulated in oocyte-depleted ovaries

For a long time it had been proposed that oocytes are essential to maintain granulosa cell fate and that the loss of oocytes could cause a switch from granulosa cells to Sertoli cells (McLaren, 1991). In this thesis the question was addressed whether the loss of oocytes resulted in a postnatal up-regulation of Sox9 expression comparable to the de-repression seen in XX TESCO:CFP; Foxl2LacZ/LacZ mice. When oocytes were specifically depleted by activation of a conditional form
of the diphtheria toxin fragment A (DTA) (Ivanova et al., 2005) using ZP3:Cre, no postnatal Sox9 expression was detected in these ovaries at any of the postnatal time points analysed. Similar results were obtained using Gdf9:Cre as well as an inducible approach which allowed the deletion of oocytes in the adult ovary (Uhlenhaut et al., 2009). These results, compared to the analyses in the XX TESCO:CFP; Foxl2LacZ/LacZ and XX Foxl2Δ/Δ mice, show that it is not the loss of oocytes, but the loss of a sole gene, Foxl2, which leads to up-regulation of Sox9 expression in granulosa cells, resulting in their transdifferentiation to Sertoli cells. Interestingly, it has also been shown in neonatal rats, that depletion of oocytes by γ-irradiation resulted in a survival of granulosa cells which developed morphological characteristics of Sertoli cells, but did not up-regulate Sox9 expression (Guigon et al., 2005). However, these experiments were not conclusive as not all oocytes were eliminated. In further experiments, the oocyte-depleted ovaries (ZP3:Cre, R26DTA) should be analysed in more detail regarding their morphological characteristics as well as further marker analyses to establish which cell types are still present upon loss of oocytes and how the granulosa cells are effected in terms of cellular and molecular properties. However, it remains possible that oocytes are required during late fetal stages to prevent a fate change from follicle to Sertoli cells. To analyse this hypothesis, oocytes have to be depleted during embryonic development, e.g. by disrupting the progress of meiosis.
7.6 WNT4 signalling partially represses TESCO activity during embryonic development

It has been shown that Wnt4, when expressed at high levels, or stabilised β-catenin can antagonise the FGF9/FGFR2 feedback loop (Kim et al., 2006b) and prevent the maintenance of Sox9 expression (Figure 7.3). This suggests an antagonism between the two signalling pathways, such that FGF9 tips the balance towards testis development and WNT4 towards ovarian development. This thesis presents the first evidence that Wnt4 is also involved in the repression of TESCO activity and Sox8/Sox10 expression during embryonic development.

Homozygous loss of Wnt4 resulted in de-repression of TESCO from 14.5 dpc onwards, suggesting that Wnt4 is at least partially responsible for TESCO repression during embryonic development. However, although TESCO:CFP was de-repressed in those gonads, no endogenous Sox9 could be detected, indicating that additional factors are still repressing Sox9 during this time.

Moreover, as TESCO activity in XX TESCO:CFP; Wnt4−/− gonads becomes de-repressed only from 14.5 dpc, WNT4 cannot be the critical factor responsible for its repression prior to this point. It is possible that other Wnts which are expressed in the gonad compensate for the loss of Wnt4. However, other factors could also be involved in the early repression of TESCO activity (and Sox9 expression). R-spondin1 is one possible candidate (Figure 7.3). RSPO1 was first identified to play a role in sex determination in human patients which display XX female-to-male sex reversal due to homozygous LoF mutations of R-SPO1 (Parma et al., 2006). In the mouse, Rspos1 is expressed in somatic cells of the
indifferent gonad in both sexes at 10.5 dpc and then becomes female-specific around 12.5 dpc ([Parma et al., 2006]). Mice with a targeted null mutation of Rspo1 (Rspo1−/−) show partial XX female-to-male sex reversal ([Chassot et al., 2008; Tomizuka et al., 2008]). Rspo1 is still expressed in XX Wnt4−/− gonads ([Chassot et al., 2008]). Thus it is possible that Rspo1 might be responsible for the repression of TESCO prior to 14.5 dpc in the XX TESCO:CFP; Wnt4−/− gonads. In future experiments, the effect of loss of Rspo1 expression on both TESCO activity and endogenous Sox9 expression should be determined in XX gonads from 11.5 dpc onwards.

