CONDITIONAL STRATEGIES TO STUDY GENE FUNCTION
DURING GONADAL DEVELOPMENT IN MAMMALS

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for the Degree of Doctor of Philosophy

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To my parents,

Moliu, Tin Tin,

Babe, and Angelina
DECLARATION

I declare that this thesis represents my own work, except where due acknowledgement is made, and that it has not been previously included in a thesis, dissertation or report submitted to this University or to any other institution for a degree, diploma or other qualification.

Signed

ANGELA J. TYE
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ABSTRACT

Sexual development in mammals involves a complex cascade of genetic events. These begin with a cell fate decision, whether to make Sertoli or follicle cells, that gives rise to the development of a male or female gonad, which is controlled by the testis-determining gene \textit{Sry}. Following the expression of \textit{Sry}, genes involved in the male pathway act to reinforce and maintain testis-specific cell fate decisions, as well as to repress the female pathway. \textit{Sox9} becomes rapidly upregulated after the onset of \textit{Sry} expression, and is expressed in Sertoli cells throughout life. From mutation studies, SOX9 is known to be essential for male development in humans and to initiate Sertoli cell differentiation in mice. However, the function of SOX9 after sex determination and the reason for its maintenance in Sertoli cells remains unknown.

In order to understand the function of \textit{Sox9} in the fetal and adult mouse testis, new tools have been generated to control gene activity in a conditional manner. This thesis mainly describes strategies to control either deletion of misexpression of \textit{Sox9}. To make the tools useful at different stages, the tamoxifen-inducible Cre/loxP system was employed. This involves the establishment of two elements: a “Cre-driver” and a “\textit{Sox9}-responder”. Cre-driver transgenes were made under the control of several gonadal-specific regulatory elements, as well as a strong, ubiquitous promoter. Responder mice allow Cre activated conditional misexpression or deletion of \textit{Sox9}. Analyses on gonad morphologies and gene expression levels were compared between animals that have altered \textit{Sox9} expression and those that have not. The results reveal that \textit{Sox9} is necessary and sufficient for the expression of \textit{Stf1} in the Sertoli cells, and suggest that \textit{Sox9} is antagonistic to the ovarian-specific gene \textit{Foxl2}.

The newly established Cre-drivers can also be applied in functional studies involving other genes implicated in sexual development.
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List of Abbreviations

$^\circ C$  
degree Celsius

%  
percent or percentage

$3\beta$HSD  
3-beta-hydroxysteroid dehydrogenase

$\beta$  
beta

$\beta$-geo  
beta-galactosidase neomycine fusion protein

$\gamma$-H2AX  
gamma-histone protein

$\Delta$  
flxed-out or deletion of allele

$\mu$  
micro

$\mu m$  
micrometre

AMH  
Anti-Mullerian Hormone

Bmp  
bone morphogenetic protein

bp  
basepair

BPES  
Blepharophimosis-ptosis-epicanthus inversus syndrome

BSA  
bovine serum albumin

CD  
campomelic dysplasia

cDNA  
complementary deoxyribonucleic acid

Ck  
cytokeratin

$cm^2$  
square centimetre

CMF  
calcium-magnesium free

CMV  
Cytomegalovirus

Cre  
Causes recombination

Cyp  
cytochrome P450 super family

DAPI  
di-amino-phenyl-indol

DAX1  
dosage-sensitive sex-reversal-adrenal hypoplasia congenital-critical region of the X chromosome gene 1

dd  
double distilled

DDS  
Denys-Drash-Syndrome

DMC-1  
dosage suppressor of mck-1 homolog, meiosis -specific homologous recombination (yeast)

DMEM  
Dulbecco’s Modified Eagle's Medium

DMRT1  
Doublesex an mab-3 related transcription factor 1

DNA  
deoxyribonucleic acid

DSS  
dosage sensitive sex-reversal

E  
embryonic day

ECFP  
enhanced cyan fluorescent protein

EDTA  
ethylene-di-amine-tetra-acetate

EGFP  
enhanced green fluorescent protein

ERT  
estrogen receptor for tamoxifen

ES  
embryonic stem

EYFP  
enhanced yellow fluorescent protein

FBS  
fetal bovine serum

FGF  
fibroblast growth factor

FGFR  
fibroblast growth factor receptor

Flox  
flanked by locus of cross-over P1
<table>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SF1</td>
<td>steroidogenic factor 1</td>
</tr>
<tr>
<td>SOX</td>
<td>Sry-like high motility group box</td>
</tr>
<tr>
<td>SRY</td>
<td>sex-determining region on the Y chromosome</td>
</tr>
<tr>
<td>Stra8</td>
<td>stimulated by retinoic acid gene 8</td>
</tr>
<tr>
<td>TDF</td>
<td>testis-determining factor</td>
</tr>
<tr>
<td>TDY</td>
<td>testis-determining region on the Y chromosome</td>
</tr>
<tr>
<td>TG</td>
<td>(presence of) transgene</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase mediated deoxyribonucleate nick end labeling</td>
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<td>Volt</td>
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<td>wingless-type mouse mammary tumour virus integration site</td>
</tr>
<tr>
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<td>wildtype</td>
</tr>
<tr>
<td>Wt1</td>
<td>Wilm's tumor suppressor gene 1</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-glucopyranoside</td>
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<td>ZP3</td>
<td>zona pellucida glycoprotein 3</td>
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LITERATURE REVIEW

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1.1 Sexual reproduction

There are two types of reproduction, asexual or sexual. Asexual reproduction involves the generation of offspring from an individual organism and without involving different sex organs. As a consequence, the offspring are phenotypically and genetically very similar or identical to the parent. This can happen by binary fission as in bacteria, by budding as in yeast, or by parthenogenesis, as in some lower plants (see review Charlesworth, 2006a), some invertebrates (e.g. snail, Johnson, 2006) and some vertebrates including certain species of reptiles, amphibians (see review Charlesworth, 2006b) and fish (Itono et al., 2006), salamanders (Bi and Bogart, 2006) and even, on rare occasions, turkeys (Cassar et al., 1998).

However, during evolution most metazoan plants and animals, including apparently all mammals, have adopted sexual reproduction, which involves two different types of gametes. Male gametes, pollen or sperm, are small and motile and tend to be produced in large numbers, whereas female gametes, ovules or oocytes, are large and sessile, and relatively few are made. In multicellular organisms with separate sexes, the soma will differ according to which type of gamete they carry.

The gametes are derived from germ cells and the existence of two different types of gamete implies requirement of two different germ cell niches to nurture their distinct needs. These are the gonads, which begin to develop during the period of organogenesis in a mammalian embryo and are the first elements of the reproductive system to show a difference between males and females. The events that underlie sexual differentiation of the gonad are critical for the generation of functional gametes. Therefore, it is important to understand how the two
different types of gonad are formed and how this relates to their function. During gonadal differentiation, a cascade of events happens at the molecular level to direct the formation of either testes in the male or ovaries in the female. Subsequently, sexually dimorphic internal and external genitalia develop to give a functional male or female reproductive system. Sexual development also involves the development of other sexual characteristics, which largely depend on secretion of sex-specific hormones, but these are all events downstream of the formation of the gonads.

The development into a male or female depends on a specific genetic signal, which becomes important mid-way through gestation in the mouse or at about 6 weeks in humans. This process is called sex determination. Almost immediately after sex determination, sexual differentiation begins in the gonad, allowing translation of the genetic sex into formation of different sex organs.

1.2 The biology of sexual development in mammals

To understand what happens at molecular and cellular levels, we must first understand the biology of urogenital development. In the following sections, the steps that lead to the formation of a complete sex organ will be described.

In the mammalian embryo, the gonad arises from the presumptive urogenital tract, which comprises both the excretory and the genital systems and is situated at the lower posterior part of the embryo (Figure 1.1A). The excretory system develops from three primordial kidneys (in Latin: nephros), pronephros, mesonephros, and metanephros, which develop
respectively in a consecutive manner. In the mouse, the nephric primordium first appears between 11-13 somites stage. From the pronephros, caudal extension of condensed intermediate mesoderm separates from the dorsal mesoderm and gives rise to the nephrogenic ridge, which in turn gives rise to the mesonephric nephrons and tubules. On the other hand, several canals project from the coelomic epithelium to the nephrogenic ridge. The Wolffian duct forms from the intermediate mesoderm at around embryonic day (E) 9.0 in mice, in craniocaudal succession. It initially forms as an extension of the pronephric duct in the region of the future forelimb buds, and grows caudally (Torrey, 1945; also see review Hannema and Hughes, 2006). Between E11.5 and 12.5, the Müllerian ducts form in parallel to the Wolffian ducts. They are paired longitudinal tubes that arise by caudal extension of an initial invagination of coelomic epithelium at the rostral end of the mesonephros, which are then surrounded as they grow by mesenchymal cells originating from overlying coelomic epithelium (Guioli et al, 2006). Later on, the Wolffian duct will give rise to the epididymis, \textit{vas deferens} and seminal vesicles in the male; whereas in the female the Müllerian ducts give rise to the uterus, oviducts and the upper portion of the vagina (Figure 1.1B).

1.3 \textbf{Sex-determination and differentiation in mammals}

Sex determination is the process by which a bipotential gonad takes up a genetic pre-decision to develop into either a testis or an ovary. The earliest morphological event in the process leading to sex determination in the mouse happens around E9.75, when the genital ridges first arise as a thickening along the inner surface of the mesonephros (Figure 1.2). Research findings support the theory that the gonadal primordium originates from cells from both the coelomic epithelium and the mesonephros (Byskov, 1986; Martineau \textit{et al.}, 1997; and see
also Sekido and Lovell-Badge, 2006). As there is no difference between XX and XY gonads at this stage, the genital ridge is often described as “indifferent” or “bipotential”, which refers to its ability to develop either into a testis or an ovary. Shortly after the genital ridges are formed, sex determination occurs.

![Diagram of developing gonads](image)

**Figure 1.1**

(A) Schematic presentation of the developing gonads.

(B) Development of the nephrogenic ducts: in the male, Anti-Müllerian Hormone produced by Sertoli cells induce Müllerian ducts regression, whereas testosterone produced by Leydig cells promote development of the Wolffian ducts, later on into the epididymis, seminal vesicles, and vas deferens. In the female, the Müllerian ducts give rise to the uterus, oviducts and the upper portion of the vagina.
Our knowledge of mammalian sex determination is based on two main areas of study. The first one is the characterization of the biological events that determine the sexual development of the individual, including patterns of gene expression. The second one is based on the study of genetic mutations in humans and mice that lead to abnormal sexual phenotypes (Reviewed by Swain and Lovell-Badge, 1999; Nikolova and Vilain, 2006). In the following sections of this chapter, I will discuss the major known events that occur during sexual development in mammals and their cellular and genetic contexts.

![Diagram](image)

**Figure 1.2** Molecular events that happen during mammalian sex determination.
1.4 Mammalian sexual differentiation: the cellular context

1.4.1 Gonadal cell lineages: Somatic cells

Somatic cells in the gonads include 5 main cell types (Figure 1.3A). The supporting cells, which are called Sertoli cells in the male and follicle, or granulosa cells in the female, form the environment for the germ cells to reside within the adult organ. The supporting cells are important for gonadal maintenance and germ cell survival in both sexes, and all available evidence suggests they have a common precursor (Albrecht and Eicher, 2001; Ito et al., 2006). Other somatic cell types include the steroidogenic cells, which are called Leydig cells in the male and theca cells in the female, which produce sex-specific steroid hormones, and the endothelial cells of the vasculature. Finally, there are the peritubular myoid cells (PMCs), which are testis–specific and together with Sertoli cells form the epithelial testis cords (Figure 1.3B).

Of all the somatic cell types in the developing testis, it is the Sertoli cell lineage that is the most critical for testis differentiation in mammals. Studies of chimaeras made by aggregating early XY and XX embryos indicate that if the early Sertoli cell number in a gonad reaches a threshold (about 25% of all supporting cells), they are able to recruit all other cell types in the gonad, whether XX or XY, towards the testicular pathway (Palmer and Burgoyne, 1991). Anti-Müllerian hormone (AMH) is a TGFβ-related factor secreted by Sertoli cells, and is important for Mullerian duct regression. It is male-specific in the embryo, but is also made by follicle cells in the postnatal ovary (Munsterberg and Lovell-Badge, 1991; Arrango et al., 1999, Salmon et al., 2005).
It has been reported that Sertoli cells have different origins in different vertebrate species. In the mouse, Sertoli cells originate from cells that have migrated from the coelomic epithelium (Karl and Capel, 1998) whereas those in the chick are derived from the nephrogenic mesenchyme (Sekido and Lovell-Badge, 2006). Three events take place to give rise to Sertoli cells: (1) proliferation, (2) migration and (3), differentiation. In the mouse, precursors...
of Sertoli cells form between E10.5 to E12.5, in response to the expression of a transcriptional signal from the Y chromosome (Koopman et al., 1991). Before arranging into testis cords, these cells are termed pre-Sertoli cells. During differentiation, pre-Sertoli cells probably signal back to the coelomic epithelium to recruit more cells into the developing testis, which then give rise to more pre-Sertoli cells (Martineau, 1997; Brennan et al., 1998; Sekido et al., 2004). The establishment of the (pre-)Sertoli cell population then influences the development of all other cell lineages in the gonad. A number of reports have suggested that cell migration from the mesonephros and coelomic epithelium into the XY genital ridge is essential for cord formation (Buehr et al., 1993; Merchant-Larios et al., 1993; Martineau et al., 1997). PMCs and endothelial cells migrate into the gonad from the mesonephros, probably also in response to signals from Sertoli cells. PMCs, possibly the only cell lineage unique to the testis, participate with Sertoli cells to form the epithelial testis cords (Martineau et al., 1997; Tilmann and Capel, 1999). The endothelial cells contribute to the characteristic vasculature of the testis, which is thought to be important to support rapid growth of the testis in comparison to the ovary, and to allow efficient transport of hormones, which masculinise the remainder of the embryo.

The origin of steroidogenic cells in the mouse is not known. They are likely to have populated the genital ridge by E11.5 and differentiate into Leydig cells later on (Figure 1.3; Morohashi 1997). It has been shown recently that Leydig cells can survive in gonads depleted of Sertoli cells (Gao et al., 2006), indicating that these cell lineages are maintained by different factors. Leydig cells secrete two male-specific hormones, testosterone and insulin-like factor 3 (INSL3). These are important to the formation of secondary sexual characteristics in the male.
The differentiation of the ovary appears relatively quiescent. There is no obvious tissue remodelling, no complex tubule and vascular structures, and the localization of the different somatic cell types within the early XX gonad is not obvious. However, changes are occurring at a molecular level during these early stages, even though the mechanisms directing ovarian development are still poorly understood (see below). The first morphological change during ovarian differentiation in the mouse appears at around E13.5, when the germ cells progress into meiosis (Byskov, 1981). Subsequently, the follicle cells aggregate around the germ cells (oocytes) to form follicles (Torrey, 1945). The follicle cells, or granulosa cells, belong to the same lineage as the Sertoli cells in the testis. These cells produce receptors for the follicle-stimulating hormone (FSH), a hormone produced by the anterior pituitary. FSH stimulates the granulosa cells to produce estrogen, with the help of theca cells, the steroidogenic cells in the ovary, which forms the outer layer of the follicle. Proliferation of, and hormone synthesis by, theca cells is stimulated by luteinising hormone, a gonadotrophin produced by the anterior pituitary (Vander et al., 1990).

1.4.2 Gonadal cell lineages: Primordial germ cells (PGCs)

Germ cell competence in mice is induced at E6.5 in epiblast cells by signals emanating from the extraembryonic ectoderm (Lawson et al., 1999). PGCs are first specified at approximately E7.2. They appear as clusters just posterior to the definitive primitive streak in the extraembryonic mesoderm. Later on, these cells move back into the embryo proper, to the mesoderm of the primitive streak and then to the endoderm (Ginsburg, 1990). They migrate along the hind gut and dorsal mesentery and then into each mesonephros, entering the genital ridges as they form. By the time of sex determination, the PGCs are already
within the genital ridges. By E12.5, the PGCs are packed inside the cords in the developing testis, and gather in small clusters that are uniformly distributed across the ovary (Fox et al., 1981).

Germ cell migration is not a sex-specific process. Differentiation of germ cells along either the male or female pathway does not depend on the sex chromosome constitution of the germ cell, but instead depends on the somatic environment of the gonad, which forms the germ cell niche (McLaren and Monk, 1981; Palmer and Burgoyne, 1991). In the mouse, germ cell identity is established around E12.5, as somatic differentiation of the gonad becomes morphologically dimorphic (McLaren, 2003, also see review Swain, 2006). Germ cells in the XX gonad undergo an anterior-to-posterior wave of differentiation that lasts approximately from E12.5 to E16.5 (Menke et al., 2003), giving rise to oogonia, which arrest in the last phase of the meiotic prophase, the diplotene stage. They then become surrounded by granulosa cells and a basal lamina, to form the follicle around each germ cell, now referred to as an oocyte. Postnatally, a subset of oocytes will be grown and upon ovulation, the oocyte (now termed secondary oocyte) resumes meiosis, completing the second meiotic division after fertilization (Peters, 1978, Figure 1.4).

At around E13.5, male germ cells arrest in mitosis, rather than entering meiosis (McLaren and Southee, 1997). These prospermatogonia resume mitosis a few days after birth, then shortly before puberty, a subset, now named spermatogonia, begin the first meiotic wave, becoming spermatocytes (Figure 1.4). In the mouse, this happens around postnatal day 10-14. Subsequently, spermatocytes undergo meiotic divisions, giving rise to spermatids, which differentiate into spermatozoa, carrying either the X or Y chromosome (Moens, 1987), and
one set of autosomes. The entry of a spermatozoan into a secondary oocyte restores the
diploid state of the zygote and subsequent fetus. As the spermatozoan carries either an X or
Y chromosome, the sex of the newly conceived embryo is pre-decided by which sex
chromosome the spermatozoan carries.

Figure 1.4  Gamatogenesis: (A) Spermatogenesis; (B) Oogenesis
1.5 Genes involved in mammalian gonadal development

Sexual development in mammals is tightly controlled by gene function. As discussed earlier, there are three major steps involved: the formation of the gonadal primordia, sex determination, and sexual differentiation. The initiation of each step and the progression into the next one is coordinated by genes that are expressed at the relevant stage. In this section, genes will be discussed according to the stage(s) when their function becomes important.

1.5.1 The formation of the gonadal primordia

The formation and maintenance of the gonadal primordia is an important process that sex determination in mammals depends upon. Several genes have been shown essential for the development and survival of the genital ridge.

Steroidogenic factor 1

*Sf1* is an example of a gene that plays roles at multiple stages and in many cell types during gonadal development. In mice, *Sf1* expression is detected around E9.0 in the cells of the coelomic epithelium. By E12.5, following sex determination, *Sf1* is downregulated in the ovary, whereas in the testis SF1-expressing cells differentiate into Sertoli and Leydig cells. At later stages, *Sf1* plays important roles in regulating steroidogenesis in both sexes.

*Sf1* encodes an orphan nuclear receptor with a highly conserved, typical zinc finger DNA-binding domain (Lynch *et al.*, 1993). Other than the developing gonad, it is also expressed in
cells in the adrenal gland, hypothalamus and pituitary.

Sfl null mutant mice lack both adrenal glands and gonads, and die by postnatal day 8 due to adrenal insufficiency (Luo et al., 1994). The gonads start to form but fail to give either ovaries or testes, undergoing instead massive apoptosis by E12.5. As XY gonads do not produce testosterone or Anti-Müllerian Hormone, Wolffian ducts do not differentiate and there is no Müllerian regression. As a result, both XX and XY Sfl" mice have female internal and external genitalia.

Wilm's tumor suppressor gene 1 (Wt1)

Wt1 encodes zinc finger protein that regulates gene transcription, and has at least 24 isoforms (Menke et al., 1998; Lee et al., 1999). The most commonly studied isoforms, + and -KTS, are splice variants resulting either in an insertion or an omission of the KTS amino acid motif between zinc fingers three and four. These isoforms are highly conserved amongst vertebrates (Kent et al., 1995, Miles et al., 1998). Mutations of the KTS domain lead to two developmental syndromes in humans (Gubler et al., 1999). Denys-Drash-Syndrome (DDS) patients display urogenital abnormalities, whereas Frasier patients display male-to-female sex reversal.

In mice, transcription of Wt1 in the genital ridge begins at E9.5 within the coelomic epithelial cell layer. Following sex determination, Wt1 is expressed in Sertoli cells in the testis, and (pre-)granulosa cells in the ovary (Armstrong et al., 1993).
$Wt1^{-/-}$ mutants were found not to express $Sfl$ and fail to develop kidneys, gonads and adrenal glands. These mice die after birth due to kidney failure (Kreidberg et al., 1993; Moore et al., 1999).

To investigate the function of $Wt1$ during gonadogenesis, mouse models were developed based on observations in human patients (Hammes et al., 2001). “KTS” mice were developed as a model for DDS patients. These mice are ablated for the $-KTS$ isoform of WT1, and the gonadal primordia undergo apoptosis by E11.5. It has been demonstrated that the $-KTS$ isoforms associate and synergize with SF1 to promote male-specific gene expression (Nachtigal et al., 1998). Analyses of WT1-binding sites within an $Sfl$ gonadal specific promoter demonstrated that the $-KTS$ isoform can bind and activate the $Sfl$ promoter (Wilhelm and Englert, 2002). This indicates that $Wt1$ may regulate development of the bipotential gonad via activating $Sfl$, and therefore gonadal defects observed in KTS mice and $Sfl$ null mice were similar.

$LIM$ class homeobox gene 9

$LIM$ class homeobox gene 9 ($Lhx9$) also functions in close relation to $Sfl$. $Lhx9$ is detected in the epithelial cells of the mouse bipotential gonad by E9.5. After sex determination, $Lhx9$ transcripts were localized in the interstitium in the differentiating testis (Birk et al., 2000).

Somatic cell proliferation in the genital ridge is greatly reduced in mice lacking $Lhx9$ (Birk et al., 2000), with $Sfl$ transcription reduced to minimal (Wilhelm and Englert, 2002). This results in the failure to form the bipotential gonad and to express testosterone and $Amh$ in XY
embryos. Therefore, XX and XY \(Lhx9^{-}\) mice have internal and external female genitalia, a phenotype similar to that of \(Sf1\) (Birk et al., 2000).