As homozygous Wnt4 mutant mice die within 24 h after birth due to kidney failure, the gonadal phenotype of those mice could only be analysed until P0. Recently, a conditional Wnt4 mutant mouse line has been described (Wnt4fl/fl), which upon cre-mediated deletion during early embryogenesis, displays a phenotype consistent with the Wnt4−/− mice ([Shan et al., 2009]). It would be very interesting to analyse the effect of later loss of Wnt4 expression on both TESCO activity and Sox9 expression. If FOXL2 is indeed the only critical factor repressing TESCO activity after birth, postnatal loss of Wnt4 should not result in a de-repression of either TESCO activity or Sox9 expression.

Although CFP expression was detected in XX gonads of both TESCO:CFP; Wnt4−/− and TESCO:CFP; Foxl2−/− mice at P0, no de-repression of Sox9 could be found in either single mutant. Interestingly, XX gonads of mice with double homozygous mutations in Foxl2 and Wnt4 not only show a stronger de-repression of TESCO activity at P0, but also up-regulation of endogenous Sox9 expression.
Moreover, XX gonads of double mutant mice show a much more severe phenotype than the single mutants including the formation of seminiferous tubule-like structures. This suggests that both FOXL2 and WNT4 are necessary to repress Sox9 expression in XX gonads around the time of birth. However, more detailed analyses of de-repression of both TESCO activity and endogenous Sox9 during the time of embryonic development are necessary in the gonads of XX TESCO:CFP; Foxl2\textsuperscript{LacZ/LacZ}; Wnt4\textsuperscript{-/-} mice to better understand the synergistic effect of the two genes.

7.7 **Sox8 and/or Sox10 expression is de-repressed in embryonic Wnt4 mutant XX gonads**

The de-repression of TESCO in XX TESCO:CFP; Wnt4\textsuperscript{-/-} gonads coincides with the activation of the expression of SoxE genes from 14.5 dpc until P0. The SOXE group of proteins comprises SOX8, SOX9 and SOX10, which share high sequence homology even outside the HMG box. Like Sox9, both Sox8 and Sox10 are known to be expressed in the XY gonad around the time of sex determination (Schepers et al., 2003; Polanco et al., 2010).

Studies in mice with double mutations of both Sox8 and Sox9 have suggested that the two genes might be involved in redundant pathways immediate after Sox9 up-regulation and during subsequent testis development (Chaboissier et al., 2004; Barrionuevo et al., 2009). It has been shown that Sox9 is essential for the initiation of testis differentiation as the homozygous deletion of Sox9 prior or at the time of sex determination causes XY sex reversal (Chaboissier et al., 2011).
Moreover, conditional deletion of Sox9 at 14.5 dpc, after the successful initiation of testis differentiation, results in normal embryonic and early postnatal testis development. These mice show a late sterility around 7 months of age due to a loss of spermatogonial stem cells and subsequent abrogation of spermatogenesis (Barrionuevo et al., 2009). On the other hand, homozygous loss of Sox8 leads to an 80% sterility in male mice, while XY Sox8+/− and XX Sox8−/− mice are reproductively normal with no sex reversal. However, about 20% of XY Sox8−/− mice are able to produce a reduced number of offspring before they become sterile at 5 months of age (O’Bryan et al., 2008). Mice carrying a heterozygous conditional mutation of Sox9 on a Sox8 null background (Sox8−/−, Sox9A/−) display a more severe phenotype than single Sox8−/− or Sox9A/− mice. XY Sox8−/−, Sox9A/− gonads show abnormally shaped testis cords and defects in the formation of the coelomic vessel. Moreover, these gonads have a reduced number of seminiferous tubules and show partial sex reversal with areas resembling ovarian structures and lacking Amh expression at 15.5 dpc (Chaboissier et al., 2004). Homozygous double mutations of the two genes after 14.5 dpc using the Sox9 conditional allele and Amh:Cre (Sox8−/−, Sox9A/Δ) result in progressive testis cord degradation with reduced numbers of testis cords containing a large amount of apoptotic cells. At 2 months of age, the testes of such mice completely lack any tubular structures and are mainly composed of Leydig cells resulting in a primary infertility. Moreover, some XY mice with a homozygous double mutation in both genes displayed a residual uterus in addition to testicular structures, the formation of which is attributed to an almost complete down-regulation of Amh expression (Barrionuevo et al., 2009). All this data indicates that Sox8 and Sox9 act redundantly during
testis differentiation as both are important for proper Sertoli cell specification and maintenance of Amh expression.

Although a targeted mutation of Sox10 has been described (Britsch et al., 2001), the effect of loss of Sox10 in the gonad has not yet been determined. Recently, it has been demonstrated that misexpression of Sox10 in XX mice under the control of the Wt1 regulatory region, causes XX female-to-male sex reversal at 13.5 dpc (Polanco et al., 2010).

Since SOX9 is still repressed in the XX TESCO:CFP; Wnt4−/− gonads analysed in this thesis, the detected SOXE signal must be due to SOX8 and/or SOX10. In future experiments, it will be necessary to determine by in situ hybridisation which of the two genes, if not both, are expressed in the mutant gonads. The de-repression of the SoxE gene(s) in XX TESCO:CFP; Wnt4−/− gonads suggests that Sox8 and/or Sox10 might normally be repressed by WNT4 signalling in XX gonads during this time of embryonic development. It might therefore be possible, that the up-regulation of Sox8 and/or Sox10 expression could be involved in causing the masculinisation described in XX Wnt4−/− gonads (Vainio et al., 1999) in the absence of Sox9. Furthermore, it would be interesting to know whether Sox8 misexpression can cause XX sex reversal similar to Sox9 and Sox10.

Recently, it has been shown that both SOX8 and SOX10 in combination with SF1 can activate TESCO in vitro, although not as strongly as SF1 and SOX9 (Polanco et al., 2010). According to this data, it might also be possible that loss of Wnt4 in XX gonads results in a de-repression of endogenous Sox8 and/or Sox10 which then in turn activates the TESCO:CFP reporter. To further analyse
this possibility, the TESCO:CFP; Wnt4 mutant mice could be crossed to the Sox8 null background, the Sox8/Sox9 double null background and the Sox10 null background. On the other hand, no gonad-specific regulatory elements for Sox8 and Sox10 have been described. As Sox8 and/or Sox10 de-repression temporally coincides with TESCO up-regulation in XX TESCO:CFP; Wnt4−/− gonads, it is reasonable to speculate that a gonadal Sox8 and/or Sox10 enhancer might be regulated similarly to the TESCO element.

7.8 Additional factors must be involved in repressing Sox9 in embryonic XX gonads

While TESCO:CFP became de-repressed in XX TESCO:CFP; Wnt4−/− gonads at 14.5 dpc, endogenous Sox9 was still repressed. This may indicate that other factors are involved in repressing Sox9 expression during embryogenesis (Figure 7.3), independently of the TESCO element, although it is also possible that the latter is integrated in a site more favourable for expression, e.g. next to an active gene. Both RSPO1 and WNT4 act by stabilising β-catenin (Komiya and Habas, 2008; Chassot et al., 2008). It has already been shown that expression of a stabilised form of β-catenin in XY gonads at 11.5 dpc led to male-to-female sex reversal (Maatouk et al., 2008). Both Sox9 and Amh expression were dramatically reduced in the XY gonads of these mice after 12.5 dpc, suggesting that β-catenin is indeed able to antagonise Sox9 expression in the gonad, although the mechanism is still unknown. In future experiments, it would be interesting to analyse the effect of gonadal loss of β-catenin on Sox9 expression and TESCO ac-
tivity by crossing conditional β-catenin null mutant mice ([Huelsken et al., 2001]) to mice expressing a gonad-specific cre-recombinase (e.g. Sf1:Cre, Amh:Cre) and also to the TESCO:CFP reporter mice.