1.5.2 Sex-determination: cell fate decisions

With respect to cell fate choices, sex determination can be divided into three different phases: (1) the operation of the genetic switch that triggers the initiation of cell fate decisions; (2) initiation of sex-specific cellular activity, and (3) reinforcement of cell fate decision. A current model of genetic pathways during this process is schematically presented in Figure 1.5.

![Figure 1.5: Schematic diagram presenting the current model of genetic pathways during early gonadogenesis. Note: the location of genes in this diagram refers to when they become upregulated and exerting function but not correspond to the time they first become expressed in the urogenital ridge.](image-url)
Amongst all gonadal cell lineages, the supporting cells are the first to adopt a sex-specific identity. Upon sex determination, supporting cell progenitors will differentiate into either Sertoli cells in the testis, or granulosa cells in the ovary. The fate of other lineages will follow that of supporting cell precursors. Steroidogenic cells differentiate after specificity of the supporting cells have been established, and secrete sex-specific hormones. In the absence of a masculinised environment, germ cells in the XX gonad adopt the female identity and start to enter meiosis at around E13.5, whereas germ cells in the XY gonad arrest in mitosis.

**Stage I: Sry – the Sertoli cell fate switch**

In the search of the testis-determining factor (TDF), efforts have been made on looking for the minimal region of the human Y chromosome that led to development of XX males. By looking within this region for sequences that were conserved on the Y chromosome of other mammals, with the mouse being special importance, few genes were put forward as the TDY candidates but eliminated. A more focused search using two sex reversing mouse models, XXSxrb males and XYtdym1 females, led to the discovery of Sry (Sex-determining region on the Y chromosome, Gubbay et al., 1990; Sinclair et al., 1990). In humans, the discovery of de novo point mutations in SRY in XY sex-reversed females made it highly likely that SRY was TDY (Berta et al., 1990). In mice, evidence equating Sry with Tdy came from mutation studies, notably XY females carrying an 11kb deletion of Sry (Gubbay et al., 1992). More critically, transgenic experiments, in which XX mice bearing an Sry transgene developed into normal, albeit infertile males (Koopman et al., 1991). This result showed that Sry was the only gene on the Y chromosome required for testis determination, and that Sry alone was sufficient to drive the formation of testes on an XX background.
SRY is the founder member of the SOX (Sry-like HMG box) gene family, members of which possess an HMG box DNA-binding domain, implicating them as transcription factors (Gubbay et al., 1990). SRY exhibits poor or even no conservation outside the HMG box among different mammalian species (Whitfield et al., 1993; Marshall Graves, 2002). Sry has a narrow expression ‘window’, it is detected shortly after the bipotential gonad is formed, specifically in supporting cell precursors in the XY genital ridge. Sry is expressed in a dynamic wave that emanates from the central and/or anterior regions of the genital ridges. This wave extends subsequently to both poles and ends in the caudal pole, and does not affect coelomic surface epithelial cells, suggesting that their proliferation is not dependent on Sry (Bullejos and Koopman, 2001). A peak of Sry expression occurs at E11.5, which coincides with increased proliferation within the coelomic epithelium and the migration of cells from the coelomic epithelium. Twenty-four hours after Sry peak of expression, transcripts are no longer detectable indicating that Sry must initiate Sertoli cell differentiation during this critical period (Hacker et al., 1995). It has been shown that all cells that once expressed Sry would commit to Sertoli cell fate (Sekido et al., 2004).

A few genes have been suggested in the involvement of upregulating Sry. A mouse model has been developed based on Frasier syndrome. Frasier mice are ablated for the WT1 +KTS splice variant, and displayed male-to-female sex-reversal resembling the phenotype seen in humans. Further examination revealed a reduction in Sry transcription, indicating that the WT1 +KTS splice variant is required to upregulate Sry. However, it was not mentioned in the report whether the cell number has been affected in this mutant. WT1 is known to cause apoptosis in somatic cells in the genital ridges (Loeb, 2006), and therefore the reduction in Sry transcription observed might be due to the loss of cells expressing Sry instead of an effect of
Wt1 directly regulating Sry expression. SF1 has been reported to bind and trans-activate the Sry promoter in vitro (Shimamura et al., 1997).

By E12.5, Sry transcripts are no longer detectable in the mouse testis. This suggests that Sry acts as a molecular ‘switch’ to specify Sertoli cell fate, but is not required for mediating testis differentiation. It is therefore reasonable to think that genes downstream of Sry will play maintenance roles to reinforce and stabilize the initial cell fate decision.

**Stage II: Sertoli cell proliferation and maintenance**

**Sox9 – Critical for Sertoli cell proliferation and testis cord formation**

Amongst the factors identified to be upregulated by Sry, the transcriptional factor Sox9 (Sry-like HMG box gene 9) is the most extensively investigated. Sox9, together with Sox8 and Sox10, belongs to the SoxE group of the Sox gene family. The Sox9 gene is highly conserved and displays testis-specific up-regulation in all vertebrates examined to date (Morais da Silva et al., 1996; Kent et al., 1996; Kobayashi et al., 2005). Sox9 is expressed at low levels in the mouse bipotential gonad. It is rapidly upregulated in the supporting cells of the XY genital ridge by the time Sry expression reaches its peak (Morais da Silva et al., 1996; Kent et al., 1996, Sekido et al., 2004). Unlike Sry, Sox9 expression persists in the sex cords of the testis throughout life. In the XX, Sox9 is down-regulated following sex determination (Morais da Silva et al., 1996) (Kent et al., 1996). Without Sox9, or with its level lower than a certain threshold, Sertoli cells either do not begin to differentiate or fail to maintain differentiation (Morais da Silva et al., 1996; Chaboissier et al., 2004).
In humans, heterozygous mutations of SOX9 cause campomelic dysplasia (CD), a pleiomorphic syndrome characterized by abnormal bone development. In 75% of cases, XY individuals develop as females (Foster et al., 1994). Duplication of Sox9 has also been shown to cause testis development in an XX patient (Huang et al., 1999). In contrast, testis development in mice harbouring heterozygous Sox9 deletion progress normally despite perinatal lethality (Bi et al., 2001), indicating that testis determination in humans is more sensitive to the dosage of the gene. Sox9 null mutation obtained through conditional gene targeting lead to absence of sex cord development and expression of female markers in XY gonads (Chaboissier et al., 2004, Barrionuevo et al., 2006). On the other hand, ectopic expression of Sox9 in the XX genital ridge leads to male development despite the absence of Sry (Vidal et al., 2001; Qin et al., 2004). These findings show that Sox9 can substitute Sry function in testis determination.

**Stage III: reinforcing the cell fate decision**

Several studies lead to a model for the regulation of Sox9 expression during gonadal development (Figure 1.5).

*Fibroblast growth factor 9 (Fgf9)*, an extracellular signalling molecule, was proven essential to maintain Sox9 expression and therefore Sertoli cell differentiation (Reviewed by Kim and Capel, 2006).

Fgf9 transcripts were first detected at E11.5 in the Sertoli cell precursors of the XY gonad (Colvin et al., 2001), and may act as an autocrine factor to maintain Sox9 expression.
However, it may also act as a paracrine factor in neighbouring cells. FGF receptor 2 (FGFR2) is the principle receptor for FGF9. In the developing gonad, FGFR2 displayed sexual dimorphic sub-cellular localization. While it was detected in the cytoplasm of ovarian somatic cells, FGFR2 becomes nuclearly localized in the XY gonad by E12.5, coinciding with the nuclear translocation of both FGF9 and SOX9 (Schmahl et al., 2004).

*Fgf9* is one of the signalling molecules identified so far that can cause complete sex reversal in null mutant mice. Organ culture of *Fgf9<sup>−/−</sup>* XY mutant gonads demonstrates testis cord formation failure in proliferation of Sertoli cell precursors (Colvin et al., 2001; Schmal et al., 2004; Kim et al., 2006). Studies on genetic interactions between *Fgf9*, *Sox9* and *Sry* revealed the importance of *Fgf9* during early stages of Sertoli cell specification. Although *Sry* activity and the initial upregulation of *Sox9* in Sertoli cells of XY *Fgf9<sup>−/−</sup>* mice are not affected, *Sox9* expression could not be maintained. As a result, Sertoli cells undergo cell fate transition causing sex reversal (Kim and Capel, 2006).

WT1 may be required for continued expression of *Sox9*. *Wt1* null mice and -KTS mice both show an early failure of gonadal development, whereas Frasier mice display sex reversal (Kreidberg et al., 1993; Hammes et al., 2001). Studies on *Wt1* function after sex determination therefore have to rely on mutant mice generated through conditional deletion. *Amh-Cre* mice were employed to conditionally remove exons 8 and 9 of *Wt1*. Mice harbouring only one functional *Wt1* allele have lost the majority of Sertoli cells by E15.5, and also Sertoli cell markers SOX8, SOX9 and AMH (Gao et al., 2006). This suggests that continual *Sox9* expression in the developing testis relies directly or indirectly on *Wt1*, and is
independent of WT1's putative function in upregulating Sox9, as shown in the -KTS mouse model (Hammes et al., 2001).

Sox8 belongs to the same group of SOX protein-encoding genes as Sox9 and functions in close relation with Sox9 during testis determination. In vitro data showed that SOX8 can bind and activate promoters of SOX9 downstream targets including Amh (Schepers et al., 2003, see below). Moreover, an attempt to use an Sfl-Cre driver to conditionally delete Sox9 in vivo failed to give complete XY sex reversal, whereas introducing one null allele of Sox8 into the same background, to give, in theory, supporting cells that would be Sox9<sup>−/−</sup>; Sox8<sup>+/−</sup>, lead to development of ovaries (Chaboissier et al., 2004). This could mean that SOX8 and SOX9 have an additive role to give Sertoli cell differentiation in the mouse. However, it seems that the Sfl-Cre driver that was used probably acts too late and/or incompletely to delete Sox9 from the early gonad, and there was evidence that Sox8 expression depends on SOX9, suggesting instead that Sox8 serves as a backup mechanism for Sox9 function, to reinforce testis determination and differentiation (Chaboissier et al., 2004).

**Mini-summary on testis determination**

Sry is required to switch supporting cell fate from that of follicle cells to Sertoli cells, characteristic of the testis, and is sufficient to initiate the male pathway. Although Sry is normally required to do this, in certain circumstances it is, however, not essential for testis determination, because Sox9 has been shown able to substitute for Sry function during testis determination. More importantly, Sox9 is required for building up the Sertoli cell population, which is a crucial event during early stages of testis development. On the other hand, Fgf9
functions to maintain SOX9 levels and cell proliferation. Without a threshold number of Sertoli cells, testis cords will not form and the testicular pathway will not be maintained.

Other than \textit{Sry} cell-autonomously switching cell fate, the paracrine-signalling molecule prostaglandin D2 (PGD2) has been postulated to induce Sertoli cell differentiation. PGD2 has been shown able to partially masculinise XX gonads in culture, probably by inducing supporting cells in the ovary to differentiate into Sertoli cells (Adams and McLaren, 2002). Recent studies suggested a possible role of PGD2 to induce SOX9 nuclear translocation in the XY gonad (Malki \textit{et al}., 2005), a sex-specific process that is important in the male sex determination cascade (Argento \textit{et al}., 2003; Smith and Koopman, 2004). PGD2 was further proposed to be sufficient in upregulating \textit{Sox9} expression in cells that did not express \textit{Sry} (Wilhelm \textit{et al}., 2005), suggesting a likely backup candidate to ensure efficient Sertoli cell differentiation, which becomes activated when cells destined to the Sertoli cell lineage failed to differentiate.

\textbf{An ovarian determinant – does one exist or is it “when \textit{Sry} is absent”?}

As \textit{Sry} acts as the genetic switch towards testis development, its absence will allow the network of transcription factors and extracellular signalling in the bipotential gonad to proceed to female development. The first significant cellular event in female differentiation happens at E13.5, when germ cells enter meiosis. However, before this happens, genetic activities in somatic cells have already adopted the female pattern and cell fate decisions appear to have been taken. Several attempts have been made to identify genes that actively promote ovarian development, or at least act as anti-testis genes. These would counteract the
idea that ovarian development is a passive pathway. This section focuses on several candidates that have proposed to be ovarian determinants.

\textit{Dax1 (Dosage-sensitive sex-reversal-adrenal hypoplasia congenital-critical region of the X chromosome gene 1)}

\textit{Dax1} is an unusual member of the nuclear hormone receptor superfamily, with an expression pattern that overlaps that of SF1 in various tissues, including the gonad, adrenal, hypothalamus and pituitary (Ikeda et al., 1996). Indeed it is thought to physically interact with SF1 and to recruit co-repressors to the complex, thereby modulating SF1 transcriptional activity (Ikeda et al., 1994). \textit{Dax1} is expressed in the bipotential gonad at the same time as \textit{Sry}, and becomes specific to ovarian somatic cells shortly after E12.5 (Swain et al., 1996, Bouma et al., 2004). DAX1 expression persists in ovarian somatic cells throughout life.

\textit{Dax1} is an X-linked gene that is involved in mammalian sex determination. In XY humans, loss of DAX1 does not affect male development, however, duplication of a genomic region encompassing DAX1 can cause male-to-female sex reversal (Bardoni et al., 1994), suggesting that DAX1 is the gene responsible for dosage sensitive sex reversal (DSS). The function of \textit{Dax1} during sex determination in mice is controversial: while its overexpression is able to antagonize \textit{Sry} function and can cause XY female sex reversal (Swain \textit{et al.}, 1998), \textit{Dax1} was also reported to be important for upregulating \textit{Sox9} in Sertoli cells, and therefore for cord formation (Bouma \textit{et al.} 2004), although only on specific genetic backgrounds. The function of \textit{Dax1} during testis/ovary differentiation will require further studies using conditional transgenesis.
Daxl transcription could be enhanced by the addition of β-catenin in vitro, indicating that a WNT signalling molecule might be involved in the upregulation of Dax1 in vivo. However, β-catenin-mediated upregulation of Dax1 occurred only in the presence of SF1 in a dosage-dependent manner (Mizusaki et al., 2003), indicating DAX1 functions through a cellular balance with SF1. The early expression of Dax1 has also been shown to depend directly on SF1 (Hoyle et al, 2002).

Wnt4

WNT4 is an extracellular signalling molecule and is conserved in all vertebrates (Hollyday et al., 1995). It is expressed in the mesonephros and bipotential gonad, but is downregulated in the XY gonad after Sry is upregulated and therefore becomes ovary specific (Vainio et al., 1999). A single point mutation within the WNT4 locus has been found in a human XX patient showing signs of masculinisation, while XX Wnt4" mice develop gonads with a mixed phenotype and with signs of androgen action on internal and external genitalia (Biason-Lauber et al., 2004; Vainio et al., 1999). Müllerian ducts were not formed, but this is due to an additional role of Wnt4 common to both sexes. The gonads display some male-specific characteristics, including coelomic vessels, and have Leydig-like cells that secrete testosterone. Although a very recent report suggests that there is transient up-regulation of Sox9 (Kim et al, 2006), testis cords do not form and there is no obvious differentiation of Sertoli cells, at least early on. The germ cells also do not follow the male pathway; instead oocytes are lost during mid-fetal stages (Vainio et al., 1999). This precedes the detection of Sertoli cell markers at perinatal stages, which was therefore attributed to a type of secondary sex reversal seen in a number of circumstances when germ cells are lost from ovaries after
they have begun to differentiate (e.g. Behringer et al., 1990; Morais da Silva et al., 1996). (If
germ cells are never present or are lost early from an XX gonad, this type of sex reversal is
not seen and the gonad simply forms a "streak" of connective tissue.)

The lack of Sertoli cell differentiation early on in Wnt4<sup>−/−</sup> XX mutants appeared to rule Wnt4
as the ovarian determinant. However, the data from Kim et al (2006), together with new work
that revealed a new candidate ovary determining gene, R-Spondin1, which is also involved in
the WNT signalling pathway (Parma et al, 2006), suggests instead a redundant mechanism
where Wnt4 is only one of several genes used to ensure ovary development in XX animals.

WNT4 acts to prevent the migration of endothelial cells from the mesonephros into the gonad,
forming vascular structures (Jeays-Ward et al., 2002). The loss of WNT4 also led to
elimination of other female specific gene expression including that of follistatin (Fst) and
Bmp2, suggesting these genes act as downstream effectors of WNT4. Other than its
importance in female development, WNT4 also appears to be required for male
differentiation. XY gonads from Wnt4<sup>−/−</sup> mice displayed reduction in Sertoli cell
differentiation, evidenced by the decreased levels of markers including SOX9 by E11.5
(Jeays-Ward et al., 2004). However, this may reflect a role for WNT4 in achieving a correct
balance between proliferation and differentiation of supporting cells.

Recently, Wnt4 was shown to be an antagonist of Fgf9: Fgf9<sup>−/−</sup> XY gonads displayed
upregulation of Wnt4 and its downstream markers including Fst (DiNapoli et al., 2006).
Ectopic expression of FGF9 in organ cultures not only induced SOX9 expression in the XX
gonad, but also blocked Wnt4 expression, demonstrating the ability of FGF9 to act non-cell-
autonomously to block signals specific to ovarian development and perhaps to recruit all the supporting cells in the gonad to follow the male pathway. WNT4 reciprocates by suppressing the male pathway: in addition to the transient effects on Sox9 expression as mentioned above, Fgf9 was upregulated in gonads of Wnt4−/− XX embryos, while ectopic Wnt4 could repress both Sox9 and Fgf9 (Kim et al., 2006). These findings show that Sox9 upregulation in the gonad does not have to depend on Sry, as in this case it happened as a result of downregulation of Wnt4. It was proposed that the antagonism between Wnt4 and Fgf9 balances the bipotential gonad between two alternative fates. In the male this balance is disturbed by the action of SRY, which rapidly leads to upregulation of Sox9, while in the female it naturally, but perhaps more slowly, tips towards the ovarian pathway (Reviewed by Kim and Capel, 2006).

Forkhead domain/winged-helix transcription factor L2 (Foxl2)

A candidate for a gene acting downstream of WNT4 is Foxl2, which encodes a forkhead domain/winged-helix transcription factor. Mutations in FOXL2 cause Blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), which is associated with premature ovarian failure (Crisponi et al., 2001; De Baere et al., 2001). The gene was also found to map to the critical region responsible for an XX male sex reversal syndrome in goats associated with the polled mutation (Pailhoux et al., 2004).

Foxl2 is specifically expressed in the ovary in mouse, chick, turtle and fish embryos at the time of sex determination (Loffler et al., 2003; Wang et al., 2004). Foxl2 transcripts start to appear in the mouse XX gonad at around E12, which coincides with the downregulation of
Wnt4 and Fst (Yao, 2005). After this stage, Foxl2 expression levels are maintained at a high level in follicle cells (Ottolenghi et al., 2005). In the adult ovary, Foxl2 expression was seen highest in the granulosa cells in early follicles, whereas expression levels decline during later stages of folliculogenesis (Schmidt et al., 2004).

Foxl2 does not appear to affect initial ovary formation, but it is essential for ovarian maintenance. Foxl2\(^{-}\) mutants display high perinatal mortality rates, as the gene is also required for pituitary function, but the survivors are pre-dominantly, phenotypically males (Ottolenghi et al., 2005), implicating perinatal sex reversal, a late sex-reversal phenotype similar to that observed in Wnt4 null mutants (Vainio et al., 1999). XX mice heterozygous for Foxl2 have an early block in folliculogenesis, with a failure to form primordial follicles, and are infertile (Uda et al., 2004). However, unlike Wnt4 null ovaries, Foxl2 null ovaries do not display massive loss of oocyte (Ottolenghi et al., 2005; Vainio et al., 1999).

While overdosage of the DSS region encompassing DAX1 and WNT4 can both lead to XY-sex reversal in humans, they are both shown to be required for proper Sertoli cell differentiation, at least in certain genetic backgrounds in mice (Meeks et al., 2003; Jeays-Ward et al, 2004). The female-specific gene Foxl2 is not upregulated in the XX until E12.0, which is significantly after the establishment of differentiation of the Sertoli cell lineage in the male, which would appear to rule out Foxl2 as a primary ovarian determinant. Moreover, Foxl2 mutant mice do not show primary XX male sex reversal, which would suggest that at best it was only part of a redundant mechanism. In order to further address the function of Foxl2 during sex determination, transgenic mice misexpressing Foxl2 are needed for the investigation of possible sex reversal phenotypes.
The list of genes implicated in sex determination seems to be ever expanding. However, for most of these genes there is still relatively little understanding of underlying molecular mechanisms and more effort has to be made to uncover their place in the pathways and networks involved in cell fate decisions and morphogenesis of the ovary and testis. Genes that are not discussed here include in particular Dessert Hedgehog, a gene that is expressed in Sertoli cells shortly after Sry but plays roles in Leydig and germ cell survival; Dmrt1, which is expressed in Sertoli and germ cells in the testis, but whose function is not yet understood; Fst and Bmp2, expression of which is exclusive to somatic cells in the developing ovary and are important for germ cell survival, Vanin1, which may be involved in extracellular matrix remodelling specifically in the testis (Grimmond et al., 2000), and many others (see review by Wilhelm and Koopman, 2006).

1.5.3 Sexual differentiation – executing the decision

The previous sub-section detailed landmark genetic activities taking place during different phases of sex determination. After the initial establishment of supporting cell identity, sexually dimorphic gonad development proceeds to a further level, with other cell lineages being induced or permitted to follow the male or female pathway, while factors made specifically in the testis instruct the rest of the embryo to follow a male pathway. In the absence of such factors or their receptors, or in the absence of any gonads, the embryo will become female (Luo et al., 1994; Adams and McLaren, 2002). As soon as they begin to differentiate, the Sertoli cells organise into cords and secrete AMH. This induces Müllerian duct regression, an early masculinisation event (Behringer et al., 1994). Testosterone and INSL3, start to be expressed shortly after Leydig cells differentiate at about E12.5 (for
reviews see Haider, 2004; Viger et al., 2005). In the female, the earliest obvious cellular event happens at around E13.5, when germ cells undergo meiosis (McLaren, 1984; Adams and McLaren, 2002).