In future analyses it will be necessary to search for additional factors which are involved in the repression of Sox9 in the XX gonad during embryogenesis. This search could be performed via different approaches, e.g. by comparing differences in gene expression between XX and XY gonads during different time points around sex determination, as done by [Nef et al., 2005], [Beverdam and Koopman, 2006] and [Bouma et al., 2007]. Another possible approach would be forward genetic screening, such as ENU mutagenesis as performed by [Bogani et al., 2009]. Subsequently, mutations of identified candidate genes should be analysed in vivo. However, mutations in genes which are important in early embryonic development could result in embryonic lethality prior to the time of sex determination and therefore conditional approaches might be necessary. As an alternative to the classical expression-base screens, more global genomic approaches have recently been applied. In these studies, differences in gene expression profiles are mapped to chromosomal regions in the genome (eQTL). Recently, an eQTL analysis compared the gene expression levels in the developing gonad between a mouse strain sensitive to XY male-to-female sex reversal (C57BL/6J) versus a non-sensitive strain (129S1/SvImJ) at 11.5 dpc and found a number of novel genes and chromosomal loci which might be involved in the regulation of sex-related genes ([Munger et al., 2009]). This approach can be used to identify genetic network interactions on a global scale, as well as finding new regulators in sex determination, including both protein-coding and non-protein coding genes.
Non-protein coding genes, e.g. micro RNAs (miRNAs), are known to play important roles in the regulation of gene expression and it is possible that they account for the unknown factor regulating Sox9 expression in the XX gonad. Micro RNAs are small non-coding RNAs of 21-23 nucleotides, which alter the expression of target genes by post-transcriptional inhibition or degradation of mRNA sequences to which they are complementary (for review see Bartel, 2004). Analyses in the adult subventricular zone of the central nervous system have shown that Sox9 is a direct target of miR-124 (Cheng et al., 2009). It is possible that miR-124 might also be involved in the degradation of Sox9 transcripts in the early XX gonad (Figure 7.3), resulting in the persistent repression of endogenous SOX9 in the XX TESCO:CFP; Wnt4−/− gonads while TESCO:CFP is de-repressed. Moreover, the brief burst of Sox9 expression seen early in XX Wnt4−/− gonads (Kim et al., 2006b) could fail to establish the positive autoregulatory loop because miR-124 has become up-regulated and prevents further translation of SOX9. However, it is not known yet whether miR-124 is indeed expressed in the supporting cell precursors of the XX gonad at the right time to be involved in the repression of Sox9. Thus, in future experiments, the precise expression pattern of miR-124 in the gonad should be investigated at different time points during embryonic development. Furthermore, the effect of miR-124 misexpression on Sox9 expression could be analysed in XY gonads or the effect of anti-miR (miRNA inhibitors) designed for miR-124 could be examined in XX gonads.
Figure 7.3: Model of genetic interactions during ovarian development. Possible genetic interactions in the XX gonad, including different mechanisms which could be involved in the down-regulation of Sox9 in the female granulosa cell lineage.
7.9 Summary

In this thesis, it has been shown that the genes Dax1, Sox4 and Foxl2 (alone or in combination with ERα) have the potential to repress TESCO activity in vitro. It has also been demonstrated that the loss of Foxl2 results in a postnatal de-repression of TESCO activity and endogenous Sox9 expression in XX gonads, which is most evident in the adult. The de-repression of both TESCO and Sox9 occurs in cells derived from granulosa cells, indicating a transdifferentiation of the female supporting cells to Sertoli cells. These data indicate that FOXL2 is the crucial factor to repress Sox9 expression in the adult ovary and that this repression is mediated via the TESCO element (Figure 7.4). On the other hand, Foxl2 is not critical for the repression of either TESCO or Sox9 during embryonic development. Moreover, it has been shown in this thesis that WNT4 signalling could be partially responsible for Sox9 repression during embryonic development from 14.5 dpc onwards via the TESCO element. As Sox9 is still repressed in XX Wnt4 mutant gonads, additional factors (e.g. Rspos or miR-124) must be involved in its initial down-regulation at 11.5 dpc and persisting repression during embryonic development. Interestingly, both FOXL2 and WNT4 seem to play a role in the repression of Sox9 around the time of birth, possibly via the TESCO element (Figure 7.4).
In the XY gonad, SF1 binds to the TESCO enhancer of Sox9 and initiates a low Sox9 expression, then SRY binds to TESCO and up-regulates Sox9 expression, which is maintained by SOX9 itself and other factors such as FGF9 and PGD2 signalling. In the XX gonad, the initial expression of Sox9 is down-regulated at 11.5 dpc and the repression is maintained, both of which could possibly involve the TESCO element. It is not known yet which genes are responsible for this early down-regulation, though both miR-124 and Rspo1 might be good candidates. WNT4 signalling seems to be partially responsible for repression of TESCO activity from 14.5 dpc onwards. Both WNT4 and FOXL2 are involved in the repression of TESCO activity and Sox9 expression at the time of birth. In the adult ovary, FOXL2 (in cooperation with ERα) is the crucial factor to repress Sox9, possibly via TESCO.
Chapter 8

Appendices
8.1 Plasmid maps

8.1.1 pcDNA-Foxl2
8.1.2 pcDNA-Dax1

pcDNA3.1 (+/-) 5428/5427 bp

EcoRI Dax1 cDNA HindIII

pcDNA3.1/BGH reverse priming site

T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010

NheI PmeI DraI ApaI XbaI XhoI NotI BsrI EcoRV EcoRI BsiEI BamHI KpnI HindIII PmeI

pUC ori

Ampicillin

pcDNA3.1 (+/-) 5428/5427 bp

5428/5427 bp

(pCMV) BGH pA f1 ori SV40 ori Neomycin

pUC ori

Strand

179
8.1.3 pcDNA-Sox4

There is an ATG upstream of the Xba I site.

BamHI XhoI Sox4 cDNA

pcDNA3 5.4 kb
8.2 PCR-protocols

8.2.1 CFP PCR

Primer

Forward: 5’-GACCCTGAAGTTCATCTGCAC-3’  
Reverse: 5’-GTGGCTGATGTAAGTTGACTC-3’  
Size: 300 bp

PCR Protocol

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</tr>
<tr>
<td>10X buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>MgCl₂ [25mM]</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>dNTP [25mM]</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>primer for [50mM]</td>
<td>0.125 µl</td>
</tr>
<tr>
<td>primer rev [50mM]</td>
<td>0.125 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.125 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>0.5 µl</td>
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</tbody>
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PCR Program

\[
\begin{align*}
94^\circ C & \quad 3 \text{ min} \\
94^\circ C & \quad 45 \text{ sec} \\
53^\circ C & \quad 45 \text{ sec} \\
72^\circ C & \quad 1 \text{ min} \\
72^\circ C & \quad 7 \text{ min} \\
\end{align*}
\]

\[=\]

30x
8.2.2 Foxl2 PCR

Primer

FWB3: 5’-CAGATGATGGCCAGCTACCCCGAGC-3’
FWB4: 5’-GTTGTGGCGGATGCTATTCTGCCAGCC-3’
FWB5: 5’-GTAGATGGGGCGCATCGTAACCGTGC-3’
Size: wild-type = 200 bp, mutant = 500 bp