Amh – eliminating the female reproductive tract

Amh is responsible for Müllerian duct regression in the male, and is therefore an essential component of the male sexual developmental pathway in mammals (Josso et al., 1993). In the mouse, the expression of Amh initiates at around E11.5, shortly after Sox9 is activated, and is expressed in increasing levels by the Sertoli cells through E19.5. Testicular Amh expression transiently declines at perinatal stages (Josso et al., 1993), and is dramatically downregulated at puberty (Münsterberg and Lovell-Badge, 1991; Hacker et al., 1995), coinciding with the onset of meiosis (Hirobe et al., 1992; Rey et al., 1996; Al-attar et al., 1997). Other than Müllerian regression, Amh also appears to have a secondary role to eliminate any germ cells that have entered meiosis early in the testis (Josso, 1990). Expression of AMH is not male-specific towards the end of gestation: it is also expressed in the XX gonads during late stages of embryogenesis and in postnatal granulosa cells (Vigier et al., 1984; Rey et al., 1996).

Apart from SOX9 and SOX8 as mentioned before, SF1 has been shown able to bind putative sites on the rat Amh promoter in gel shift assays (Hatano et al., 1994) and mutating its binding site upstream of Amh in vivo significantly reduces expression (Arango et al., 1999).
Embryonic germ cell meiosis – a cellular landmark of ovarian development

Morphological landmarks of ovarian development are not obvious until late gestation, when follicles start to surround the oocyte. Before this, changes in the germ cell lineage have already begun. Germ cells in the XX gonad enter meiosis at around E13.5; this process was recently shown to be related to Retinoic acid (RA) signalling, which acts to regulate the timing of meiosis, and therefore determines germ cell sex. Stra8, a gene that positively responds to RA, displays XX-specific expression by E12.5, just a day prior to the onset of meiosis (Menke et al., 2003). In the male, CYP26B1, the enzyme that catabolizes RA to an inactive form, was found to be specifically expressed in somatic cells in the XY gonad at E13.5, coinciding with the onset of germ cell meiosis in the XX (Menke et al., 2002; Koubova et al., 2006; Bowles et al., 2006). This suggests that RA signalling is a likely inducer of germ cell meiosis, which in the XX is promoted by Stra8 and in the XY is inhibited by Cyp26b1 (Reviewed by Swain, 2006).

Sexual hormone production

Sex hormone synthesis is a process downstream of sex determination. In the embryonic testis, male-specific hormones including AMH and testosterone start to be produced during gestation. In the ovary, follicle-stimulating hormone is produced by granulosa cells, whereas estrogen is synthesized by theca cells during folliculogenesis.

Six distinct forms of cytochrome p450 enzymes are involved in the synthetic pathway of steroid hormones from cholesterol. At least three of these P450 enzymes have been identified
in the steroidogenic cells in the gonads: side chain cleavage enzyme (P450scc), aromatase (CYP19), and reductase (P450c17 or CYP17). P450scc is required for testosterone synthesis in the male. In females P450scc is expressed in theca cells and the corpus luteum. Biosynthesis of estrogens from androgens is catalyzed by P450 aromatase, which is important in follicular growth, especially towards ovulation. It has been postulated that estrodiol blocks expression of male-specific genes including Sox9 and Fgf9 in granulosa cells (Richards, 2001). P450 reductase (P450c17, CYP17) is expressed at high levels in the embryonic testis during late gestation and in theca cells of pre-ovulatory follicles, and is responsible for catalyzing the synthesis of androgens (Reviewed by Richards, 2001).

Sf1 is linked to these steroidogenic P450s by their coexpression pattern in various steroidogenic tissues (Honda et al., 1993). The expression of Sf1 seems to correlate with the timing of hormone synthesis in gonads of both sexes. After sex determination, Sf1 expression levels increase and are maintained in Leydig cells in the testis, but are decreased in the ovary. This refers to a phase of testosterone and INSL3 production by the Leydig cells. Puberty sees a dramatic change in the Sf1 expression pattern: it becomes expressed at high levels in the theca and granulosa cells in the postnatal ovary around the first ovulation cycles (Hatano et al., 1994). This coincides to the expression of P450scc and aromatase in granulosa cells and reductase in theca cells (Reviewed by Richards, 2001), both of them synthesizing hormones including estrodial and progesterone.

Putative SF1-binding sites were found in the cis-regulatory elements of P450scc and aromatase (Rice et al., 1991; Morohashi et al., 1992). In vitro assays demonstrated that SF1 is able to activate the transcription of genes encoding both enzymes even in non-
steroidogenic cells (Morohashi et al., 1993). Using Amh-receptor-Cre, XY Sfl null mice were achieved, and were depleted for SF1 specifically in Leydig cells and granulosa cells. Embryonic Leydig cells from these mice do not express P450scc, and therefore failed to produce testosterone and INSL3. Although embryonic Sfl−/− ovaries look normal, fewer follicles are formed and corpora lutea are absent in female adults, indicating that ovulation is impaired in these mice (Jeyasuria et al., 2004). However, further studies will be required to address the function of Sfl in ovarian development.

Other than Sfl, several other genes were found important in regulating P450s. P450scc is also regulated by at least Fgf9 in the XY gonad. It has been shown that Fgf9−/− mice failed to upregulate Cyp11a1, the gene encoding P450scc, resulting in failure in Leydig cell development (Colvin et al., 2001). During late folliculogenesis, aromatase is upregulated by Wnt4 and Foxl2, and is expressed in granulosa cells (Pannetier et al., 2006).

1.6 Technologies developed for conditional mutagenesis in the mouse

The ability to produce mice that carry altered genomic loci or foreign transgenes has facilitated the study of many biological processes. Undoubtedly, studying gene function during embryogenesis is one of these. However, the complexity of development demands further control of gene alteration in vivo to facilitate gene function analyses. In many cases, introducing genetic changes to the germ line also have severe developmental consequences, complicating or even precluding the desired experimental analysis. For example, the loss-of-function of a gene might cause early embryonic lethality, rendering the study of its function
in other later-developed tissues impossible. Conditional mutagenesis is therefore required to overcome these undesired limitations.

The establishment of several conditional technologies in mice has enabled considerable sophistication to be applied to gene function research. Many of these apply the concept of a 'genetic switch', which ideally results in zero basal gene alteration when switched 'off' and high levels of activity when 'on'. These criteria have largely been met by binary transgenic systems, in which gene expression is controlled by two components: an 'effector' transgene, which acts on a target, or 'responder' transgene. The most commonly applied effector involves a recombinase that rearranges the responder gene, by either activating or silencing it (for reviews see Lewandoski, 2001; Ryding et al., 2001; Bockamp et al., 2002).

Cre (for "causes recombination") recombinase, originally from bacteriophage P1 (Sternberg and Hamilton, 1981), and Flp recombinase from Saccharomyces cerevisiae (Kilby et al., 1993) are both powerful tools to make site-specific changes in the genome. Both have become popular in conditional mutagenesis in the mouse, especially during embryogenesis (Figure 1.6). Cre and Flp operate in similar manners: both mediate effects on their DNA target sequences depending on the orientation, location and frequencies of the recognition molecules. Recombinase-mediated changes range from excision, inversion, duplication, and chromosomal translocation. For the interest of my project, I will focus on the Cre/loxP system.
1.6.1 Cre/loxP system

The Cre/loxP system has attracted a lot of attention in recent years (see Nagy A's Cre transgenic database). Cre recombinase has now evolved to become more suitable for in vivo usage in the mouse by changing a few codons in cis. This improved version, called iCre (for improved-codon Cre; Shimshek et al., 2002), was proved to function better than the original Cre recombinase (Casanova et al., 2002). Both of them recognize a 34 basepair DNA sequence (loxP, for locus of cross-over P1) in the same orientation, and are believed to gain access to chromatin either through diffusion or following the transient breakdown of the nuclear membrane during mitosis (Orban et al., 1992). Upon homologous recombination, any DNA sequence bound between these loxP sequences would be deleted (Abremski et al., 1983; Review by Kwan, 2002). In the classic set up, the Cre-recombinase would be driven by a regulatory element, which defines where and when homologous recombination takes place, i.e., the effector, or 'Cre-driver'. On the other hand, the 'responder' allele would be 'floxed' by two loxP sequences (flanked by loxP). This can either be a genomic sequence or a transgene driven by an exogenous regulatory element. In the latter case, it is usual to have a gene of interest, or otherwise a 'reporter gene', to follow the floxed transgene. This genetic set-up allows loss- or gain-of-function studies based on the time and space specific Cre recombination profile, by conditional alteration of gene expression patterns in vivo.

1.6.2 Tamoxifen-inducible Cre

The generation of a tamoxifen-inducible Cre system is achieved via fusing Cre with a mutated ligand-binding domain (LBD) from estrogen receptor. This domain no longer
recognizes endogenous estrogen 17β-estradiol but an exogenous drug called tamoxifen, and therefore specially termed ERT (Danielian et al., 1993; Brocard et al., 1997). The function of the ERT domain is to hold the Cre inactive in the cytoplasm by forming a complex with heat-shock protein 90. In the presence of the catabolized form of tamoxifen (4-hydroxytamoxifen, or OHT) and its binding to ERT, the Cre-ERT fusion protein translocates into the nucleus, where it can trigger homologous recombination between target \(loxP\) sequences. By doing this, although Cre expression is controlled by a tissue-specific promoter, its activities are prohibited until tamoxifen is introduced into the system.

Different versions of the ERT domain have been developed. The improved version ERT2 has been made more sensitive to OHT, which does not require hepatic catabolism (Indra et al., 1999).

1.6.3 Other conditional or inducible systems

Apart from Cre/\(loxP\) and the inducible forms of it, other conditional systems for gene expression are available, and are also accompanied with inducible versions. The most commonly used one is the tetracycline responsive system, in which the effector is a fusion between sequences that encode the VP16 transactivation domain and the \(Escherichia coli\) tetracycline repressor (TetR). TetR specifically binds to both tetracycline and the 19bp operator sequences (\(tetO\)) of the \(tet\) operon in the target sequence, which results in its transcription (Gossen and Bujard, 1992). The TetR system is later on modified to enhance its efficiency and reduce basal transcription levels (Gossen et al., 1995). Also available now is a
doxycycline (Dox)-inducible system (Baron and Bujard, 2000; Hasan et al., 2001), which applies similar concepts to the tamoxifen-inducible Cre system. This inducible TetR system is also applied in several cases to temporally control Cre activity (Saam and Gordon, 1999; Utomo et al., 1999; Holzenberger et al., 2000).

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**Figure 1.6 Controlling gene expression by DNA recombination.**

(a) The 34-bp loxP and FRT sites each consist of two 13-bp inverse repeats (black) that flank an 8-bp core sequence (red). This core sequence confers directionality to these sites (red arrows).

(b) Dimers of Cre or Flp (pink) catalyse in cis the conservative recombination between two directly repeated loxP or FRT sites (red arrowheads), resulting in the formation of a synaptic structure, the excision of region B and the juxtaposition of regions A and C. If region B is an essential region of a gene, then the recombination event results in gene inactivation. Recombination can also activate gene expression. For example, transcription from a promoter in region A could fail to reach protein-coding sequences in region C if polyadenylation sites exist in region B; excising region B would therefore activate transcription of region C.

(c) Use of three loxP sites and a 'partial' recombination event (between loxP sites labelled 1 and 2) to remove a neomycin resistance (neo) selection cassette and leave an essential region floxed. The other two products of recombination between loxP sites 2 and 3, and between 1 and 3 are also shown.

(pA, polyadenylation site; TSP, tissue-specific promoter.)
1.7 Project outline

Existing data suggests that both Sry and Sox9 need to be expressed in a highly coordinated temporal and cell-specific manner in order to initiate testis formation in the mouse. The aim of my project is to manipulate the timing and cell-specificity of Sry and Sox9 expression, and to test the effect of this manipulation on sex determination. For this purpose I am using the tamoxifen-inducible Cre/loxP system, which allows gene expression in a highly controlled manner.

In my experiments, gonad specific regulatory elements will be employed to control iCre expression, thereby controlling the conditional expression profiles of Sry or Sox9 (Figure 1.7). Following the next chapter, which outlines all materials and general techniques in use in my project, I describe how Cre-driver and responder elements were chosen and how these transgenes were made and tested (Chapters 3-5). I then describe the tools chosen for studies on the possible function of Sox9 (Chapter 6). Briefly, an inducible iCre driven by a ubiquitous promoter is used to study the effects of loss- or gain-of-function of Sox9 in the developing mouse gonad. Experiments are performed in different systems, including cell cultures, in vivo or explant cultures. We proved that our newly established tamoxifen-inducible Cre system, called CAGGSET, has no basal activity, but can produce recombination activity in different cell types in the gonad, as well as many other tissues. Using CAGGSET, Sox9 was partially knockdown in the XY gonad, giving rise to changes in gene expression patterns. On the other hand, misexpression of Sox9 by either CAGGSET or β-actin-Cre (Lewandoski et al., 1997a) led to the formation of cord-like structures in gonads
of both sexes. Using the new tools developed, we can explore the function of \textit{Sox9}, as well as many other genes involved in gonadal development in the mouse.

\textbf{Figure 1.7} Schematic diagram presenting the project. An example here is the conditional misexpression of \textit{Sry} or \textit{Sox9} in the developing mouse gonad, which is mediated by a tamoxifen-inducible Cre. Upon tamoxifen administration, the iCre protein, which is flanked by two ERT domains, translocates into the cell nucleus, allowing DNA recombination to remove the floxed (yellow triangles) stop codon, thereby allowing gene expression. (TAM: tamoxifen).
CHAPTER 2

GENERAL MATERIALS AND METHODS

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2.1 **Reagents**

Preparation of all reagents followed a published guide (Roskams and Rodgers, 2002).

2.2 **Molecular cloning**

*Reagents for DNA manipulation:* Restriction enzymes and their buffers were purchased either from Roche Diagnostic Inc, Germany, or New England Biolabs, UK. Klenow enzyme (Promega, UK) was used for generating blunt-ended DNA. Ligase and shrimp alkaline phosphatase were purchased from Invitrogen, UK. The DNA polymerase used to generate fragments for sub-cloning purposes was Pfx (Invitrogen, UK). The BIO101 GeneClean system was used for agarose gel purification. Plasmid preparation was done with the Quantum Prep Plasmid Miniprep Kit (BIORAD, USA). Large quantity DNA preps were done with the QIAGEN-tips 500 (Qiagen, Germany). DNA concentration was measured by optical density using a DNA spectrophotometer. *Reagents for bacterial growth:* Escherichia coli strains used included DH5α, XL1-Blue, Top10 and Stbl2. Cells were grown in Luria Broth (LB) that contains antibiotics, either 100µg/mL (w/v) Ampicillin or 30µg/mL (w/v) Kanamycin. For plating, cells were spread onto LB agar that contained the same amount of antibiotics.

2.2.1 **Sub-cloning**

All steps in molecular sub-cloning followed standard protocols (Sambrook and Russell, 2001). Up to 1µg DNA was used in 50µL of reaction buffer for endonuclease digestion, which was carried out at the designated temperature according to the enzyme for no more
than 24 hours. For partial digestion, 0.1 unit of enzymes were used and digestion was carried out for 15-30 minutes. For 5' DNA-flanks removal, digested DNA was treated with Klenow enzyme at 37°C for 1 hour. Before proceeding to the next steps, digested DNA was checked by electrophoresis. Digested fragments were purified and diluted for ligation. Sticky end ligation took place at room temperature from 5 hours to overnight, whereas blunt-ended ligation took place at 16°C overnight. In both cases, the ligation mixture is 20μL.

2.2.2 Transformation and bacterial culture

After ligation, the DNA was transformed either chemically or by electroporation. *Chemically transformation:* the ligated mixture was mixed with 200μL freshly defrosted chemically competent bacteria on ice and incubated for 10 minutes. Transformation took place at 42°C for 90 seconds, after that the transformed cells were placed on ice for 2 minutes, and then added with 800μL warm LB. The mixture was then incubated at 37°C for 1 hour, and spread out on LB agar plates that contain the relevant antibiotic. *Electroporation:* ligation DNA was ethanol precipitated and resuspended in with 50μL electrocompetent cells (Appendix A). The DNA-cell mixture was then transferred into a 0.1mm electroporation cuvette (Bio-Rad, UK) and transformed at 1.8V. Cells were immediately transferred to 200μL warm LB and incubated at 37°C for 1 hour, before plating out.

2.2.3 Plasmid extraction and amplification

*Mini-DNA-prep:* A single colony was inoculated into 3mL LB-antibiotic and agitated at 37°C for 6 hours. Small scale DNA preparation was carried out using a commercial available
kit, following manufacturer’s protocol. **Large-scale DNA prep:** For plasmid maintenance and pronuclear injection, large amount of DNA was needed. Maxi-DNA prep. was carried out using Qiagen Tip 500 (Qiagen, Germany), following the kit protocol. DNA was kept at 4°C.

2.2.4 Preparation for microinjection

For linearization and to get rid of the bacterial backbone of the plasmid, the transgene was digested with restriction endonucleases. 20-50μg DNA was digested overnight, and then purified using the QIAQuik kit (Qiagen, Germany). Microinjections were carried out by the Biological Service Team at the NIMR.

A list of all imported plasmid, and plasmids generated during my PhD in Lovell-Badge lab is listed in Appendix F.

2.3 Tissue culture

**Media:** The culture medium base used was standard DMEM (Sigma, UK), with 1% (v/v) L-glutamate, 1% 9v/v Penecillin/Streptomycin, and 5-10% (v/v) fetal bovine serum (FBS). To ensure that the pH is maintained outside the incubator, the dissection medium for culture purposes was supplemented with 20mM HEPES buffer (Gibco, BRL, UK). **Washes:** Cells were washed with PBS, calcium-magnesium free (CMF), Tissue culture grade (Gibco BRL, UK). **Trypsinization:** The trypsinization reagent used was ready-to-use Trypsin-EDTA (Gibco BRL, UK). **Transfection:** Lipofectamine 2000 (Invitrogen, UK) was used to transfect
COS7 cells (ATCC No. CRL-1651). FuGene6 (Roche, USA) was used to transfect granulosa cells. All transfections were carried out in the serum-free OptiMEM medium (Gibco BRL, UK). *Freezing:* The freezing medium consists of FBS and 5% dimethyl sulfoxide (Sigma, UK). *Plastics:* Plasticware, including culture dishes and plates, were purchased from either Costar Inc. or Nunc. For harvesting and mounting purposes, cells were cultured on cover slips, either of 22 x 22 cm² or 13mm diameter (VWR or BDH).

### 2.3.1 Immortalized cell lines

COS7 (ATCC# CRL-1657) cells were cultured in standard medium with 10% FBS. Cells were split between 70-80% confluence, by rinsing with PBS-CMF, and then incubating in Trypsin-EDTA until cells become detached from the plasticware. To quench trypsinization, three fold of culture medium was added. Cells were then centrifuged at 1000rpm at 4°C for 5 minutes and the pellet was resuspended with culture medium and split to a desired confluency. For transfection purpose, cells were resuspended in OptiMEM and seeded onto a 22X22cm² cover slip in 6-well culture plates between 80-90% confluency. Lipofection was carried out 6 hours later using Lipofectamine 2000, following manufacturer’s protocol. For each well, a total amount of 4μg DNA was used. Cells were cultured for 36-48 hours after transfection. In case of tamoxifen administration, 4-hydroxytamoxifen (OHT, Sigma H-7904, USA), or an equal amount of ethanol, were added to the culture medium at least 6 hours after transfection. There was no medium change following tamoxifen administration.
2.3.2 **Granulosa cell culture**

To collect granulosa cells, female mice between 21 to 28 days of age were superovulated by PMSG, 5I.U. Cells were centrifuged in culture medium containing HEPES at 1000rpm at 4°C for 10 minutes, and the pellet was resuspended with normal culture medium containing 5% FBS, and then seeded onto 13mm diameter cover slips in 24-well plates. Either OHT or ethanol was added 6 hours after seeding. Following tamoxifen administration, cells were cultured for a further 24-36 hours before harvest.

2.3.3 **Explant organ culture**

Gonads were dissected in culture medium containing 5% FBS. For conditional expression studies, each pair of gonad was grown separately on different agarose blocks, which were soaked in medium containing either OHT or ethanol. Medium was discarded two days later, and changed to OHT free medium. Gonads were cultured until harvest, with a change of medium every two days.

2.4 **Animal colony maintenance**

2.4.1 **Legal declaration**

All experiments were carried out in accordance with the UK (Scientific Procedures) Animal Act 1986, under the project licence 70/5042 and its renewed version, 80/1949A. During
licence transition, part of the protocols were carried out using licences 80/1761 and 80/1653. I declare that all procedures undertaken in my project follows the regulations stated in the above licences, and are legal procedures listed in my personal licence (80/8481).

2.4.2 Mouse line maintenance

The mouse colony is held in the Laidlaw Green (LLG) unit. Day-to-day maintenance works, including husbandry, feeding, plug-checking, weaning and tailing, were mainly carried out by the LLG animal technicians. Post-mortal investigation, embryo transfer, mouse line re-derivation, and pronuclear micro-injection, were general services provided by Procedural Services of the institute. All reagents, chemicals, and drugs, unless otherwise specified, were provided by LLG or Procedural Services.

Mouse lines CAGGSET (Line B), Flox-Sox9, Rosa26 Reporter LacZ (R26R LacZ) and Rosa26 Reporter EYFP (R26R EYFP) were maintained as homozygotes. To generate a null allele for Sox9, subsequent breeding were set up between CAGGSET (Line B) and Flox-Sox9. Only heterozygotes could be maintained for Z/Sox9 lines so far.

2.4.3 Matings for vaginal plug check

For the interest of studies during embryonic stages, time matings were set up between 17:00 and 00:00, and vaginal plug (VP) checks were carried out between 07:00 and 09:00. The morning that a VP was detected is referred to embryonic day (E) 0.5. For postnatal studies, the day when the pups were born would be designated as postnatal day (P)0.
2.4.4 Genotyping

For mouse line maintenance, weaning and tailing were carried out between P16 to P22 of newborns. For embryos, the yolk sac was kept for genotyping. With the exception to Z/Sox9 Line 10F, C and M, which were genotyped using X-gal staining (See section 2.7), all genotyping were done using polymerase chain reaction (PCR) method.

Tissue samples for PCR genotyping were digested in 100μL tail lysis buffer (Appendix B) at 55°C overnight before 1:1 phenol-chloroform (v/v) extraction. Extracted DNA samples were centrifuged for 20 minutes at room temperature and 1μL of the supernatant is used for template in the PCR reaction. In specified cases, supernatants obtained from phenol extraction were further precipitated with 1:1 (v/v) isopropyl alcohol, washed with 75% ethanol, and resuspended in 100μL water.

For CAGGSET, DaxCSET, SfIET and S9iCre mouse lines, PCR were done against the presence of the iCre transgene. Sox9:ECFP and AngelBLUE-Sry were genotyped using ECFP-specific primers. R26R LacZ and Z/Sox9 lines were genotyped using LacZ-specific primers, or X-gal staining. De-floxed Z/Sox9 embryos were genotyped with EGFP, using its specific primers. To genotype the sex of the animals, Y-chromosome-specific primers ZFY/YNLS were used. To ensure homozygous status for all R26R mice, three primers were used for PCR (Soriano, 1999). For Flox-Sox9, homozygous, heterozygous and knock-out alleles were genotyped using published primers (Akiyama et al., 2002). PCR conditions are briefly summarized in Table 2.1.
Table 2-1 Primers and PCR programs used for mouse genotyping. Unless specified in NB, all conditions followed standard PCR protocols.

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<th>Size (bp)</th>
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<td></td>
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<td>ODB43: 5’ CaC gggC gCg Atg Tag C A 3’</td>
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<td>ECFP</td>
<td>CYAN</td>
<td>cyanF: 5’ gAc cCT gAA gTT CTT cTg CAC -3’</td>
<td>53</td>
<td>~400</td>
<td>ISOP; Mag</td>
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<tr>
<td></td>
<td></td>
<td>cyanR: 5’ gTg gCT gAT gTA gTT gTA CTC -3’</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EGFP</td>
<td>iZ/EG</td>
<td>GT-EGFPF: 5’ ATg gTg AgC AAa gggC gAg gA 3’</td>
<td>57-58</td>
<td>~400</td>
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<tr>
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<td>FLOX S9</td>
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<td>TG: ~450</td>
<td>WT: ~400</td>
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<td></td>
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<td>Δ: ~400</td>
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<td></td>
<td>FloxS9-3': 5’ Tgg TAA TgA gTC ATA CAC AgT A 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iCre</td>
<td>iCre2</td>
<td>iCre2F: 5’ AGA TgC CAg gAC ATC Agg AAC Ctg 3’</td>
<td>60-62</td>
<td>~350</td>
<td>no DMSO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>iCre2R: 5’ ATC AgC CAC ACC AgA CAC AgA gAT C 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LacZ</td>
<td>iZ/EG</td>
<td>LacZ5: 5’ Cgg AAa TCC ggg CgT TAA CTC ggc gTT TCA T 3’</td>
<td>57-58</td>
<td>~400</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LacZ3: 5’ AAC TgC Agg TTA ACg CTC CgA ATC A 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosa26</td>
<td>R26R2</td>
<td>R1: 5’ AAA gTC gCT Ctg TagTgT Tat 3’</td>
<td>50</td>
<td>TG: ~340</td>
<td>WT: ~650</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R2: 5’ gCg Aag AgT GTC CTC CAA ACC 3’</td>
<td></td>
<td>Δ: ~1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R3: 5’ gGa gCg gga gAA ATg gAT gTg 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sex</td>
<td>ZFY/YNLS</td>
<td>ZFY: 5’ gAc Tag ACA TgT CTT AAC ATC TgT CC 3’</td>
<td>58-60</td>
<td>~300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>YNLS: 5’ CCT ATT gCA Tgg ACA gCA gCT TAT g 3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1 Primers and PCR programs used for mouse genotyping. Unless specified in NB, all conditions followed standard PCR protocols.

1. Akiyama et al., 2002

A(°C) Annealing temperature
ISOP: Isopropanol precipitation required.
Mag: Magnesium concentration reduced to 1.25mM
Sep: Reactions for differentiation primer combination has to be set up separately.

2.4.5 Drugs and chemicals: Preparation and administration

Tamoxifen: Tamoxifen was purchased from Sigma, Europe (T-5648). The stock concentration of tamoxifen was 20mg/mL (w/v), unless otherwise stated. To prepare the stock concentration, tamoxifen powder was dissolved in 1 part of absolute ethanol and then mixed with 9 parts of autoclaved sunflower oil (TESCO Ltd, UK). The mixture was dissolved in a 37°C water bath and then aliquoted into sterilized tubes (Falcon 2059), and kept in 4°C for one experimental period (5-10days) only. OHT: OHT for in vivo injection
was purchased from Sigma, Europe (H-6278). The stock solution for OHT is 10mg/mL (w/v). To prepare the stock concentration, the OHT powder was dissolved in 1 part of absolute ethanol and then mixed with 9 parts of autoclaved sunflower oil (TESCO Ltd, UK). The mixture was sonicated and then aliquoted directly into 1mL syringes, and kept frozen in -20°C for no more than 3 months. **Anesthetics**: Hypnorm, which contains Fentanyl citrate 0.315mg/mL (w/v) and Fluanisone 10mg/mL (w/v) (Vetapharma, UK) were used.

Administration of tamoxifen was either by oral gavage (carried out only under project licences 70/5042, 80/1949 and 80/1653) for embryonic stages, or intraperitoneal injection to adults. OHT was only administrated by intraperitoneal injection. Hypnorm was administered through intraperitoneal injection at a dosage of 0.1mL/10g mouse body weight. A summary of induction protocols was tabulated in Table 2-2.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tamoxifen</th>
<th>OHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7.5-E8.5</td>
<td>10mg*</td>
<td>3mg</td>
</tr>
<tr>
<td>E9.0-E12.5</td>
<td>25mg*</td>
<td>6mg</td>
</tr>
<tr>
<td>P1-P9</td>
<td>N.U.T.</td>
<td>1mg</td>
</tr>
<tr>
<td>P21&gt;</td>
<td>5 X 4mg</td>
<td>N.U.T.</td>
</tr>
</tbody>
</table>

**Table 2-2**  Tamoxifen induction protocol for mice/embryos at different stages.

*induction by oral gavage.

N.U.T.: not under test.
2.5 Animal harvest

2.5.1 Embryo harvest

Pregnant females were sacrificed by schedule-1 killing methods between 11:00 to 14:00. Embryos were dissected into PBS-CMF. For genotyping purposes, either a small piece of tail or the yolk sac was kept.

2.5.2 Dissecting embryonic gonads

Gonads, together with the mesonephros, were dissected in PBS-CMF. For embryos exceeding stage E12.0, phenotypic sex of the gonads were recorded and compared with the genotypic sex, if required.

2.5.3 Harvesting adult gonads

Ovaries for granulosa cell culture: To induce supervulation, females were intraperitoneally injected with PMSG (5I.U.) between P21 and P28, and sacrificed by cervical dislocation 40-48 hours later. Ovaries were dissected and dissociated in culture medium containing 20mM HEPES, using two 25G needles held by 1mL syringes. General dissection of gonads: Gonads were generally isolated from the rest of the body and rinsed with PBS-CMF. Postnatal and adult testes collected were either processed for sectioning (Section 2.6) or cord dissociation. For cord dissociation, testes were broken through with a 25G needle and cords were fixed in 4% (w/v) paraformaldehyde (PFA) at room temperature for 10 minutes. Cords were then split into small portions and transferred onto SuperFrost Plus slides, and then
squashed with a thumb through a cover slip. The slides were then quick-frozen in liquid nitrogen. After pulling off the cover slip, the slides were kept at -80°C until use.

2.6 Sample processing

2.6.1 Fixation by immersion

Embryos, or tissues from the embryo or adult, were immersed into different fixatives for different purposes.

Glutaraldehyde: For X-gal staining, samples were fixed with a solution that contains 0.2% (v/v) glutaraldehyde and 2% formaldehyde (v/v) in phosphate-based buffer (Full recipe listed in Appendix C). Paraformaldehyde (PFA): For cryosectioning or whole-mount immunohistochemical staining, samples were fixed with 4% (w/v) paraformaldehyde in PBS. Bouin: For histology wax sectioning, samples were fixed with Bouin, which contains picric acid, formaldehyde and glacial acidic acid (provided by Histology Service of the NIMR), and then rinsed and stored in 70% ethanol at 4°C.

2.6.2 Fixation by cannulation of the heart

Adult mice (1 to 2 month old) were anesthetized using Hypnorm, and intracardially perfused with chilled 4% (w/v) PFA at room temperature. Samples from the fixed animals were removed and washed in PBS-CMF.
2.6.3 Cryosectioning

Duration of PFA fixation depends on the size and type of the tissue. For wholemount embryos at or bigger than E11.5, embryos were pinched at the back of the neck with a 25G needle to facilitate penetration of the fixative, and fixed for 30 minutes. E13.5 embryos were fixed for 1 hour. Embryonic gonads up to E14.5 days were fixed for 30 minutes, whereas E15.5 – E18.5 gonads were fixed for 1 hour. Postnatal testes, up to P14, were fixed for 1 hour. Adult testes from P14 onwards were fixed at 4°C overnight. For cryoprotection, fixed samples were rinsed with PBS-CMF and then equilibrated in 30% (w/v) sucrose at 4°C overnight, and then embedded in OCT compound (BDH). Embedded tissue blocks were kept frozen at -20°C and sectioned within a week, using a Leica cryostat (model 3050S). Sections were collected on Superfrost Plus slides and kept at -20°C until further processing.

2.6.4 Cutting free floating sections

Samples perfused with 4% PFA were rinsed in PBS-CMF and then embedded in agarose 2.5% (w/v). 70μm free floating sections were made using a vibratome (VT1000S, Leica, Germany), and collected into 24-well plates.

2.6.5 Histology

Sectioning: Samples were fixed in Bouin and rinsed with an increment percentage of ethanol, and were kept in absolute ethanol before handed over to the Histology Service Group of the NIMR. For sectioning, samples were embedded in wax and cut at 6micron thickness with a
microtom (Leica). *Haematoxylin-Eosin (H&E) staining:* H&E staining was performed by the histology Service group after paraffin sectioning.

2.7 **Analyses and information technology**

2.7.1 **β-galactosidase activity assay**

Duration of glutaraldehyde fixation depends on the size of type of the tissue. For wholemount embryos at or bigger than E11.5, embryos were pinched at the back of the neck with a 25G needle to facilitate fixative penetration. All fixations take place at 4°C with agitation. E11.5 embryos were fixed for 20 minutes. E13.5 embryos were fixed for 30 minutes. Embryonic gonads up to E14.5 days were fixed for 15 minutes, whereas E15.5 – E18.5 gonads were fixed for 30 minutes. Samples were washed two times, 5 minutes each, with phosphate-based buffer L o. β-galactosidase activities were detected in X-gal solution at 37°C, until signal develops, for no more than 48 hours. The composition of all reagents used for X-gal staining is listed in Appendix C.

2.7.2 **Immunocytochemical staining**

Upon harvest, cells were fixed with 4% (w/v) PFA for 20 minutes at room temperature, following a brief rinse with PBS-CMF (tissue culture grade). Following fixation, cells were rinsed two times with PBS-CMF containing 0.1% (v/v) Triton (PBT), and then immersed in blocking solution, which is 2% (w/v) bovine serum albumin (BSA), for a minimum of 30 minutes. Primary antibodies were diluted in BSA and incubated either at room temperature
for 2 hours or at 4°C overnight, unless otherwise specified. To get rid of excessive antibodies and non-specific binding, cells were washed three times, 5 minutes each, with PBT. Signals were visualized with fluorescent secondary antibodies, which were incubated in darkness at room temperature for 1 hour. After three washes, 5 minutes each, cells were incubated with 1:10,000 DAPI (gift from François Guillemot Lab, origin unknown) for 5 minutes at room temperature. After a 5-minute wash, the coverslip that contained the cells were then transferred onto a glass slide and mounted with AquapolyMount (PolyScience, UK). The details of the antibodies used are summarized in Table 2.3.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Host</th>
<th>Dilution</th>
<th>From</th>
<th>Secondary antibodies used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Host</td>
<td>Dilution</td>
<td>From</td>
<td>Hosts</td>
</tr>
<tr>
<td>3βHSD</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>Gift²</td>
<td>Goat a Rabbit</td>
</tr>
<tr>
<td>AMH</td>
<td>Goat</td>
<td>1/100</td>
<td>scbt</td>
<td>Goat a Mouse</td>
</tr>
<tr>
<td>c-myc</td>
<td>Rabbit</td>
<td>1/500</td>
<td>scbt</td>
<td>Donkey a Rabbit</td>
</tr>
<tr>
<td>Cre</td>
<td>Mouse</td>
<td>1/100</td>
<td>Covance</td>
<td>Donkey a Goat</td>
</tr>
<tr>
<td>DMC-1</td>
<td>Goat</td>
<td>1/200</td>
<td>scbt</td>
<td>Goat a Chick⁶</td>
</tr>
<tr>
<td>FOXL2</td>
<td>Rabbit</td>
<td>1/250</td>
<td>Gift⁴</td>
<td>Donkey a Chick⁶</td>
</tr>
<tr>
<td>GFP</td>
<td>Sheep</td>
<td>1/1000</td>
<td>Covance</td>
<td>Donkey a Sheep⁷</td>
</tr>
<tr>
<td>GFP</td>
<td>Chick</td>
<td>1/500</td>
<td>Chemicon</td>
<td>Donkey a Rabbit</td>
</tr>
<tr>
<td>Laminin</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>Gift⁵</td>
<td>Goat a Mouse</td>
</tr>
<tr>
<td>PECAM</td>
<td>Rat</td>
<td>1/500</td>
<td>Pharmingen</td>
<td>Donkey a Goat</td>
</tr>
<tr>
<td>SCP3</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>Upstate</td>
<td>Goat a Rabbit</td>
</tr>
<tr>
<td>SCP3</td>
<td>Mouse</td>
<td>1/1000</td>
<td>Upstate</td>
<td>Goat a Mouse</td>
</tr>
<tr>
<td>SF1</td>
<td>Rabbit</td>
<td>1/400</td>
<td>Gift¹</td>
<td>Goat a Rat</td>
</tr>
<tr>
<td>SOX9</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>Gift³</td>
<td>Donkey a Rabbit</td>
</tr>
<tr>
<td>Stella</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>Abcam</td>
<td></td>
</tr>
<tr>
<td>β-gal</td>
<td>Mouse</td>
<td>1/500</td>
<td>Sigma</td>
<td></td>
</tr>
<tr>
<td>β-gal</td>
<td>Goat</td>
<td>1/500</td>
<td>Gift⁹</td>
<td></td>
</tr>
<tr>
<td>yH2AX</td>
<td>Rabbit</td>
<td>1/1000</td>
<td>Upstate</td>
<td></td>
</tr>
<tr>
<td>yH2AX</td>
<td>Mouse</td>
<td>1/1000</td>
<td>Upstate</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-3 Antibodies used in this study. Unless specified, fluorescent secondary antibodies were purchased from Molecular Probes.

¹K Morohashi  ²⁻⁴J Mason  ⁵⁻⁷P Goodfellow  ⁶⁻⁸J Cocquet  ⁹Ang Lab (N.B. commercially available)
2.7.3 Immunohistochemical staining

*On cryosections:* Sections were brought to room temperature and washed in PBS-CMF to remove the OCT compound, and then blocked with BSA for at least 30 minutes. All primary antibody incubation was done at 4°C overnight. Washing and secondary antibodies incubation procedures were the same as for immunocytochemical staining. For enhance nuclear staining signal, sections were incubated with a DAPI solution (1:10,000 v/v DAPI in PBST) at room temperature for 5 minutes, before mounted by Vectorshield and covered by a coverslip. *On paraffin sections:* Sections were de-waxed by immersing in histoclear solution for 5 minutes, and then re-hydrated with washes in decrement in percentage of ethanol, 75%, 50%, 25%, 5 minutes each. Re-hydrated sections were then rinsed in PBT, and blocked in BSA. All following procedures were the same as that of on cryosections.

2.7.4 *In situ* hybridization

The *iCre* in situ hybridization described in Chapter 4 is performed on 10 microns cryosections, following the protocol published by Wilkinson’s lab. Hydridization temperature of the *iCre* riboprobe was 70°C. The riboprobe was synthesized as follows: plasmid pBlue.iCre (Appendix E) was cut with restriction endonuclease *Bgl* II and transcribed with the T7 polymerase. The ~800bp RNA obtained was purified and used as the probe for *iCre*.
2.7.5 **Microscopy and softwares**

*Light microscopy:* Pictures of non-fluorescent wholemount embryos, tissues, or sections were taken by a light microscope, using the software IM50 (Leica, Germany) or OpenLab (Improvision, UK).

*Fluorescent imaging:* Fluorescent sections were handled with the following systems: DeltaVision; Leica systems including Confofal SP2 with LCS software, Confocal SP5 with LAS software, or with a Leica microscope with the software FW4000. Pictures of fluorescent wholemount samples were taken with a Leica microscope, using softwares either FW4000 (Leica, Germany) or OpenLab (Improvision, UK). Where necessary, images were processed using Adobe Photoshop CS2. All figures presented in this thesis are produced by Adobe Illustrator CS2. Softwares used for texts for this thesis include Microsoft Word 2003 and Excel 2003. This thesis is printed out in Adobe Acrobat (.PDF) format. Soft copies of all data, versions of this thesis, and the final corrected proof will be saved in a directory called “atye” in the Lovell-Badge lab archive.
CHAPTER 3

ESTABLISHING TOOLS TO ALTER GENE EXPRESSION DURING GONADAL DEVELOPMENT IN MAMMALS

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3.2 Materials and Methods: Molecular cloning
  3.2.1 Sox9:ECFP
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3.5 Discussion
3.1 Introduction

For the purpose of the project, several tools had to be made. As described in the general introduction of this thesis, my experiments are based on two components, a “Cre-driver” mouse and a “responder” mouse. Previous members of the lab have generated several mouse lines for these purposes, however, none of them turned out to be appropriate to drive reporter gene expression in the gonads. For example, an $Sry$:Cre transgene made previously in the lab did not show evidence of functionality (Claire Canning, unpublished and data not shown), whereas $Dax1$:Cre was shown to be active in pre-implantation embryo (Amy Johnson, PhD thesis, 2002). For my interest, I have chosen new materials to create the transgenic constructs. For the “Cre-drivers”, I have chosen regulatory elements of Sox9, Daxl and Sfl, which are all gonad-specific (at least at mid-gestation stages). Apart from gonad-specific sequences, a ubiquitous, strong promoter sequence was employed to drive the expression of Cre. This latter system will be described in detail in the next chapter.

Figure 3.1 A 3.2kb Sox9 enhancer exhibits activity in the embryonic testis. (R. Sekido, unpublished)

A X-gal staining of a E12.5 embryonic testis from a 3.2Sox9:LacZ embryo.

B ECFP autofluorescence of the same stage from a Sox9:ECFP
Ryohei Sekido, a current member of the lab has characterized aspects of the mouse Sox9 upstream regulatory region, defining an element that is gonad specific (unpublished). He established transgenic lines using a 3.2kb enhancer to drive reporter LacZ, which displayed strong activity in the developing testis, where it showed expression equivalent to that of the endogenous Sox9 gene (Figure 3.1). Based on his work, I made a transgene using this 3.2kb Sox9 enhancer region to drive the expression of ECFP (3.2S9ECFP), which also showed a similar, gonad-specific ECFP expression pattern in vivo (Figure 3.1). This suggests that the 3.2kb region would be a good element to drive Cre expression in Sertoli cells.

Daxl is expressed initially in the supporting cell lineage in the indifferent gonad of both XX and XY embryos. It is then downregulated in the testis at about E12.0, but remains expressed in the ovarian supporting cell lineage throughout development, and during folliculogenesis (Swain et al., 1996; Swain et al., 1998). An 11kb regulatory region just 5' to the Daxl open reading frame has been characterized previously in the lab (Swain et al., 1998, Hoyle et al., 2002). In Daxl:LacZ transgenic mice, this region was consistently able to drive LacZ expression in a pattern identical to that of endogenous Daxl. Reasoning that this regulatory region will allow, for example, misexpression of male-specific genes in the XX supporting cell lineage, it was used to drive the expression of Sry. The Daxl:Sry transgene resulted in female-to-male sex reversal at the same frequency as LacZ expression was seen in the Daxl:LacZ mice (Swain et al., 1998), proving this Daxl regulatory element to be both functional and efficient. This 11kb fragment was therefore used to construct the Daxl Cre-driver.
An Sfl regulatory element was also selected. Sfl is expressed in both the supporting and steroidogenic cell lineages at early stages, and while it is downregulated in the ovary, it is maintained in differentiated Sertoli cells and Leydig cells. In theory an appropriate regulatory region will be useful in gene alteration studies both cell lineages. For example, it could be used to ask if the expression of a Sertoli cell-specific gene in steroidogenic cells will lead to a change in cell fate. A 674bp Sfl promoter region was shown to be sufficient to activate LacZ reporter gene expression in E11.5 mouse gonads, spinal cord, and weakly in the hindbrain (Wilhelm and Englert, 2002). In transgenic mice, this Sfl promoter driving Cre successfully deleted a conditional Sox9 allele in the gonads (Chacoissier et al., 2004) and was therefore chosen to generate the Sfl Cre-driver.