PCR Protocol

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<tr>
<td>DMSO</td>
<td>1.25 µl</td>
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<td>dNTP [25mM]</td>
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<tr>
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<tr>
<td>primer FWB4 [10mM]</td>
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</tr>
<tr>
<td>primer FWB5 [10mM]</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.1 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>

PCR Program

\[
\begin{align*}
94^\circ C & \quad 3 \text{ min} \\
94^\circ C & \quad 30 \text{ sec} \\
58^\circ C & \quad 30 \text{ sec} \\
72^\circ C & \quad 45 \text{ sec} \\
72^\circ C & \quad 5 \text{ min}
\end{align*}
\]

\[\text{35x}\]
8.2.3 Wnt4 PCR

Primer

Wnt4-E3for: 5’-CTTCACAACAACGAGGCTGGCAGG-3’
Wnt4-E4rev: 5’-CACCCGCATGTGTCAAGATGG-3’
Wnt4-neo-rev: 5’-GCATTGTCTGAGTAGGTGTCATT-3’
Size: wild-type = 700 bp, mutant = 400 bp

PCR Protocol

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<th>Component</th>
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</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>17.05µl</td>
</tr>
<tr>
<td>10X CAT-buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dNTP [25mM]</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>primer E3for [20mM]</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>primer E4rev [20mM]</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>primer neo-rev [20mM]</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>DNA</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

PCR Program

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>4 min</td>
</tr>
<tr>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>65°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
</tbody>
</table>

45x
8.2.4 Cre PCR

Primer

Forward: 5’-GGCGGATCCGAAAAGAAAA-3’
Reverse: 5’-CAGGGCGGCGAGTTAGTAGC-3’
Size: 400 bp

PCR Protocol

\[
\begin{align*}
\text{H}_2\text{O} & : 17.62 \mu\text{l} \\
10\text{X buffer} & : 2.5 \ \mu\text{l} \\
\text{MgCl}_2 [25\text{mM}] & : 1.5 \ \mu\text{l} \\
\text{DMSO} & : 1.25 \ \mu\text{l} \\
\text{dNTP [25mM]} & : 0.2 \ \mu\text{l} \\
\text{primer for [50mM]} & : 0.31 \ \mu\text{l} \\
\text{primer rev [50mM]} & : 0.31 \ \mu\text{l} \\
\text{Taq DNA polymerase} & : 0.31 \ \mu\text{l} \\
\text{DNA} & : 1.0 \ \mu\text{l}
\end{align*}
\]

PCR Program

\[
\begin{aligned}
94^\circ\text{C} & \ 3 \text{ min} \\
94^\circ\text{C} & \ 45 \text{ sec} \\
54^\circ\text{C} & \ 45 \text{ sec} \\
72^\circ\text{C} & \ 45 \text{ sec} \\
72^\circ\text{C} & \ 7 \text{ min}
\end{aligned}
\]

\[
\{ 35x \}
\]
### 8.3 TESCO sequence alignment

<table>
<thead>
<tr>
<th>SeqA Name</th>
<th>Len(nt)</th>
<th>SeqB Name</th>
<th>Len(nt)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>TESCO_human</td>
<td>1389</td>
<td>TESCO_dog</td>
<td>1502</td>
<td>78</td>
</tr>
<tr>
<td>TESCO_human</td>
<td>1389</td>
<td>TESCO_mouse</td>
<td>1293</td>
<td>62</td>
</tr>
<tr>
<td>TESCO_human</td>
<td>1389</td>
<td>TESCO_rat</td>
<td>1378</td>
<td>60</td>
</tr>
<tr>
<td>TESCO_dog</td>
<td>1502</td>
<td>TESCO_mouse</td>
<td>1293</td>
<td>42</td>
</tr>
<tr>
<td>TESCO_dog</td>
<td>1502</td>
<td>TESCO_rat</td>
<td>1378</td>
<td>43</td>
</tr>
<tr>
<td>TESCO_mouse</td>
<td>1293</td>
<td>TESCO_rat</td>
<td>1378</td>
<td>86</td>
</tr>
</tbody>
</table>