To enhance Cre recombination efficiency, the improved codon-Cre (iCre, Shimshek et al., 2002) was used in all cases. For Dax1, Sfl, and the ubiquitous Cre-drivers, an inducible version of iCre (ERT2iCreERT2, Casanova et al., 2002) was used. This should allow control of Cre activation through the administration of the inducer tamoxifen at the desired embryonic stage. In the nomenclature for the constructs, DaxCSET and SflET, the “ET” that follows the name of the regulatory element stands for Estrogen receptor-Tamoxifen inducible. The “CS” in the Dax1 driver DaxCSET refers to the shuttle vector pCS2+ used for subcloning.
3.2 Materials and Methods: Molecular cloning

3.2.1 Sox9:ECFP

The hsp68:LacZ plasmid was used as the backbone for constructing 3.2Sox9:ECFP (Sox9:ECFP). The first step involved swapping the LacZ encoding gene to the one that encodes ECFP (Figure 3.2A). To facilitate linearization for microinjection, the NotI restriction site between the ECFP sequence and the polyadenylation signal was destroyed by partial digestion and then blunt end ligation. This resulted in the transgene, hsp68:ECFP, which was subcloned into a plasmid that contained the 3.2kb Sox9 enhancer sequence. The resulting construct has the 3.2kb enhancer coupled to the hsp 68 minimal promoter driving ECFP (Figure 3.2B).

3.2.2 Sox9:iCre

The iCre fragment was excised and used to replace the ECFP fragment in Sox9:ECFP by blunt-end ligation. This Sox9 Cre-driver transgene is named S9iCre (Figure 3.3).

3.2.3 Daxl:ERT2iCreERT2

To facilitate subcloning via compatible end ligation, pCS2+ (Appendix) has been used as a shuttle vector. The sequence encoding the ERT2iCreERT2 fusion protein was inserted into pCS2+ to generate pCSET, and from this the Dax1:ERT2iCreERT2 transgene was made. This Dax1 Cre-driver transgene was designated as DaxCSET (Figure 3.4).
Figure 3.2  Construction of the 3.2kb Sox9-enhancer driving ECFP transgene.

A  An ECFP-encoding sequence from pECFP (Promega) was inserted into the hsp68:LacZ plasmid via the NcoI/XbaI site. Due to multiple existence of the XbaI, partial digestion was done for subcloning. The NotI site between the ECFP and polyA signal in this resulting plasmid was removed blunt-ended self-ligation.

B  The 3.2kb Sox9 enhancer was then inserted via compatible-end ligation (NotI to NotI/XhoI to SalI). The Sox9:ECFP transgene was released for pronuclear microinjection by NotI digestion. The hsp68:LacZ plasmid was used as the backbone for constructing 3.2Sox9:ECFP
Figure 3.3  Construction of the 3.2kb Sox9-enhancer driving iCre transgene.

The sequence encoding iCre from pBlueiCre (Shimshek et al., 2002) was excised by HindIII/Xhol partial digestion and replaced the ECFP fragment in the Sox9:ECFP plasmid via blunt-ended ligation, resulting in the Sox9:iCre transgene. For microinjection, this transgene was released by Sall/NotI double digestion.

Figure 3.4  Construction of the Dax1 promoter driving inducible iCre transgene.

The BamHI in the multiple cloning site in PBS.ERT2iCre was destroyed by blunt-ended self ligation. The ERT2iCreERT2-human growth hormone polyA (hGHpA) fragment was subcloned into the shuttle vector pCS2+ via compatible-end ligation (Sall to Xhol/NotI to NotI). The resulting plasmid, pCSET, was opened with BamHI/ClaI for the insertion for the 11kb Dax1 promoter. The resulting transgene was named DaxCSET. For microinjection, this transgene was released by BamHI/NotI double digestion.
3.2.4 \( Sf1:ERT2iCreERT2 \)

The short \( Sf1 \) regulatory region was amplified by PCR and subcloned directly into the 5' of the plasmid containing the \( ERT2iCreERT2 \) fragment (pBS-\( ERT2iCrehGHpA \), Figure 3.5). Restriction endonuclease linkers were added to the 5' end of both primers to facilitate sticky-end ligation.

![Diagram](attachment:image.png)

**Figure 3.5** Construction of the \( Sf1 \) promoter driving inducible \( iCre \) transgene.

The 674basepair \( Sf1 \) promoter was amplified by PCR. \( SalI \) linkers were attached to flanking ends to facilitate ligation into the plasmid PBS.ERT2iCre-hGHpA. The resulting transgene was named \( Sf1ET \), and was released for microinjection via \( SalI/NotI \) double digestion.

3.3 Establishment of stable founder mice

All constructs were linearized for injection into pronuclear stage mouse embryos. \( Sox9:ECFP \) and \( S9iCre \) were injected by Ryohei Sekido in the host lab. For the former, 6 founders were identified by PCR for \( ECFP \), whereas 7 founders were generated for the latter.
DaxCSET and Sf1ET constructs were injected by the Procedural Service Section at the NIMR (directed by Sophie Wood). Five DaxCSET founders were identified from microinjection using transgene Dax1:ERT2iCreERT2 and three Sf1ET founders were generated from transgene Sfl:ERT2iCreERT2. S9iCre, DaxCSET and Sf1ET transgenic founders were identified by iCre PCR. All transgenic mice were maintained on a mixed CBA/C57BL10 background by mating to CBA10/F1 mice, and kept as heterozygotes unless otherwise specified.

3.4 Characterization of founder mice

3.4.1 Sox9:ECFP

Three out of six Sox9:ECFP lines showed strong ECFP expression in the genital ridges. ECFP could be detected in the genital ridge as early as E11.5, and was restricted to SOX9-expressing cells by E12.5 (Figure 3.6). Expression was also seen in the Wolffian and Müllerian ducts of both sexes (Figure 3.7). SOX9 has been detected in Wolffian and Müllerian ducts of several mammals, including marsupials (Pask et al., 2002) and mice (R. Behringer, personal communication; Guioli et al., 2006 and unpublished data from various lab members). These results show that Sox9:ECFP labels SOX9 expressing cells in the urogenital ridge.
Figure 3.6   For figure legend please see opposite page.
Figure 3.7  *Sox9:ECFP* mice display ECFP expression in the nephrogenic ducts.

**A – C** Wholemount E13.5 ovary and mesonephros showing ECFP in the latter.

**D - F** 10μm cryosection of the above. Antibodies used for co-labelling were chick anti-GFP (for ECFP) and Pax2 (for epithelial cells in Müllerian duct).

To investigate whether the 3.2kb *Sox9* enhancer is still active in the adult testis, male *Sox9:ECFP* mice of different ages were studied. The results show that ECFP was still detectable in a one-year-old testis. However, unlike at embryonic stages, and unlike endogenous SOX9, ECFP expression was detected in both Sertoli and Leydig cells in the adult. The activity of this enhancer seems therefore to lose its Sertoli cell specificity (Figure 3.8). This requires further work, however, as only one line has been studied. Moreover, it
may be worth exploring the onset of Leydig cell expression within this line to see if it coincides with replacement of fetal by adult Leydig cells.

3.4.2 Sox9:iCre (S9iCre)

To examine the recombination pattern of the S9iCre mice, founder lines were crossed to ROSA26 Reporter (R26R) LacZ animals (Soriano, 1999). Embryos were harvested at E11.5 or E13.5 for X-gal staining. The intensity of X-gal staining correlates with Cre-mediated recombination. Embryos negative for iCre by PCR did not display X-gal activity (data not shown). Unexpectedly, five out of the 7 lines showed extensive X-gal activity throughout the embryo. Examples of these are lines A and C, where the former shows mosaic expression and the latter appears ubiquitous (Figure 3.9). Line V displayed mosaic expression throughout much of the embryo, but particularly strong expression in the embryonic testis where it marks all Sertoli cells by E14.5 (Figure 3.10). Line E displayed variable staining patterns on different genetic backgrounds: on a CBA/C57BL10 mixed background, X-gal activity was localized largely to the adrenal gland, whereas after backcrossing to C57BL/6 (as in Figure 3.10), X-gal activity was detected in various tissues. In any case, no X-gal activity was detected in the embryonic testis for Line E.
Figure 3.8  Sox9:ECFP is active in both Sertoli and Leydig cells in the adult.

Antibody labelling involved anti-Sox9 for Sertoli cells and 3βHSD for Leydig cells. CFP was detected by autofluorescence.
Figure 3.9  Four stable S9iCre mouse lines displayed Cre-mediated activities.

S9iCre-R26R LacZ embryos were dissected at E11.5 (A – D) and E13.5 (E – H) for wholemount X-gal staining. I – L Gonads were dissected from E13.5 embryos showed in (E – H). Note that all of these gonads are testes, whereas in ovaries from the corresponding litters, none stained for X-gal. In all cases, embryos or gonads that are negative for iCre and therefore the S9iCre transgene does not give any X-gal staining.
Figure 3.10  

*S9iCre* (Line V) displays high iCre activity in Sertoli cells.

*S9iCre* (V) males were set up with *R26R EYFP* females, which were sacrificed at different stages for detection of Cre activity in the embryos. Gonads from the embryos harvested were dissected out and cryosectioned for immunohistochemistry against *SOX9* and GFP (for EYFP). The figure shows iCre positive testes collected from stages between E12.5 to E15.5. Note that the expression of EYFP becomes Sertoli cell-specific by E14.5, as marked by Sertoli cell marker *SOX9*. No expression of EYFP could be observed in gonads that are negative for iCre, or in ovaries.
Due to the extensive staining patterns observed, it was suspected that the 3.2kb enhancer is active in early embryonic stages. Blastocysts were extracted from pregnant R26R EYFP females (Srinivas et al., 2001) mated to S9iCre mice. EYFP was detected in the blastocysts from crosses with Lines A (50%) and C (65%), indicating the 3.2kb Sox9 enhancer is active during preimplantation development in these lines. Line V (20%) displayed sporadic expression in blastocysts (Figure 3.11). Line E was not examined. These results show that the 3.2kb fragment from Sox9 has a strong tendency to be active at blastocyst stages, making it inappropriate for use with non-inducible forms of Cre.

![Figure 3.11 Three S9iCre lines demonstrated activity at blastocyst stages.](image)

Blastocysts were isolated from uteri of R26R EYFP mice mated to S9iCre mice. EYFP was observed by autofluorescence and compared to the brightfield image captured by a light microscope.

3.4.3 *Dax1;ERT2iCreERT2 (DaxCSET)*

*DaxCSET* mice were first tested for iCre expression and activity in granulosa cell culture. Founder animals were crossed to homozygous R26R EYFP mice. To increase granulosa cell number, adult females were induced for superovulation between 3-4 weeks of age.
Granulosa cells of both iCre positive and negative females were plated out in culture, in the presence of either OHT or an equal concentration of the vehicle (ethanol) alone. The presence of EYFP should reveal cells in which Cre-mediated recombination has occurred. Immunostaining using an antibody specific for Cre showed that only 3 of the 5 founder lines expressed the iCre protein in cultured granulosa cells. These founders responded differently to tamoxifen: two founder lines, F and M, displayed nuclear localized iCre only in the presence of OHT, and at the same time expressed EYFP (Figure 3.12A and 3.13A). However, Line P displayed nuclear localized iCre and expressed EYFP with or without OHT, indicating the nuclear translocation of iCre in this line is not regulated by the inducer (Figure 3.14).

Following this observation, lines F and M were investigated further to find out if Cre-mediated recombination can be induced in utero. DaxCSET mice were crossed to homozygous R26R mice and tamoxifen was administered to pregnant females by oral gavage at E11.5. Gonads were dissected from the embryos at E12.5 and examined for β-galactosidase activity by immunostaining. Gonads positive for both iCre and reporter LacZ by PCR were compared to those positive only for the reporter. Double positive gonads of both sexes from Line M embryos displayed reporter protein staining in cells located between the mesonephros and the gonad, a pattern similar to that in Dax1:LacZ mice (Figure 3.13B, Swain et al., 1998). Line F did not show any significant difference in reporter protein expression between the iCre positive and negative gonads (Figure 3.12B). In parallel, several mice were given the vehicle (oil) only. No β-galactosidase activity was detected in any gonad from either line, indicating the appearance of the reporter protein in Line M was a result of tamoxifen-induced Cre-recombination events.
Figure 3.12  Cre-mediated recombination in DaxCSET Line F.

A Dissociated granulosa cells isolated from DaxCSET (F)-R26R EYFP ovaries were plated in culture, in the presence of either 2μM OHT or same volumetric amount of ethanol. Antibodies used for co-labelling were anti-Cre and chick anti-GFP (for reporter EYFP). B in utero recombination in DaxCSET (F)-R26R EYFP. 25mg tamoxifen was administered to the pregnant female by oral gavage at E11.5, and gonads of the embryos were dissected at E13.5, cryosectioned for immunohistochemical staining using sheep anti-EYFP. M: mesonephroi; G: gonads.
Figure 3.13 Cre-mediated recombination in DaxCSET Line M.

**A** Dissociated granulosa cells isolated from DaxCSET(M)-RYFP ovaries were plated in culture, in the presence of either 2μM OHT or same amount of ethanol for 48 hours. Antibodies used for co-labelling were anti-Cre and chick anti-GFP.

**B** In utero recombination in DaxCSET(M)×R26R LacZ. 25mg tamoxifen was administered to the pregnant female by oral gavage at E11.5; gonads of the embryos were dissected at E12.5, cryosectioned and stained using a mouse anti-β-gal antibody to label cells that display recombination activity.

**Legend**
- **Testis**
- **Ovary**
- **DAPI**
- **β-gal**
- **G**: Gonads
- **M**: Mesonephroi

**Caption**
75
Figure 3.14  Cre-mediated recombination in DaxCSET Line P.

Dissociated granulosa cells isolated from DaxCSET (P)-RYFP ovaries were plated in culture, in the presence of either 2µM OHT or same volumetric amount of ethanol. Antibodies used for co-labelling were anti-Cre and chick anti-GFP (for reporter EYFP).

3.4.4  Sfl:ERT2iCreERT2 (SflET)

One of the SflET founders (Line M) died at the age of ten weeks. This founder failed to mate with females and never produced any offspring. The remaining 2 lines were investigated for Cre-mediated recombination activities during embryonic stages. Lines C and P were crossed to R26R LacZ mice and tamoxifen given via oral gavage. Several cells displayed β-galactosidase activity in Line P iCre; LacZ double positive gonads (Figure 3.15). No β-
galactosidase activity was detected in \(iCre\) negative gonads or in gonads of embryos that received vehicle alone, indicating that any \(\beta\)-galactoside activity obtained must have been due to tamoxifen-induced Cre-recombination events. Unfortunately, Line P mice, including the founder itself, stopped transmitting the \(Sf1ET\) transgene later on during the study. Line C did not show \(\beta\)-galactosidase activity after induction \textit{in utero} or \textit{ex vivo} (data not shown). New founders have been generated by pronuclear microinjection, and will require characterization.

![Figure 3.15](image)

\textit{Figure 3.15}  \textit{in utero} Cre-mediated recombination in \(Sf1ET\) Line P.
3.5 Discussion

The establishment of the Sox9:ECFP mice provides a simple way to detect both fixed and live Sox9 expressing cells in the gonad. It can also be used to discover genetic factors that modulate the activity of this enhancer, which are likely to be required for regulation of Sox9 expression in the gonads (see, for example, Kim et al 2006).

However, the widespread expression of S9iCre in R26R LacZ mid-gestation embryos revealed wider activity of the 3.2kb Sox9 enhancer at earlier embryonic stages. Indeed, EYFP was detected in blastocysts, indicating enhancer activity in the pre-implantation embryo. The apparent discrepancy can be explained by the differences in experimental approach in characterizing these transgenes. The reporter ECFP directly reports the activity of the 3.2kb enhancer only at the stage being examined, whereas, enhancer activities in S9iCre mice are reflected by the expression of a reporter activated by the earliest Cre-mediated recombination, which in this case happened at blastocyst stages. This was unexpected as Sox9 expression has not been detected in preimplantation embryos, indeed it is first seen during gastrulation.

The S9iCre mice were created to study gene function in the Sertoli cell lineage. S9iCre Line V displayed significant Cre-mediated activity in the embryonic testis, suggesting that the enhancer does drive Cre expression efficiently in Sertoli cells, but there was too much activity outside the gonad to make even this line useful. Clearly it would have been better to have used the inducible version of iCre. This would also permit multistage tests.
In contrast, \textit{DaxCSET} and \textit{SflET} mice both carry the inducible version of \textit{iCre}, allowing control of recombination at a specific time of interest. \textit{DaxCSET} Line M displayed Cre-mediated activity in the embryonic gonad that could be induced \textit{in utero}. Although \textit{in vivo} induction during postnatal stages failed to give recombination in any \textit{DaxCSET} line (data not shown), studies at stages after birth can make use of the granulosa cell culture system, for which Lines F and M showed efficient, inducer-specific Cre activity.

\textit{SflET} Line P showed recombination in the embryonic testis after \textit{in utero} induction, proving this transgene functional. Unfortunately, not many founders were obtained from microinjection, and Line P could not be maintained. However, more transgenic lines are being made and characterized.

The activities obtained from gonad-specific inducible Cre-driver mice were not as strong as those obtained from reporter transgenes driven by the same promoter elements (Swain \textit{et al.}, 1998; Wilhelm and Englert, 2002). This could be due to induction efficiency, which needs further optimization. The tests done and described in this chapter followed standard protocols published previously (Feil \textit{et al.}, 1997; Joyner Lab Online protocols, http://saturn.ned.nyu.edu/research/dg/joynerlab/protocols.html), but could be modified according to our own requirements giving sufficient recombination. Also, the experience gained with \textit{DaxCSET} and \textit{SflET} suggests that it would be very beneficial to use \textit{ERT2iCreERT2} with the 3.2kb \textit{Sox9} regulatory region, to make \textit{S9ET}.

Recently, a mouse line using \textit{Sry} regulatory sequences to drive \textit{Cre} expression was published (Ito \textit{et al.}, 2006). Recombination activities of this \textit{Sry/Cre} mouse were detected in fetal and
adult Sertoli cells and in granulosa cells, although very few, which would make it of limited use to mediate gene expression or deletion in these lineages. *Sry* is also expressed in preimplantation stage embryos (Boyer and Erickson, 1994) and it is possible that the only line obtained with specific expression in gonadal supporting cells was subject to strong negative influences from sequences flanking the transgene integration site.

There are many other genes expressed in the developing gonads that could provide appropriate regulatory sequences for driving inducible or non-inducible Cre. These would include regulatory sequences specific to granulosa cells, e.g. *Foxl2*, or those that are expressed in the bipotential gonad, and stay expressed in differentiating gonads. The latter include *Wt1*, regulatory sequence of which was previously employed to drive expression of *Sox9* in the XX leading to sex reversal (Vidal et al., 2001). *Sfl*, for which a BAC clone has been recently reported to produce stronger activity compared to the 674bp gonad-specific promoter (Bingham et al., 2006). *Lhx9* is probably the earliest acting gene known that is essential for the formation of the gonadal primordia. However, its regulatory region has not been defined, and therefore, the generation of an inducible Cre-driver using *Lhx9* will require subcloning into the BAC that contains *Lhx9*.
CHAPTER 4

CAGGSET – A UBIQUITOUS, INDUCIBLE CRE-DRIVER

4.1 Introduction
4.2 Molecular cloning of CAG:ERT2iCreERT2
4.3 In vitro assays for CAGGSET
  4.3.1 CAGGSET functions correctly in immortalized COS7 cells
  4.3.2 CAGGSET is active in primary culture cells
4.4 Establishment of CAGGSET stable founder mouse lines
4.5 Detailed in vivo characterization of CAGGSET founder lines
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  4.5.5 CAGGSET is active in different cell lineages in the gonads
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4.6 Discussion
4.1 Introduction

There have been several attempts to establish mice ubiquitously expressing an inducible Cre recombinase. The first used the CMV regulatory region to drive Cre-ERT (Feil et al., 1996, Brocard et al., 1997). However, these CMV-Cre-ERT mice produced Cre activity in only a few tissues (F. Guillemot; D. Metzger; C. Galichet, personal communication), with the highest activity (~40%) found in skin cell types. A similar line of transgenic mice, termed CMV-Cre-ER™, produced Cre activity in a wider variety of tissues (Hayashi and McMahon, 2002). It is not clear if these mice actually harbour the same version of inducible Cre. According to the original report and experience of several others (Casanova et al., 2002), this Cre-ER™ mouse displayed low levels of Cre activity even in the absence of the inducer. However, tamoxifen induction did not lead to any Cre-activity in the gonad (R. Behringer; personal communication). Because these Cre-driver mice were unsatisfactory, it was important to attempt to establish lines that have more widespread expression and better control of recombinase activity.

It was decided to use the ERT2iCreERT2 (Casanova et al., 2002) for making the ubiquitous inducible Cre. The CMV regulatory region, as tried by others, is known to express well in various cell lines in vitro, but in transgenic mice it tends to give rather variable expression. We therefore chose instead to use the ubiquitous regulatory element in the expression vector pCAGGS, which comprises the CMV enhancer linked to the chicken β-actin promoter (Niwa et al., 1991), and was reported to drive high levels of gene expression. This was used to make the CAG:ERT2iCreERT2 transgene, called CAGGSET. It was found to produce high levels of Cre activity in many tissues, including different gonadal cell types, and to be highly responsive to tamoxifen in all the systems under test.
In this chapter, the establishment of CAGGSET, as well as the characterization in transgenic mice will be described in detail.

4.2 Molecular cloning of CAG:ERT2iCreERT2

The promoter/enhancer fragment of pCAGGS (Niwa et al., 1991) was released and inserted into the pBS-ERT2iCreERT2-hGHpA plasmid (Casanova et al., 2002) via SalI/XhoI compatible-end ligation (Figure 4.1). This transgene is designated as CAGGSET. The construct has been verified by sequencing the full-length of the transgene.

![Diagram](image)

**Figure 4.1** The molecular construction of the transgene CAG:ERT2iCreERT2.

A ubiquitous 1729 basepair CMV enhancer/chicken beta-actin promoter complex was released from plasmid pCAGGS by SalI and XhoI double digestion. This fragment was inserted into the SalI site of the plasmid pBS-ERT2iCrehGHpA via compatible sticky-end ligation. This resulted in the ERT2iCreERT2 transgene under-driven by the ubiquitous regulatory complex. The recombinant transgene, CAG:ERT2iCreERT2, designated as CAGGSET, was removed from the vector backbone for pronuclear microinjection by SalI and NotI digestion.
4.3  *In vitro* assays for *CAGGSET*

*CAGGSET* function was first assessed *in vitro*, both in an immortalized cell line and in primary cell culture.