Result:

- **TESCO_mouse**: CATATGTCACATACCTAAGGTAAGGAATATACGAGCCCTGTCTAAATCAGAACGCCCAACCA 60
- **TESCO_rat**: CAGATGTCACATACCTAAGGTAAGGAATATACGAGCCCTGTCTAAATCAGAACGCCCAACCA 102
- **TESCO_human**: CAGATGTCACATACCTAAGGTAAGGAATATACGAGCCCTGTCTAAATCAGAACGCCCAACCA 102
- **TESCO_dog**: CAGATGTCACATACCTAAGGTAAGGAATATACGAGCCCTGTCTAAATCAGAACGCCCAACCA 259

# 185
<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>GAAC-TCGGACTGCGGTTGCATTTGGACTGGTAAATGTGGTCAGTCACATAGCAAGG---</td>
</tr>
<tr>
<td>Rat</td>
<td>GAGT-TTGGACTGTGGTTGCATTTGGGCTAGTAAATGTGGCCAGCCCCAAGCGAGG---</td>
</tr>
<tr>
<td>Human</td>
<td>GAAT-TAAGAATGAAGTTGCCCTTGGGTTGGTAAATTTGGGTAGTCGGATAGCACGC---</td>
</tr>
<tr>
<td>Dog</td>
<td>GGATGTAAAAATGCCATTGCCCTTGAGCTGGTCCATGTGGGTAGTTGTACAGCACGGCGG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>---CAGGACTC----AGACACT---------------GCA GAAATGCAC</td>
</tr>
<tr>
<td>Rat</td>
<td>---CAGGACTC----AGACACT---------------GCAAA AATGCACCA</td>
</tr>
<tr>
<td>Human</td>
<td>AGCCAGGCCTCTCCTAGCCGTTCAAGAAATGTTTGTGGAA TGAATGTGCACTG</td>
</tr>
<tr>
<td>Dog</td>
<td>AGCCAGGCCTCTCCTAGCCGTTCAAGAAATGTTTGTGGAATGACAGACCTTT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>GAA GGCTTGT TGACCCTGATAAAGCTTGTGG---CCCTTCTAGAAG-AGGTGT------</td>
</tr>
<tr>
<td>Rat</td>
<td>AAA GGCTTGT TGACGCTGATAAAGCTCATGGG--CTTTTCTAGAGG-AGGAGT------</td>
</tr>
<tr>
<td>Human</td>
<td>AAA GAGCAGAACATTGAGAAGTTGCTGACGCTTCCCCCACATCCTACCTCGCAGGAGTTTTAAC</td>
</tr>
<tr>
<td>Dog</td>
<td>AAA GAGCAGAACATTGAGAAGTTGCTGACGCTTCCCCCACATCCTACCTCGCAGGAGTTTTAAC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>ATCC----TTGTCCCACCTCCCACCT-----------CCA GCCTTCCTGGCTT-CCTGAG</td>
</tr>
<tr>
<td>Rat</td>
<td>ATTT----TTGCCCCACCTCCCACCT-----------CCAGC CTTCCTGGCTT-TCTGAG</td>
</tr>
<tr>
<td>Human</td>
<td>ATTTAAATTTTTTCATTGTTCTTGCCCTCCCACTCCCCTCACCTCTCGCAGGAGTTTTAAC</td>
</tr>
<tr>
<td>Dog</td>
<td>ATTTGAAGTCGTCATCATTCCTGTGC--------CCAGTAGG TTCCCTGGCTCACCTGAG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>AGCAATCTGTGCTCAGGGCCAGTCCACACAGTGTGCTACTGAGTTGAAATGACCTT-GTC-</td>
</tr>
<tr>
<td>Rat</td>
<td>AGCAATCTGAGCTCAGGGCCAGTCAGCACACAGTGTGCGACTGATCGAATGACCTT-GTC-</td>
</tr>
<tr>
<td>Human</td>
<td>AGGGGCCTGAGCTCAGGGAAAGTCAATAAAATATATTCTTGAATAGATCGTTCCTTT</td>
</tr>
<tr>
<td>Dog</td>
<td>AGGGAATCTGAGCTCAGGGAAAGTCAATAAAATATATTCTTGAATAGATCGTTCCTTT</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>--CTCTCGAACTCCCCTGTCCCCATTCAGTACACCATTGTTC TGCAATCTCCACCAGCAT</td>
</tr>
<tr>
<td>Rat</td>
<td>--CTCTTGAACTCTCCTGTCCCCATTCTGTACACCATTGTTC TTCAGTATCCACCAGCAT</td>
</tr>
<tr>
<td>Human</td>
<td>TTTTCATGAAGTCCCCTGTCCTCATTCTGTCTAT-ATTCACTGATAGCAATCTTGAGC</td>
</tr>
<tr>
<td>Dog</td>
<td>--CTCTGGAAATCTGCTGTCCTTATTCTGT--AT-ATTCACTGATAGCAATCTTGAGC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>TGGTTCAAGGACCCTCTCTATAGCTACAAAAGTCCAGGGACCCCAAGTCTCATATAAAACA</td>
</tr>
<tr>
<td>Rat</td>
<td>TGGTTCAAGGACCCTCTCTATAGCTACAAAAGTCCAGGGACCCCAAGTCTCATATAAAACA</td>
</tr>
<tr>
<td>Human</td>
<td>T-GCCCTCACCCCATCCCTTGCTCTAGCTCAATAAAAAAC</td>
</tr>
<tr>
<td>Dog</td>
<td>TGGCCCATTTACCCCCACTTCTTCATATAGCTCAATAAAAAAC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organ</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>C 1293</td>
</tr>
<tr>
<td>Rat</td>
<td>C 1378</td>
</tr>
<tr>
<td>Human</td>
<td>-</td>
</tr>
<tr>
<td>Dog</td>
<td>-</td>
</tr>
</tbody>
</table>
8.4 TESCO deletion constructs