4.3.1  *CAGGSET* functions correctly in immortalized COS7 cells

To verify that the construct is able to express the Cre-recombinase correctly, and that Cre-mediated recombination could be induced by tamoxifen administration, plasmid CAGGSET (p*CAGGSET*) was transfected into COS7 cells, either on its own or with plasmid *iZ/EG*, a transgene that expresses EGFP as a reporter of Cre activity (Novak *et al.*, 2000). Cells were cultured in the presence of the inducer, 4-hydroxytamoxifen (OHT), or the vehicle (ethanol) alone. An anti-Cre antibody was used to label cells that express the *ERT2iCreERT2* transgene. Without the inducer, iCre was detected in the cell cytoplasm, whereas cells cultured in the presence of OHT displayed nuclear localized iCre. Transfected cells were also co-labelled with an antibody against GFP, which was observed only in iCre positive cells that were cultured in the presence of OHT (Figure 4.2A). The sub-cellular localization of the iCre protein and the expression of EGFP indicate that the nuclear translocation of iCre and its activity were highly dependent on the inducer.

4.3.2  *CAGGSET* undergoes Cre-recombination in primary culture cells

To test the possibility of recombination in primary cell culture, p*CAGGSET* and *iZ/EG* were transfected into pre-ovulatory granulosa cells. As in the test carried out in immortalized cells,
iCre was only detected in the cell nucleus in the presence of OHT, and reporter EGFP was only detected in cells where iCre was nuclear localized. This result shows that CAGGSET is functional in \textit{ex vivo} cell culture (Figure 4.2B).

### 4.4 Establishment of CAGGSET stable founder mouse lines

Having proven functional \textit{in vitro}, the CAGGSET transgene was removed from its vector backbone via \textit{Sall}/\textit{NotI} double digestion (Figure 4.1) for pronuclear microinjection (for which I am grateful to Sophie Wood, NIMR Procedural Services Division). Five individual transgenic mouse founders (out of 35 animals born in total) were positive for iCre by PCR. All founders were maintained for characterization.

To ask whether the transgenic lines can respond to tamoxifen and trigger Cre-mediated recombination \textit{in vivo}, CAGGSET mice were crossed to Rosa26 Reporter (R26R) LacZ animals (Soriano, 1999). Pregnant females were injected either with OHT or the vehicle (oil) alone at Embryonic day (E) 11.5 and embryos were harvested 48 hours later (E13.5) for X-gal staining. The presence and intensity of LacZ expression reflects the levels of CAGGSET-mediated Cre activity. In the absence of OHT, no LacZ expression was detected in the embryos from any of the five lines (data not shown). However, three lines (Lines B, M and N) displayed significant LacZ expression (Figure 4.3A) after OHT induction, suggesting CAGGSET is active in recombination. We have focused on Line B, the line that showed the most intense X-gal staining, to conduct further experiments. Despite displaying mosaicism, line B also shows highest levels of transcript and the most intense X-gal staining in the gonads, and will benefit the studies in gonadal development (Figure 4.3B and C).
Figure 4.2 CAGGSET-mediated recombination in vitro.

Plasmid CAGGSET was transiently transfected into either (A) COS7 or (B) rat granulosa cells. In both cases, a mouse monoclonal antibody against Cre was used to detect the presence of iCre (red). EGFP protein (green) was detected using a sheep anti-GFP antibody. DAPI was used to stain cell nuclei.
Figure 4.3 Three out of 5 transgenic CAGGSET founders display Cre-mediated LacZ reporter activity.

CAGGSET founder mice were crossed to R26R LacZ animals, pregnant females were induced by 1mg of OHT at E11.5 and embryos were harvested at E13.5.

(A) Out of the 5 founders, B, M and N showed significant X-gal staining, whereas only patches of X-gal staining were observed in Line A. No X-gal staining was observed in Line J.

(B) In situ hybridization of iCre on Line B gonads.

(C) X-gal staining patterns of Line B gonads.
4.5 Detailed characterization of CAGGSET mice

4.5.1 CAGGSET mice display mosaic iCre expression

Granulosa cells from CAGGSET-R26R-EYFP (Srinivas et al., 2001) females were dissociated and cultured in the presence of either OHT or the vehicle (ethanol) alone. EYFP could be detected by autofluorescence as early as 24 hours after OHT administration (data not shown). Cells were co-labelled with antibodies against Cre and GFP (for EYFP). iCre was localized in the cytoplasm of cells cultured without OHT, and in the nucleus in the presence of OHT (Figure 4.4). Not all cells were positive for iCre, suggesting the expression of the transgene CAGGSET displayed mosaicism. The reporter protein was detected only in the presence of OHT, and specifically in iCre positive cells. No reporter protein was detected in cells negative for iCre or in the absence of OHT, indicating the expression of the reporter protein was due to CAGGSET-mediated activities (Figure 4.4). This experiment showed that CAGGSET is functional in an ex vivo culture environment. However, not all the cells were iCre positive, indicating mosaic expression of the transgene, at least in ovarian cell types.

![Figure 4.4](image_url)

*Figure 4.4*

For figure legend see opposite page.

scale bar: 20um
Figure 4.5  For figure legend see opposite page.
4.5.2 **CAGGSET induces recombination in explant gonad culture**

To examine if *CAGGSET* can mediate recombination after induction in whole organs in culture, *CAGGSET-R26R EYFP* gonads were dissected from embryos at E12.5 and placed on agar blocks in DMEM. EYFP autofluorescence was detected 48 hours after OHT was administered into the medium (Figure 4.5A-C). To label the cells that have undergone recombination, antibodies against GFP and lineage-specific markers were used together for immunohistochemical staining. EYFP expression was widespread in the gonad, and in many cases co-localize with SOX9 (Figure 4.5D-F) or FOXL2 (Figure 4.5H-I), indicating Cre-mediated recombination events have occurred in the supporting cell lineage of both sexes. No recombination event was observed in the absence of OHT, proving *CAGGSET*-mediated recombination highly specific in this experimental system (Figure 4.5J-L).

4.5.3 **CAGGSET activity at different embryonic and postnatal stages**

To find out if *CAGGSET* can be used to induce recombination in embryos at different stages, pregnant females from *CAGGSET-R26R EYFP* matings were injected intraperitoneally with OHT at different time points between E7.5 and E11.5. Sufficient recombination levels were detected at all stages, indicating that *CAGGSET* may be of general use throughout embryogenesis.

*CAGGSET* also functions during postnatal stages. OHT was administered by intraperitoneal injection to *CAGGSET-R26R EYFP* pups less than 10 days of age, while 3-week old *CAGGSET-R26R EYFP* mice were injected with tamoxifen each day for a consecutive 5-day
period. As a control, an equal volume of the vehicle (oil) was administered in parallel for all experiments. Analyses show that significant levels of reporter EYFP expression were only detected from animals that have received OHT or tamoxifen, but not the vehicle alone (Figure 4.6). This shows that CAGGSET-mediated recombination is highly specific to the inducer at all stages.

CAGGSET-R26R EYFP mice were induced postnatally, and the testes were collected, cryosectioned and examined for immunohistochemistry using sheep anti-GFP (for EYFP) and SOX9 antibodies. For testes harvested at P5 and P11, in vivo induction took place two days earlier. For the adult testes harvested at 6 weeks of age, tamoxifen induction began in vivo at the age of 4 weeks. The inserts of each panel show staining patterns for testes that are negative for the CAGGSET transgene, where the expression of EYFP was not detected.

Figure 4.6 CAGGSET-mediated activity after birth.

CAGGSET-R26R EYFP mice were induced postnatally, and the testes were collected, cryosectioned and examined for immunohistochemistry using sheep anti-GFP (for EYFP) and SOX9 antibodies. For testes harvested at P5 and P11, in vivo induction took place two days earlier. For the adult testes harvested at 6 weeks of age, tamoxifen induction began in vivo at the age of 4 weeks. The inserts of each panel show staining patterns for testes that are negative for the CAGGSET transgene, where the expression of EYFP was not detected.
Figure 4.7  Recombination activities of CAGGSET are dependent on the dosage of inducer.

CAGGSET males were crossed to R26R EYFP females. Different amounts of OHT were given in utero at E11.5 by intraperitoneal injection, whereas tamoxifen was administered by oral gavage. Gonads were dissected at E13.5 and cryosectioned for immunohistochemistry for EYFP using a sheep anti-GFP antibody (green).
4.5.4 Recombination activities of CAGGSET are dependent on the dose of the inducer

Next it was asked how Cre activity responded to the dosage of the inducer. To examine Cre-mediated recombination levels in the gonads, pregnant females from the CAGGSET-R26R EYFP cross were administered with different amounts of OHT by intraperitoneal injection, or tamoxifen via oral gavage. Similarly for the adult, CAGGSET-R26R EYFP mice were induced with different amounts of tamoxifen via oral gavage. Levels of reporter EYFP expression were examined by immunohistochemical staining using an anti-GFP antibody. Figure 4.7 shows that at all stages studied, the levels of recombination were positively modulated by the amount of inducer given, with highest recombination activities achieved at the highest dosage under test (6mg of OHT for embryonic stage, 20mg of tamoxifen for adults). However, not all cells were positive for reporter EYFP, suggesting a random event of recombination. As expected, no reporter EYFP protein was detected in the absence of the inducer.

4.5.5 CAGGSET is active in different cell lineages in the gonads

Due to the mosaicism of Cre-mediated activity, we investigated whether CAGGSET-mediated recombination has lineage preferences. To examine this at the cellular level, induced CAGGSET-R26R EYFP gonads were co-labelled with antibodies against GFP and cell-type specific markers. As shown in Figures 4.8, all lineages in the gonads under test displayed CAGGSET activities, although at different levels. These results showed that recombination has taken place at least in all the major cell types we could test, although for some, not all the cells showed CAGGSET activity.
Figure 4.8  CAGGSET mediates recombination in different cell lineages in the developing gonad.

CAGGSET males were crossed to R26R EYFP females. OHT was given in utero E11.5 by intraperitoneal injection and gonads were harvested at E13.5, cryosectioned and examined for immunohistochemistry. Antibodies used for co-labelling were Sheep anti-GFP for EYFP and for lineage markers: Sox9 for Sertoli cells (A – D), 3βHSD for Leydig cells (E – H), and Stella for germ cells (I – L).
4.5.6 Position in utero contributes to the efficiency of tamoxifen-induced recombination after intraperitoneal injection

It was noticed that reporter activity varied between embryos within a litter. This was in contrast to the explant or organ culture experiments mentioned above where the inducer was applied in vitro. Therefore it was decided to test whether the relative position of embryos in the uterus would affect recombination efficiency. To ensure all embryos in a litter harbour equal transgene copy number, homozygous CAGGSET males were crossed to homozygous R26R LacZ females. With all induction time points tested, highly variable intensities of X-gal staining were detected across the same litter, indicating tamoxifen-administration did not distribute OHT equally to each embryo in utero (Figure 4.9). Although it may be difficult, further investigation would be required to address whether this is due to specific injection sites or to patterns of blood flow within the uterus, or both. However, this result needs to be borne in mind when interpreting results from embryos treated with inducer in utero.

4.5.7 CAGGSET activity was detected in a variety of tissues

Other than the gonads, recombination activities using CAGGSET were also observed in other tissues, including the brain and heart, at different stages. As these tissues were not the principle interests of this thesis, I will not discuss the results in detail. Example tissues that were positive for CAGGSET activities are presented in Figure 4.10.
Figure 4.9 Intraperitoneal injection does not deliver the inducer evenly in utero.

The 7 embryos obtained from this litter harbour the same transgene dosage. In utero induction took place at E9.5 by intraperitoneal injection of 0.5mg OHT into each side of the abdomen of the pregnant female. Embryos were harvested at E11.5.
FIGURE 4.10 For figure legend see opposite page.

<table>
<thead>
<tr>
<th>Gut (mid gut)</th>
<th>Heart (Ventricle)</th>
<th>Brain (hippocampus)</th>
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<td><img src="image2" alt="Heart Image" /></td>
<td><img src="image3" alt="Brain Image" /></td>
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Embryonic (E13.5)

Adult
4.6 Discussion

*CAGGSET* offers a Cre-mediated recombination system for conditional mutagenesis in a tightly controlled manner. Its activities were obtained in all the systems under test: *in vitro* culture, *in utero* induction, *in vivo* induction after birth, and explant culture. *CAGGSET* can also mediate recombination in developing chick embryos (collaboration with Martin Cheung and James Briscoe, data not shown) and will therefore be a useful tool in this system. As the focus of this thesis is mammalian gonadal development, the successful achievements in the chick model will not be discussed here. However, *CAGGSET* could well be of use to study gonadal development *in ovo*, and also in *ex ovo* gonad culture.

The *CAGGSET* plasmid was transiently expressed in culture systems and proved functional. This is particularly useful to test responder transgenes in an *in vitro* environment, providing a quick and reliable way to test newly created constructs (see Chapter 5).

*CAGGSET*-mediated recombination can be induced at different stages during development and in postnatal mice. It is the first inducible-Cre driver shown to display recombination activity at neonatal stages. *CAGGSET* allows studies of gene function in a highly time-controlled manner: it can delete an already expressing floxed gene in different tissues at a desired time; it can also mediate ectopic gene expression.

Induction via intraperitoneal injection *in utero* demonstrated highly variable X-gal staining within a litter, suggesting uneven distribution of OHT. The two embryos showing highest staining intensity could have been situated closest to injection sites, and have therefore
received OHT more efficiently. An alternative method for \textit{in utero} induction is oral gavage, which requires assimilation of the drug into the circulation system of the pregnant female. Comparison between these two types of induction methods revealed that although oral gavage distributes the inducer more evenly to all embryos, less recombination was observed within 24 hours after induction compared to intraperitoneal injection. However, similar levels of recombination were achieved 48 hours after induction, indicating that both methods resulted in recombination activity, but at different efficiencies. This has also been noted by others using Cre-ERT2 based systems (A Joyner, personal communication).

\textit{CAGGSET}-mediated recombination displayed mosaicism, which perhaps reflects the biology of recombination and tamoxifen half-life. Mosaic transgene expression is also a common problem in transgenic mice generated by random integration and usually reflects position effects. There are two subtypes of position effect, stable, or variegation. Stable position effects mean that the integration site influences the transcriptional activities of a transgene. Under this influence, the same transgene integrated at different loci will harbour different transcriptional efficiencies, and therefore display different expression levels. The 5 different \textit{CAGGSET} mouse lines generated are good examples of this effect: the injected embryos carried the same transgene but displayed different activities due to different sites of chromosomal integration (Kioussis and Festenstein, 1997).

Variegation position effects occur when a transgene is influenced by local chromatin structure, which can vary between the same cell type (for example if integration occurred at a boundary between heterochromatin and euchromatin), between different cell types, and/or between different stages. All of these can lead to gene silencing in an unexpected or random
fashion. As the Cre antibody or probe did not work on sections or wholemount tissues, it was not possible to examine whether the mosaic pattern of CAGGSET-induced reporter activity was due to position effect variegation or to the dynamics of iCre-mediated recombination within specific cell lineages. However, it is possible to conclude that all of the cell lineages studied displayed significant levels of CAGGSET-mediated iCRE activity, proving it useful for developmental studies.

Furthermore, the mosaicism can be advantageous for specific tests; notably CAGGSET allows comparison between mutant and wild type cells within the same environment, and consequently the different downstream cellular events that occur due to the mis-expression of a transgene, or an altered genetic event. For example, multiple attempts have been made to study gene function in the Sertoli cell lineage. However, these generally give rise to sex reversal or severe testis cord disorganization, due to the complete extinction of Sertoli cells (e.g. Barrionuevo et al., 2006; Gao et al., 2006). In these circumstances it is difficult to know whether a particular effect is due to cell autonomous action of a gene or not, and whether disorganised tissue architecture leads to secondary effects on the cell lineage being studied. It is therefore useful or necessary to use an effector, or a Cre-driver in our case, which does not alter gene expression in the whole lineage. In this way it should be possible, for example, to investigate how specific genes act within Sertoli cells, build genetic pathways within the lineage, and ask how Sertoli cells influence other cell lineages. Mosaic Cre-mediated recombination can also be useful for lineage studies. Observations from co-labelling experiments have proved CAGGSET a good tool for all these purposes, within the gonad or in other tissues.
Loss- or gain-of-function of a gene can lead to embryonic lethality, which might render in vivo gene function studies unfeasible. Sox9 provides a good example of this limitation: it has been shown that embryos homozygous for Sox9 deletion die around E11.5, a stage before the sex of the embryo could be morphologically distinguished (Akiyama et al., 2002). In such a case, CAGGSET could serve to bypass embryonic lethality either by inducing Cre-mediated recombination later, or by reducing recombination efficiency by modulating inducer dosage. Another option is explant culture, in which CAGGSET displayed higher recombination efficiencies.

CAGGSET activity is highly specific to the inducer, which is an absolute requirement for studies involving cell lineage tracing or gene function at a particular time point. Any undesired activity will abdicate the principle of the inducible Cre system and impair the accuracy of the experiments. In this respect, CAGGSET is certainly advantageous over previously established lines, which displayed low levels of recombination activity in the absence of tamoxifen (e.g. CMV-CreERT2; CMV-CreER™).

Recombination using CAGGSET could be activated not only in all gonadal cell lineages under test, but also in various tissues and cell lineages in the mouse. For example, CAGGSET demonstrated recombination activities in the adult brain (data not shown), which has not been reported for other tamoxifen-inducible Cre mouse lines. Tissue-specific recombination studies in various tissues and at various stages would benefit from the use of CAGGSET.
CHAPTER 5

CREATING RESPONDER TRANSGENES FOR MISEXPRESSSION STUDIES DURING GONADAL DEVELOPMENT IN MAMMALS

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5.6.3 Z/Sox9 on a C57BL6 background are poor breeders

5.7 Discussion
5.1 Introduction

The term "responder" is used here to refer to an allele that has been genetically engineered to respond to the activity of a site-specific recombinase in such a way as to trigger an alteration in gene activity, usually loss or gain of function. Although it has been published that loss or gain of function of *Sry* or *Sox9* can lead to sex reversal phenotypes in both humans and mice (Chapter 1), it is not known if altering gene expression after sex determination can lead to cell-fate reversal. To address this question, conditional alleles for *Sry* and *Sox9* become necessary. So far, conditional loss-of-*Sox9*-expression alleles have been generated in mice (Chaboissier et al., 2004; Barrionuevo et al., 2006). *Sry* is expressed briefly in the genital ridge, which would mean there a conditional loss of function allele. This leaves conditional misexpression alleles for *Sry* and *Sox9* to be created. This chapter describes the design, establishment, and tests of conditional misexpression alleles for *Sry* and *Sox9*.

An ideal conditional misexpression allele, which can be introduced into mice as a transgene or through gene targeting (but see below), requires three elements: (1) the promoter, which controls where and when the gene of interest is expressed; (2) a "stop" cassette that is flanked by recombinase-recognizable sequences to block transcription of the conditional allele, i.e., the gene of interest. This can be the stop codon on its own, or include a reporter gene before the stop codon. The presence of this reporter reflects promoter activity, whereas its disappearance indicates a successful recombination event; (3) the responder allele, expression of which is not allowed until the removal of the reporter allele. A schematic representation is shown in Figure 5.1.
As mentioned in Chapter 1, the Cre/loxP conditional system was chosen for gene alteration studies. For the responder transgenes, cDNA sequences of Sry and Sox9 were used for subcloning. The Sry gene was discovered in the host lab (Gubbay et al., 1990) and its expression in XX embryos led to sex reversal XY female sex reversal (Koopman et al., 1991). To misexpress Sox9, a previously reported cDNA was employed (Wright et al., 1995), but in addition, a transgenic construct that allows conditional expression of the mouse genomic Sox9 DNA (Z/Sox9, Sarah Wynn et al., unpublished) was also adopted. In all cases, transgenes were driven by a ubiquitous promoter. The floxed “stop” cassette included an ECFP reporter protein, which allows live detection of cells displaying promoter activity. Under the activity of the ubiquitous promoter, the reporter ECFP will be expressed in all cell types and at all times, whereas the expression of the responder gene will be induced by cellular Cre-activity, which is in turn defined by the regulatory elements that drive the expression of Cre (Chapters 3 and 4).
5.2 Creating responder transgene alleles

5.2.1 CMV:ECFP/Sox9 (COND-FlagSox9)

To create the floxed ECFP-reporter allele, the \textit{pGK-neo-pGK} fragment in the vector \textit{pBigT} (Srinivas \textit{et al.}, 2001) was replaced by \textit{ECFP} via blunt-ended ligation. Using this as a backbone, the cDNA of Sox9, which has a flag tag inserted into its 5' end, was inserted after the floxed \textit{ECFP} cassette to replace an existing \textit{EYFP}, generating the shuttle vector \textit{TS9C2} (Figure 5.2A). The floxed \textit{ECFP}/Sox9 fragment from \textit{TS9C2} was then subcloned into the plasmid \textit{pCS2+}, to generate a conditional Sox9 cDNA expression plasmid, \textit{COND-FlagSox9} (Figure 5.2B). As a control for this plasmid, a ubiquitous \textit{FlagSox9} construct was also made by removing the floxed \textit{ECFP} fragment in \textit{COND-FlagSox9}, generating \textit{CMV:FlagSox9} (Figure 5.2C).

5.2.2 Building a conditional Sry-responder:

Due to the problems encountered during molecular cloning, parallel strategies were used to make an \textit{Sry}-responder transgene. In both attempts, an \textit{Sry}^{Myc} transgene was used. This encodes a Myc tagged SRY protein, with 6 copies of the Myc tag inserted at the 3-prime end of the \textit{Sry} open reading frame. This was proved functional, as it caused sex reversal in mice (Sekido \textit{et al.}, 2004).
Figure 5.2  For figure legend see opposite page.
(I) **CMV:ECFP/SryMyc6 (COND-SryMyc6)**

In the first strategy to generate the Sry-responder, the floxed ECFP-triple pA fragment from TS9C2 (Figure 5.2A) was inserted between the CMV promoter and the SryMyc sequences in the pcDNA3 plasmid to generate COND-SryMyc6 (Figure 5.3).

![Figure 5.3 Molecular cloning of the COND-SryMyc6 construct.](image)

To generate a conditional Sry allele, the floxed ECFP-triple pA sequence from earlier made plasmid TS9C2 was excised by NheI/SpeI double digestion and inserted into the XbaI site of plasmid pcDNA-SryMyc6 (Lovell-Badge lab plasmid stock 0076). This transgene, CMV:ECFP/SryMyc6, is designated as COND-SryMyc6.

(II) **CAG:ECFP/SrvMvc6 (AngelBLUE-Sry)**

Another strategy to make the Sry-responder transgene was to use the CAG promoter to drive conditional expression of Sry. To start with, the floxed ECFP-triple pA fragment was inserted into the plasmid pCAGGS (Niwa et al., 1991), generating a vector called AngelBLUE (Figure 5.4A). The SryMyc sequences were amplified by PCR, and inserted into the region after the floxed ECFP-triple pA sequence of AngelBLUE, giving AngelBLUE-Sry (Figure 5.4B).
Figure 5.4 Molecular cloning of the AngelBLUE-Sry construct

(A) To make the shutter vector AngelBLUE, the floxed ECFP-triple pA sequence from earlier made plasmid TS9C2 was excised by Nhel/Spel double digestion and inserted into the Xhol site of plasmid pCAGGS (Niwa et al., 1991) via compatible-end ligation.

(B) PCR cloning was employed to bring in the SryMyc6 cDNA: Primers for Sry was designed with SalI linkers attached to flanking ends. The amplified fragment was then digested by SalI and inserted into the Xhol site of AngelBLUE via compatible-end ligation. This transgene, CAG:ECFP/SryMyc6, is designated as AngelBLUE-Sry.
5.3 *In vitro* functionality tests for responders

After sequence verification at all cloning junctions, constructs were tested *in vitro*. As the aim was to observe gene misexpression effects at different stages using the same Cre-driver, a tamoxifen-inducible Cre/loxP system was employed. The responder constructs were therefore co-transfected with plasmid CAGGSET into immortalized COS7 cells.

5.3.1 *CMV*:ECFP/Sox9 (COND-FlagSox9)

COND-FlagSox9 and *CMV*:FlagSox9 were tested in parallel. Both were co-transfected with CAGGSET and treated with either 4-hydroxytamoxifen (OHT) or the vehicle (ethanol) alone. In any experimental condition, neither of the Sox9 constructs expressed SOX9, even though iCre became nuclearly localized in the presence of OHT in the culture medium (data not shown). This was found to reflect a problem in the Sox9 cDNA itself. Sequencing the full length of the construct revealed a missing basepair in the Flag sequence, which shifted the translation initiation codon and therefore no flag or SOX9 protein could be produced (data not shown).

5.3.2 Plasmid SW88 (Z/Sox9)

![Diagram](Z/Sox9.png)

*Z/Sox9:*

CMV enh Chicken β-actin promoter

Figure 5.5 Schematic diagram of the Z/Sox9 transgene.
The alternative conditional Sox9 construct, derived using the plasmid SW88 contains the CAG ubiquitous promoter driving a floxed β-geo-stop/mouse genomic Sox9-encoding region transgene, termed Z/Sox9 (Figure 5.5, Sarah Wynn, unpublished). SW88 was transiently transfected into COS7 cells together with the CAGGSET plasmid. EGFP, which reports recombination activity, could be detected as early as 12 hours after administration of OHT. SOX9 was detected in cells that had nuclear localized iCre, indicating the expression of the transgene is due to CAGGSET-mediated activity (Figure 5.6). SOX9 or EGFP was not detected in non-transfected cells or in transfected cells in the absence of OHT, indicating that the expression of Sox9 and EGFP is specific to Cre activity (Figure 5.6). Transfected cells cultured without OHT showed X-gal activity, indicating the floxed β-geo gene is also functioning correctly (data not shown).

**Figure 5.6**  
For figure legend see opposite page.

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5.3.3 \textit{CMV:ECFP/SryMyc6 (COND-SryMyc6)}

Similar experiments were performed using \textit{COND-SryMyc6}. ECFP was detected 12 hours after transfection in cells cultured in the presence of OHT. Antibody co-labelling revealed that in the absence of iCre or its nuclear localization, ECFP and low amounts of MYC were expressed. However, cells cultured in the presence of OHT displayed much higher levels of MYC (Figure 5.7), indicating \textit{CAGGSET}-mediated recombination gives a significant expression of \textit{Sry-myc} and ‘leakage’ is a minor problem.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.7.png}
\caption{In vitro functionality test of the construct \textit{COND-SryMyc6}}
\end{figure}

Immunostaining was done on COS7 cells co-transfected with \textit{CAGGSET} and \textit{COND-SryMyc6}. A and D: DAPI staining on cell nuclei; B: in the absence of OHT, iCre was detected in the cytoplasm, ECFP autofluorescence was observed in the cell nucleus, however, small number of cells expressed myc (C); E: in the presence of OHT, iCre was detected in the cell nucleus, which co-localized with myc expression in several cells (F).
5.3.4 CAG:ECFP/SryMyc6 (AngelBLUE-Sry)

AngelBLUE-Sry was also co-transfected with CAGGSET into COS7 cells. Like COND-SryMyc6, ECFP was detected by autofluorescence after OHT administration. In the absence of OHT, iCre was detected in the cell cytoplasm and ECFP was co-localized with several iCre positive cells. Trace levels of MYC were detected in these cells, indicating minor leakage problems (Figure 5.8A-C). However, in the presence of OHT, most of the cells that were positive for nuclear-localized iCre also displayed MYC expression (Figure 5.8F). Background Myc expression was seen in some cells which are iCre negative, indicating the possible leakage problem was due to the responder construct but not CAGGSET (Figure 5.8D-F, white arrows).

Figure 5.8  For figure legend see opposite page.
5.4 Establishing stable transgenic founder mice

Following the functionality tests of the responder alleles *in vitro*, transgenic mice were made using *AngelBLUE-Sry*, and *SW88* for conditional misexpression of *Sry* and *Sox9*, respectively. Pronuclear microinjection was carried out by the Procedural Services Team of the NIMR (with thanks to Sophie Wood).

5.4.1 *AngelBLUE-Sry*

To remove the transgene from the vector backbone, plasmid *AngelBLUE-Sry* was digested by *SalI* and *HindIII*. The resulting 5.5kb transgene fragment was then purified for pronuclear microinjection.

5.4.2 *Z/Sox9*

Plasmid *SW88* encodes the transgenic *Z/Sox9* allele. The schematic diagram of this construct is presented in Figure 5.5. To remove the transgene from the vector backbone, plasmid *SW88* was digested by *SalI* and *NotI*. The resulting 11.5kb transgene fragment was then purified for pronuclear microinjection.

5.5 The *AngelBLUE-Sry* transgenic mouse

Only one transgenic founder (out of 47 genotyped born pups) was obtained from microinjection with *AngelBLUE-Sry*. This line did not display detectable ECFP, either by
autofluorescence or immunostaining on gonad sections, and was not characterized further.
The most likely reason to explain this is a chromosomal insertion effect. In this particular
animal, the transgene might have been inserted into a locus that is transcriptionally silent.

5.6 **Characterization of Z/Sox9 founder mouse lines**

5.6.1 *Z/Sox9 mice expressed the floxed \( \beta\)-geo transgene*

Six pups (out of 71) generated by pronuclear microinjection were positive for *LacZ* by PCR.
Five (Lines B, J, M, S, and V) of these founders were generated on the CBA/B10 F1 background, and one (Line C) was generated on the C57BL6 background. During characterization of these transgenic founders, we received *Z/Sox9*-10F mice from the Cheah lab (unpublished). This line carried only one copy of the transgene, and was created by germline transmission from chimaeras made by injecting host blastocysts with transfected embryonic stem cells. It was maintained on a mixed 129Sv/J; C57BL6 background. All *Z/Sox9* lines were characterized for transgene expression.

Of the 7 lines generated by pronuclear injection, only 4 displayed X-gal activity in embryos positive for the *Z/Sox9* transgene (Lines B, C, J, M, Figure 5.9A-J). Alongside Line C, 10F, which is generated by injecting blastocysts, showed highest intensities of X-gal staining. X-gal activities were also seen in both gonad subtypes of these lines, with the exception of Line M, which did not show any X-gal activity either in wholemount XX embryos or their ovaries (Figure 5.9K-T). The founder male of Line M had only one transgenic pup (out of 70 genotyped). This was a female, and her offspring did not display X-gal staining. We also
observed that as the stage of harvest (between E9.5 and E13.5) proceeded, fewer transgenic embryos compared to wildtypes were obtained from breeding the founder, which suggested embryonic lethality. This could also explain why only one transgenic pup was obtained.

![Diagram of transgenic expression](image)

*Figure 5.9  For figure legend see opposite page.*
5.6.2 \( Z/Sox9 \) expression varies in different genetic backgrounds

It has been shown that several phenotypes related to gonadal development in mice were more readily seen on the C57BL6 genetic background (e.g. \( Fgf9 \), Schmal et al., 2004; DiNapoli et al., 2006). For comparison purposes, backcrossing was set up for individual \( Z/Sox9 \) lines in parallel to their characterization. Changes in the X-gal staining pattern were observed in at least Line B. On the mixed CBA/BL10 background, embryos positive for \( LacZ \) by PCR showed a consistent staining pattern (Figure 5.9B and G). However, in crosses between a male homozygous for Line B on this mixed-background and C57BL6 females, embryos from the same litter displayed highly variable X-gal staining in different tissues (Figure 5.10). As each embryo carried equal transgene copy numbers and no recombination event was involved, it seems likely that the variable genetic background was the most likely cause for the difference in X-gal staining pattern.

\[
\begin{array}{c}
\text{Z/Sox9:} \\
\text{CMV enh Chicken \( \beta \)-actin promoter} \\
\text{\( \text{LacZ} \)} \\
\text{\( \text{Sox9} \)} \\
\text{IRES} \\
\text{\( \text{EGFP} \)}
\end{array}
\]

Figure 5.10 X-gal staining intensities vary within a litter of \( Z/Sox9 \) (B) of mixed genetic background.

Crosses were set up between a \( Z/Sox9\)-(B) male with a C57B1/6 female. Embryos were harvested at E11.5 for X-gal staining.
5.6.3 Z/Sox9 on a C57BL6 background are poor breeders

Another observation during back-crossing onto C57BL6 was the loss in number of transgenic pups. By the first backcrossing step onto C57BL6, transgene transmission rate of Line 10F dropped to 25%. No transgenic pup was obtained by the second generation. Also, several transgenic pups were much smaller in size and weaker compared to wildtype littermates. Several transgenic mice were also lost postnatally. Similar findings were obtained from Lines J during the backcrossing process. Line C was the only line generated by pronuclear injection on C57B1/6 and displayed strong X-gal staining. It has produced extremely low numbers of transgenic pups so far. Altogether, these results suggest that the Z/Sox9 transgene might not be well maintained on C57B1/6. For maintenance of the transgenic lines, Lines 10F and C are now maintained on a mixed background with 129Sv/J.

5.7 Discussion

Due to the complexity of the design for the responder transgenes, it is essential to make sure every part of the transgene function correctly before using them to make mice. During the tests *in vitro*, minor leakage problems were encountered. This could be due to the use of immortalized cells in the culture system. As the number of cells displaying leaky expression patterns was less than 5% in all cases, it was concluded that this should not be of concern in making transgenic mice.
Z/Sox9 mice were generated using two strategies: We have generated several lines (Lines B, C, J and M) by pronuclear microinjection. The copy number of the transgene and its expression levels were not known before the generation of the founder animals. On the other hand, the imported 10F line was generated by electroporation into embryonic stem (ES) cells, clones of which were screened for high β-galactosidase activity and to ensure that there was only a single copy of the transgene. Undoubtedly, transgenic mice harbouring loxP sites with unknown copy number could raise concern. For example, if multiple insertion of the transgene happened at one single site, Cre-mediated removal of segments will become a random event, which may not consistently express the conditional allele in all cases (Figure 5.11A). Rarer events include transgene insertion into multiple loci, which may happen within the same chromosome (Figure 5.11B), or different chromosomes, or events where one copy is not head-to-tail as usual, but has been inserted in an inverted orientation relative to other copies. All such cases may lead to chromosome translocations (Figure 5.11C). On the other hand, it has previously been reported that there was a good correlation between the levels of transgene expression observed in ES cell clones and the resulting expression seen in transgenic mice (Novak et al., 2000). However, there were also cases when transgene expression levels in mice did not correspond to those in the ES cell clone (James Critchley, PhD thesis, 2005). In our hands, both Line C (generated by microinjection) and 10F (generated by ES cell screening and blastocyst injection) displayed robust β-galactosidase activity, indicating feasibility of both methods to achieve desirable results.

The third alternative to generate high expressor transgenic mouse lines is by gene-targeting. Different loci have been identified to produce robust transgene expression without influencing or being influenced by intrinsic factors. Several of these, including Rosa26 on
chromosome 6, were made susceptible for gene targeting (Soriano, 1999; Srinivas, 2001). Inserting the driver transgene into the Rosa26 locus should overcome the uncertainty of position effects, and allow more appropriate conditional transgene expression patterns to be generated.

Figure 5.11 Possible genomic insertion events of a transgene introduced by pronuclear microinjection.

(A) Multiple copy number of the transgene inserted into the same genome locus. Upon Cre mediated recombination, loxP sites will be removed by random events. In certain cases, this will not lead to Sox9 expression, but instead another LacZ cassette.
(B) Copies of transgene inserted into multiple loci on the same chromosome. This is a rather rare event, but if this occurs it can lead to deletion of the genes situated between the transgene insertion loci.
(C) Chromosomal translocation between loci on different chromosomes. This is another rare event, which can affect not only transgene expression pattern, but also intrinsic gene expression.

The progeny of Z/Sox9 line M could not be maintained. The absence of transgene expression in the XX suggested gene silencing. The only transgenic female obtained from the founder bred normally, however her offspring of either sex did not display β-galactosidase activity. Also, strong expressors of the transgene might have died during embryogenesis, as the
number of transgenic embryos obtained declined as the stage of harvest progressed. This also explained why only one transgenic pup was obtained out of 70 born and genotyped.

Lines 10F and C displayed intense X-gal staining. Both comprised a high proportion of the C57BL6 genetic background. This background seems favourable for improving transgene expression, as Line B displayed stronger expression patterns when mixed with C57BL6. It has been shown that several phenotypes related to sex determination gene mutations are more robust in a C57BL6 background (e.g., Fgf9\textsuperscript{-/-}, Schmal \textit{et al.}, 2004; DiNapoli \textit{et al.}, 2006), and we therefore wanted to expose Z\textit{Sox}9 to this genetic background. However, during backcrossing, it was impossible to maintain Z\textit{Sox}9 lines on this background, perhaps with the exception of Line B. Strong expressing lines 10F and C suffered from loss of pups and general weakness under the influence of C57BL6. Instead, both lines are now being kept on a mixed background with 129Sv/J, which seemed successful with Line 10F so far. It will require further characterization during outbreeding to monitor the influence of different genetic backgrounds. Backcrossing will continue on Line B, with all offspring being examined.
CHAPTER 6

ALTERING Sox9 EXPRESSION IN THE MOUSE GONAD

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6.6 Discussion
6.1 Introduction

During gonadal development in the mouse, Sox9 is weakly expressed in the genital ridge of both sexes until the onset of Sry expression, from when it is rapidly upregulated in the XY but downregulated in the XX genital ridge (Morais da Silva et al., 1996). Testicular expression of Sox9 then persists throughout life, specifically in Sertoli cells. The upregulation of Sox9 was found to coincide with the onset of Sry expression in supporting cell precursors and the protein products of the two genes can be seen to co-localise within a subset of these cells prior to Sry switching off (Sekido et al., 2004). These data strengthened the belief that Sox9 is a direct downstream target of SRY.

Sox9 is essential for testis determination in both humans and mice. Heterozygous mutations in SOX9 in Campomelic Dysplasia patients are often associated with XY female sex reversal (Foster et al., 1994). In mice harbouring heterozygous loss of function mutations in Sox9, the testes develop normally despite perinatal lethality (Bi et al., 2001). Mice harbouring a homozygous deletion of Sox9 die around embryonic day (E) 11.5 (Akiyama et al., 2002), rendering studies of gene function at later stages feasible only by organ culture or conditional mutagenesis. Both approaches lead to XY female sex reversal (Chaboissier et al., 2004; Barrionuevo et al., 2006). Conversely, ectopic expression of a Sox9 transgene in the XX gonad led to male development (Vidal et al., 2001) as does disregulated expression of Sox9 due to a transgene insertion in Oddsex mice or duplication of the SOX9 locus in humans (Qin et al., 2004). Taken together, Sox9 is not only essential for initiating testis differentiation, but also able to do so in the absence of Sry. However, the precise function of SOX9 at different stages and the reason for its persistent expression in Sertoli cells remain unknown. Using CAGGSET, as an inducible-Cre driver that can mediate gene expression or deletion in the
developing gonad at any desired stage, the aim was to ask what happens if Sox9 expression is altered after sex determination has already taken place.

6.2 Materials and Methods

Experiments were performed using established mouse lines that have been described in previous chapters. CAGGSEfT and Flox-Sox9 (Sox9\textsuperscript{FloxFlox/}, Akiyama et al., 2002) mice are kept on the CBA/BL/10 mixed background. Z/Sox9-10F mice (Cheah Lab, unpublished) were generated on a 129Sv/J X C57BL/6 mixed genetic background, and were maintained by backcrossing to C57BL/6. With the exception of Line C, other Z/Sox9 lines were maintained on a mixed CBA/C57BL/10 background. Line C was generated and maintained in C57BL/6. Subsequent backcrossing events will be discussed where relevant. \textit{\beta}-actin-Cre mice (Lewandoski et al., 1997a) were maintained as homozygotes and were provided by the Specific Pathogen Free (SPF) unit of the NIMR, and have a mixed genetic background. To allow cells, tissues or animals carrying the homozygous null allele of Sox9 to be generated in a conditional/inducible manner, Flox-Sox9 and CAGGSEfT mice were bred together. Male and female offspring were then intercrossed until animals homozygous for the flox allele (Sox9\textsuperscript{FloxFlox}) were obtained. For analyzing Z/Sox9 mice that were heterozygotes (Z/Sox9\textsuperscript{Loc2WT} or Z/Sox9\textsuperscript{GFPWT}), homozygous CAGGSEfT (CAGGSEfT\textsuperscript{Cre/Cre}) mice were used to increase the probability of obtaining double transgenic mutants.

The nomenclature for transgenes used in this chapter is summarised in Appendix D.
6.3 **Conditional Sox9 misexpression mediated by β-actin-Cre**

The first steps of characterizing the Z/Sox9 mouse lines involved testing their ability to respond to Cre-mediated activity. To do this Z/Sox9 lines were crossed to β-actin-Cre mice, which provide robust Cre activity throughout gestation. Analyses were based on observation of possible sex reversal phenotypes. However, as Sox9 displays widespread expression pattern in numerous tissues, phenotypic observation in some of these tissues would also be briefly described in this section.

6.3.1 **Ubiquitous misexpression of Sox9 is embryonic lethal**

To test whether Z/Sox9 mice can respond to Cre-mediated recombination, Lines C, J and 10F were bred to β-actin-Cre. Embryos were dissected at E13.5 for examination. Embryos positive for both Cre and the Z/Sox9 transgene from Line J were not fluorescent (data not shown) indicating insufficient transgene expression. 16 embryos from four litters were obtained by crossing Line 10F with β-actin-Cre. None were positive for the Z/Sox9 transgene. No positives were identified amongst 13 pups from Line 10F. Similar results were obtained from Line C. The small litter sizes and failure to obtain double transgenic embryos/pups indicated possible embryonic lethality. To investigate this assumption, analyses were carried out at earlier embryonic stages.

Small numbers of Cre; Z/Sox9 double positive (Cre+; Z/Sox9GFP/WT) embryos from Lines 10F and C were obtained between E10.5 and E12.5. These embryos were positive for EGFP as observed under a fluorescent microscope (Figure 6.1), but not for LacZ by PCR
genotyping (data not shown), indicating that the floxed β-geo reporter gene has been successfully removed and Sox9-iRES EGFP activated. We also observed that the number of transgenic embryos obtained decreased as the stage of harvest progressed, supporting the hypothesis that overexpression of Sox9 causes embryonic lethality.

Figure 6.1 Wholemount embryos of β-actinCre; Z/Sox9 at E12.5. EGFP was detected by autofluorescence under a fluorescent microscope.

(A) Line 10F
(B) Line C
Figure 6.2  Gonads everexpressing Sox9 at E12.5. β-actin Cre mice were crossed with Z/Sox9-1OF mice. Embryos were harvested at E12.5 and gonads dissected out for examination.

(A) Wholemount gonads under a fluorescent microscope with a GFP filter.

(B) Gonads were cryosectioned afterwards for immunostaining. SOX9 and EGFP were ectopically expressed in β-actin Cre; Z/Sox9 XX gonads at E12.5. (A;C) Gonads wildtype for the Z/Sox9 transgene; (D;F) Gonads transgenic for Line 10F; (G;I) Gonads transgenic for Line C. For co-labelling a chick anti-GFP and an antibody against SOX9 were used. The inserts on the bottom left corner show a magnified view of the indicated area of the gonad shown in each panel.

MC: mesonephroi; G: gonads
6.3.2  *Sox9* misexpression leads to cord formation in the XX genital ridge

EGFP positive embryos from Lines 10F and C were analysed further. Initial observation indicated that XX double positive embryos display gonads resembling ovaries (Figure 6.2A), which are also positive for EGFP under a fluorescent microscope. However, immunohistochemistry revealed the presence of SOX9 and EGFP in these XX gonads, although not all SOX9-positive cells were positive for EGFP (Figure 6.2B). Strikingly, SOX9 positive cells seemed to be arranged in structures resembling testis cords, implicating partial sex reversal (Figure 6.2B).