TESCO deletion constructs

| C7  | GTAAATGTGG TCAGTCACAT AGCAAGGCAG GACTCAGACA CTGCAGAAAT GCAC TGCCCT | T/C A A G G T/C C G/A |
| C6  | TGGCTGGCC TTTCTCTCTC TTACCTTTTT ATTCAAAGTT TCCAACACAC AAAGCGCTTG |                                   |
| C5  | GTGCAGGAGT TCCCAGGGTC AAACACAAGT GCCTGGCTTC TTGGTGAGAG GAATTAGACA |                                   |
| C4  | AGGAAGGGCC TTGCTCCCCAG GAACCGAAAA CCCCCCACCC CCACCTCCCG TGCCCAATCA |                                   |
| C3  | AGAAGAGTCGC AAGGATCTTC GAAACATCCT CTTACACATT CTGGGTATGT TTGCAGCTGG |                                   |
| C2  | GCAGCCCTGG ATCAGCAGGG GACCTGTGGA GCTTTCCAAG ACTCTGCGGT TTAGAGTTTG AGTGAGCTTG |                                   |
| C1  | GTGGCTGGCC TTTCTCTCTC TTACCTTTTT ATTCAAAGTT TCCAACACAC AAAGCGCTTG |                                   |

**XXX SF1 binding sites**

| SF1 binding sites | T/C A A G G T/C C G/A |
| XXX SOX binding sites | A G A A C A A T G G |
| XXX FOXL2 core binding site | G/A T/C C/A A A C/T A |
Bibliography