6.3.3  *Sox9* misexpression upregulates *Sfl* and *Amh* in the XX genital ridge

Following the observation of cord-like structures in XX gonads that are ectopically expressing *Sox9*, it was asked if SOX9 downstream targets were upregulated in these XX gonads. SOX9 has been shown to activate the *Sfl* promoter *in vitro* (Shen and Ingraham, 2002). It has also been proved that SOX9 is essential for *Amh* expression *in vivo* (Arrango *et al.*, 1999). We therefore examined if these factors were expressed in the β-actin-Cre; Z/Sox9 XX gonads following the misexpression of Sox9. At E12.5, AMH was detected in the coelomic epithelial cell layer of XX 10F mutant (Cre⁺; 10F<sup>GFP/WT</sup>) gonads, but not in wildtype (Cre⁺; Z/Sox9<sup>WT/WT</sup>) gonads (Figure 6.3A-F). Line C mutant gonads (Cre⁺; C<sup>GFP/WT</sup>) also expressed AMH, but at much lower levels compared to that of 10F (Figure 6.3G-I). Interestingly, the presence of AMH did not always co-localize with SOX9.

On the other hand, significant levels of SF1 could be detected in XX Cre⁺; C<sup>GFP/WT</sup> gonads, some of which co-localized with EGFP, within the testis cord-like structures. SF1 was not
detected, or at very low levels, in wildtype XX gonads (Figure 6.4), indicating the presence of SF1 in Cre\(^{+}\); \(C^{GFP/WT}\) gonads was due to the ectopic presence of SOX9.

**Figure 6.3** For figure legend see opposite page.
Figure 6.4  *Sf1* is expressed at much higher levels in XX gonads overexpressing *Sox9*.

B-actin Cre mice were crossed to Z/Sox9-C mice. Embryos were harvested at E12.5 and gonads were dissected out, cryosectioned and examined for immunohistochemistry for SF1. To indicate which cells might be ectopically expressing Sox9, an antibody against GFP was also used for co-labelling. SF1 was found highly expressed in gonads transgenic for the Z/Sox9 transgene (D-F), but at only low levels in the gonads that are negative for the transgene (A-C). Note that the sub-cellular localization of EGFP is both cytoplasmic and nuclear.

M: mesonephroi; G: gonads.

6.3.4  *Sox9* misexpression leads to developmental defects in other tissues

*Cre*+/10Pt<sup>GFP/WT</sup> embryos also displayed other morphological phenotypes by E12.5. Mutants displayed various levels of craniofacial defects, ranging from gaps around the oral area (Figure 6.5A-E) to internal bleeding of the forehead (Figure 6.5A). These embryos were also
smaller, more fragile, and more hunched (Figure 6.1). Compared to their wildtype \((Cre^-; 10F^{WT/WT})\) littermates, the lungs and heart also displayed various levels of abnormalities (Figure 6.6). This includes reduction in size of the lungs, and incomplete formation of the heart, especially the auricles. In one embryo, the lungs were entirely absent (data not shown). The limbs were shorter compared to those of wildtype littermates, and in some cases were still at limb-bud stages, and had failed to start forming digits (Figure 6.1). These observations suggested that \(Sox9\) overexpression might have led to developmental retardation in several organs, although the primordial of these organs did form. It is highly likely that embryonic lethality was caused by heart defects, but further investigation is required to confirm this assumption.

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**Figure 6.5**  For figure legend see opposite page.
Figure 6.6  For figure legend see opposite page.
6.4 **CAGGSET-mediated misexpression of Sox9 in the developing gonads**

With the purpose of bypassing embryonic lethality, the effects of misexpressing Sox9 were examined after crossing Z/Sox9 mice with CAGGSET, and subsequently inducing CRE activity with tamoxifen or 4-hydroxytamoxifen (OHT). More importantly, this would allow the effects of Sox9 expression in the XX gonad after sex determination to be tested. Experiments were carried out using Z/Sox9-10F.

**Figure 6.7** For figure legend see opposite page.
Figure 6.8 SOX9 and AMH expression in CAGGSET; Z/Sox9-10F XX gonads.

CAGGSET mice were crossed with Z/Sox9-10F mice. Pregnant females were injected with OHT at E8.5 and embryos were harvested at E14.5. Gonads were dissected out, cryosectioned and examined for immunohistochemistry for SOX9 and AMH. Low levels of SOX9 and AMH could be detected in gonads transgenic for both iCre (for CAGGSET) and GFP (for Z/Sox9) by PCR (D-F), but not gonads that were positive for iCre but negative for GFP (A-C). Inserts at the bottom-left corners show a magnified view of an area that misexpresses SOX9. Note that the strong staining obtained from both transgenic and wildtype gonads were non-specific staining, whereas the area highlighted were specific for both SOX9 and AMH.

M: mesonephroi; G: gonads.

6.4.1 Induction prior to sex-determination leads to cord formation in the XX gonad

First, the effects of CAGGSET-induced Sox9 expression were examined prior to sex determination. Pregnant females from CAGGSET X 10F crosses were administered with
OHT between E8.5 and E9.5 by intraperitoneal injection, and embryos were harvested between E13.5 and E14.5. Under a light microscope, no cord-like structures could be detected in XX \textit{CAGGSET}^{\text{Cre/WT}}; \textit{1GF}^{\text{GFP/WT}} gonads (Figure 6.7). However, examination at cellular levels revealed SOX9-positive cells in these XX double positive gonads, forming structures resembling testis cords (Figure 6.8). AMH expression was also examined, but found to be not significantly different between \textit{CAGGSET}^{\text{Cre/WT}}; \textit{1GF}^{\text{GFP/WT}}- and \textit{CAGGSET}^{\text{Cre/WT}}; \textit{1GF}^{\text{WT/WT}} (wildtype) gonads (Figure 6.8).

6.4.2 Induction prior to sex-determination leads to more cord-like structures in the XY gonad

XY \textit{CAGGSET}^{\text{Cre/WT}}; \textit{1GF}^{\text{GFP/WT}} positive gonads were not significantly different from wildtype gonads by wholemount observation (Figure 6.7). However, immunohistochemistry revealed widespread expression of EGFP, both within and outside testis cords, as marked by AMH (Figure 6.9D-F). Surprisingly, EGFP positive, AMH negative cells also seemed to form cord-like structures (Figure 6.9F). Double antibody staining for SOX9 and AMH was also performed, in which SOX9 displayed similar expression patterns to EGFP. Like EGFP, SOX9 was also found within and outside testis cords (Figure 6.9J-L), with the latter displaying a less intense staining for SOX9 (Figure 6.9L). It is possible that these cords are premature at the stage of examination, and may become proper testis cords later, or alternatively, they may never fully differentiate further because of the low levels of SOX9. These cord-like structures also resemble those observed in the \textit{CAGGSET}^{\text{Cre/WT}}; \textit{1GF}^{\text{GFP/WT}} gonads. Further investigation will be required to confirm the fate of these ectopic cords.
6.4.3 **Induction after sex determination leads to formation of cord-like structures and reduced expression of the ovarian-specific marker Foxl2 in the XX gonad**

To address whether misexpression of Sox9 in the XX gonad after sex determination could change cell fate and cause sex reversal, pregnant females from the CAGGSET X 10F cross were intraperitoneally injected with OHT at E11.5. Gonads of XX double positive (CAGGSET\textsuperscript{CreWT}; 10F\textsuperscript{GFPWT}) embryos were morphologically female at E16.5. However, examination at the cellular level revealed the presence of SOX9-positive cells, which appear in structures resembling testis cords. Also, AMH was detected at low levels in these gonads within SOX9-positive cells (Figure 6.10), indicating at least weak activation of AMH.

*CAGGSET* induction was also carried out in organ culture. Pairs of CAGGSET\textsuperscript{CreWT}; 10F\textsuperscript{GFPWT} XX gonads were separated for culture in the presence of either OHT or the vehicle (ethanol) alone. Ectopic Sox9 expression was found only in gonads cultured in the presence of OHT but not the vehicle alone (Figure 6.11A-F). Similar to the results obtained from *in utero* induction experiments, SOX9-positive cells formed cord-like structures. However, AMH was not detected in this case.

The expression patterns of female-specific markers were also examined. *Foxl2* becomes expressed specifically in ovarian somatic cells by E12.5, and has been proven to be important in follicular maintenance (for review see Uhlenhaut and Treier, 2004). In XX gonads cultured in the presence of OHT, FOXL2 was detected at reduced levels, suggesting downregulation of the gene by the ectopic presence of SOX9 (Figure 6.11G-L). FOXL2
expression was also examined in the gonads that have received OHT *in utero*, but preliminary data did not reveal significant differences in expression levels (data not shown).

6.4.4 *Sox9* misexpression in granulosa cells leads to *Foxl2* downregulation

The granulosa cell culture system was employed to study the effects of *Sox9* misexpression in the adult ovary. *CAGGSET-10F* females were induced for superovulation between 3-4 weeks of age. Dissociated granulosa cells were cultured in the presence of either OHT or the vehicle (ethanol) alone. Strong EGFP could be detected in cultured cells as early as 12 hours after OHT induction (data not shown), indicating successful recombination events. Immunocytochemistry demonstrated the presence of SOX9 only in cells that were treated with OHT and had iCre nuclearly-localised, indicating the presence of SOX9 is due to *CAGGSET*-mediated recombination (Figure 6.12). SOX9 positive cells are also positive for EGFP (data not shown), indicating that EGFP can act as a reliable reporter for SOX9 expression in granulosa cell culture.

Next it was investigated whether the ectopic expression of SOX9 in granulosa cells would lead to downregulation of female markers in these cells. To address this question, immunocoo-labelling experiments were performed using antibodies against EGFP and two markers, FOXL2 and DAX1, the expression of which usually persists in granulosa cells throughout folliculogenesis. In the presence of OHT, fewer cells were positive for FOXL2, but all cells were still constitutively expressing DAX1 compared to those cultured in the presence of the vehicle alone (Figure 6.13). Therefore, the ectopic expression of SOX9 in granulosa cells selectively leads to downregulation of FOXL2.
Figure 6.9  For figure legend see opposite page.
Figure 6.10  SOX9 and AMH expression in CAGGSET; Z/Sox9-10F XX gonads.

CAGGSET mice were crossed with Z/Sox9-10F mice. Pregnant females were injected with OHT at E11.5 and embryos were harvested at E16.5. Gonads were dissected out, cryosectioned and examined for immunohistochemistry for SOX9 and AMH. The inserts at the bottom-left corner of the bottom right panel shows a magnified view of an area that misexpresses SOX9.

M: mesonephroi; G: gonads.
Figure 6.11  For figure legend see opposite page.
Figure 6.12  Ectopic Sox9 expression in granulosa cell culture.

CAGGSET<sup>Cre</sup><sup>WT</sup>; 10<sup>Cre</sup><sup>GFP</sup><sup>WT</sup> females were sacrificed between 21-28 days for granulosa cell culture. Cells were cultured either in the presence of 2μM OHT or ethanol for 48 hours. Antibodies used for immunolabelling were anti-SOX9 and mouse anti-Cre.
Figure 6.13  For figure legend see opposite page.
6.5 **CAGGSET-mediated deletion of Sox9 in the developing gonads**

Conditional knock-down of Sox9 was carried out during different embryonic and postnatal (P) stages. As in the misexpression studies, initial tests were carried out prior to sex determination.

6.5.1 Deletion of Sox9 prior to sex-determination leads to cord structure damage and downregulation of Sf1 in Sertoli cells

To generate embryos homozygous for the inducible Sox9 null allele, mating pairs were set up between Flox-Sox9 and CAGGSET-Flox-Sox9 mice. Pregnant females were injected intraperitoneally with OHT at E8.5, and sacrificed at E14.5 for embryo harvest and gonad dissection. Comparisons were made between iCre positive (CAGGSET<sup>Cre/WT</sup>; Sox9<sup>F/d</sup>; referred to as “mutant” hereafter) and negative (CAGGSET<sup>WT/WT</sup>; Sox9<sup>FloxFlo</sup>; referred to as “wildtype” hereafter) gonads. All XY, iCre positive gonads were morphologically testes, indicating that no obvious sex reversal events have occurred. However, testis cords seemed less tightly packed in mutant XY gonads compared to the wildtype by histology (Figure 6.14A-B). Immunohistochemistry for laminin revealed disorganised cord structures in mutant compared to wildtype testes (Figure 6.14E-F), indicating that cord structures have been damaged. Also noticed was a different organization of the tunica (Figure 6.14E-F), which looked slightly ruptured. Further examination by immunohistochemistry revealed a reduction in SOX9 levels, but AMH did not seem affected (Figure 6.15A). Strikingly, SF1 was extinguished in Sertoli cells, but not in interstitial (Leydig) cells (Figure 6.15B), further
supporting the earlier suggestion from in vitro studies that Sfl is regulated by SOX9 (Shen and Ingraham, 2002).

Figure 6.14  For figure legend see opposite page.
Figure 6.15  For figure legend see opposite page.
No significant effects were observed in germ cells in the gonads with partial loss of Sox9.

CAGGSET-Flox-Sox9 mice were crossed to Flox-Sox9 mice. Pregnant females were injected with OHT at E8.5 and embryos were harvested at E14.5. Gonads were dissected out and cryosectioned for immunohistochemistry for Stella, which marks the male germ cells but not female germ cells at this stage. M: mesonephroi; G: gonads

6.5.2 The effects of Sox9 deletion prior to sex-determination on germ cells

As it is known that germ cell sex is defined by the micro-environment surrounding the germ cells (McLaren and Monk, 1981; Palmer and Burgoyne, 1991; Bowles et al, 2006, Kourbova et al., 2006), it was examined whether the germ cell lineage was affected along with the
changes in cord structure and the reduction in Sertoli cell markers. *Stella*, also called *PGC7*, starts to be expressed exclusively in germ cells at E7.25 and lasts until E15.5 in the XY gonad and around E13.5 in the XX (Saitou *et al.*, 2002; Sato *et al.*, 2002). Immunohistochemistry for Stella revealed no obvious difference between mutant and wildtype XY gonads (Figure 6.16). However, histology of gonad sections revealed gaps between the Sertoli cell layer and the core region of the cords in mutant XY gonads, indicating possible germ cell loss. The nuclei of germ cells also seemed abnormal compared to those in the wildtype gonads (Figure 6.14C-D). It was also asked whether germ cells have entered meiosis following the disruption of the somatic environment. Meiotic markers used for immunohistochemistry included SCP3, γ-H2AX and DMC-1, and comparisons were made between mutant and wildtype gonads of both sexes from embryos within the same litter. None of these markers was detected in XY mutant gonads, indicating no significant entry into meiosis by germ cells within the XY mutant gonads (data not shown).

6.5.3 Deletion of *Sox9* prior to sex-determination leads to ectopic expression of the female-specific marker *Foxl2*

Next it was examined whether female markers were present following downregulation of *Sox9*. The ovary-specific gene *Foxl2* has been proposed to antagonize *Sox9* expression, as SOX9 was detected in the ovaries of *Foxl2* null mutants (Ottolenghi *et al.*, 2005). Conversely, as described in section 6.4.4 above, the ectopic expression of SOX9 led to *Foxl2* downregulation. The expression of FOXL2 was therefore examined in the gonads of our *Sox9* mutants. FOXL2-expressing cells were detected in the XY mutant gonads (Figure 6.17A), some of which were located in the interstitial space of the testis, but many of these
were found within testis cords and are also positive for AMH. Several also displayed Sertoli cell morphology (Figure 6.17B).

Figure 6.17  For figure legend see opposite page.
6.5.4 Deletion of Sox9 after sex determination leads to changes in cord structure

The upregulation of Sox9 in the developing testis by E11.5 is essential to specify Sertoli cell fate. The deletion of Sox9 after this point will test whether the gene is also required to maintain Sertoli cell differentiation. To induce Sox9 deletion, OHT was delivered *in utero* via intraperitoneal injection between E11.5 and E12.5. Gonads were harvested either at E18.5 or after birth.

Histology at E18.5 demonstrated abnormalities in the mutant testes: cords were more loosely packed compared to those of wildtype testes, with more interstitial space (Figure 6.18A). Examination at higher magnification revealed gaps within cords, consistent with possible germ cell loss (Figure 6.18B). However, no meiotic events were seen, either by histology (Figure 6.18) or by analysis of meiotic markers (data not shown).

To investigate long-term effects of Sox9 loss, pups that had been induced *in utero* at E12.5 were monitored from weaning age for 9 weeks. By three weeks of age, both XX and XY mutant pups were significantly smaller and weighed less compared to their wildtype littermates. However, the growth rate of mutants did not seem affected (Figure 6.19). These mutants were fertile: all of them mated with the female mice set up with them within 4 days, and all females produced litter sizes comparable to those provided by the control wildtype littermates (Figure 6.20A), indicating that the mutant testes were still functional. These males were killed to examine testis morphologies. Preliminary results showed that the body weights of the mutants were significantly lower than those of wildtype animals. Comparisons of the weight of testes relative to body weight showed that the testes of mutants were smaller than
those of wildtypes by about 15% (Figure 6.20B). Analysis using histology and immunohistochemistry will be required to investigate the reason for this at a cellular level.

![Diagram of CAGGSET and Sox9 genotypes](image)

**Figure 6.18**  Histology of XY gonads with partial loss of Sox9.

*CAGGSET-Flox-Sox9* mice were crossed to *Flox-Sox9* mice. Pregnant females were injected with OHT at E11.5 and embryos were harvested at E18.5. Testes were dissected out for either paraffin sectioning. The red arrow-head highlights a gap found in a testis positive for iCre.
Figure 6.19  For figure legend see opposite page.
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Figure 6.20  Fertility of CAGGSET^{CreWT}, Sox9^{L/L} mice and wildtype littermates that have been induced at E12.5 in utero.

(A) Male mice were set up with wildtype females and plug-checked daily. Pregnant females were separated after plugged and have littered down, with their litter size recorded. These males were later sacrificed to examine testicular phenotypes. Mice were weighed before schedule-1-killing and testes were taken out for weighing. Testis-to-body weight ratio (TW/BW) was recorded.

(B) Graphical presentation demonstrating normalized TW/BW ratio using the highest score obtained by a wildtype.

6.5.5  Sox9 deletion at neonatal stages leads to changes in testis cord structures and reduction in body size

We also looked at effects of CAGGSET-mediated loss of Sox9 function after birth. OHT was administered to CAGGSET^{CreWT}, Sox9^{Flox/Flox} males by intraperitoneal injection at P3, and testes were examined at P14. Pups treated with OHT did not display levels of SOX9 different from those of littermates that received the vehicle (oil) alone, although testis cords seemed less organized in the former (Figure 6.21A). If induction took place 6 days later (at P9),
SOX9 levels and cord structures in the testes did not seem significantly affected at P22. However, the body size of the OHT-treated mice was notably reduced by the same stage when compared to littermates that have received the vehicle alone (Figure 6.21B). To investigate if this is an artefact of induction instead of a general loss of Sox9 function, similar studies were conducted in CAGGSET-R26R EYFP male pups of the same age. No difference was observed between littermates that have received either OHT or the vehicle alone, or between transgenic and wildtype pups that have both received OHT (data not shown). This indicates that the reduction in size of CAGGSET-Flox-Sox9 mice that have received OHT was due to the loss of Sox9 and not an artefact of OHT administration. It is highly likely that the reduction of body size is due to effects on non-gonadal tissues, as Sox9 plays a general role in multiple tissues, and CAGGSET is not a gonad-specific Cre-driver.

6.5.6 Sox9 deletion can be induced by CAGGSET in postnatal Sertoli cells

To ask if CAGGSET can mediate Sox9 deletion in Sertoli cells, four-week old CAGGSET^{Cre/WT}; Sox9^{Flox/Flox} males were induced by intraperitoneal injection of either tamoxifen or the vehicle alone. Unlike induction studies carried out at early postnatal stages, CAGGSET-induction did not lead to a reduction of body weight or testicular weight. As the antibody against Cre did not work well on sections, testes cords were dissociated with cells spread onto glass-slides for examination at a cellular level. Immunohistochemistry for SOX9 and iCre on these testicular cells revealed that the two proteins co-localized only in the absence of the inducer (Figure 6.22), indicating a certain population of Sertoli cells are expressing ERT2iCreERT2. This observation also suggests that the ERT2iCreERT2 fusion protein could be activated in Sertoli cells upon the administration of tamoxifen. This
assumption is also supported by the observation of several iCre-positive cells displaying Sertoli cell morphology that were negative for SOX9 (Figure 6.22). Further experiments will be required to investigate if iCre-positive cells express female markers, for example, FOXL2.

Figure 6.21  *For figure legend see opposite page.*
**Figure 6.22**  *CAGGSET*-mediated recombination can take place in the Sertoli cell lineage.

*CAGGSET*-Fl ox-Sox9 mice were injected with 4mg of tamoxifen or an equivalent concentration of oil for a consecutive 5-day period (total dosage 20mg). Injected mice were sacrificed 10 days after the last injection. Testes were dissected out and tubules were dissociated and cryopreserved for immunocytochemistry for iCRE and SOX9. Note that in the absence of tamoxifen (upper panels, labelled Oil), iCRE is localized in the cytoplasm and co-localization of iCRE and SOX9 could be observed. In the presence of tamoxifen (lower panels), iCRE is localized in both the nucleus and cytoplasm. In this case no cell displayed co-localization between iCRE and SOX9, suggesting successful recombination events by *CAGGSET* to delete Sox9. The white arrow-head points to an iCRE positive, SOX9 negative cell that displays Sertoli cell morphology, which might have Sox9 deleted due to the activity of *CAGGSET*. 
6.6 Discussion

Embryonic lethality due to overexpression of Sox9

In the experiments involving Z/Sox9 mice, ubiquitous, ectopic expression of Sox9 leads to embryonic lethality by E13.5. At present it is not possible to make conclusions about the cause of lethality, as phenotypic observation of the embryos at E12.5 revealed defects in various vital organs. Some of these organs had much reduced sizes and seemed retarded in their development. Further investigation of the effects of ectopic Sox9 expression in different organs will require tissue-specific Cre mice to mediate the expression of Z/Sox9.

When Sox9 expression is altered

Sox9 overexpression mediated by β-actin-Cre led to testis cord-like structures in the XX gonad, in agreement with the sex reversal phenotypes obtained using Wt1 to drive Sox9 (Vidal et al., 2001) or in Oddsex mice (Qin et al., 2004).

Using CAGGSET, Sox9 was partially deleted or misexpressed at different time points. Induction of Sox9 misexpression at E8.5 led to cord formation within the XX gonad, although a complete sex reversal phenotype was not obtained. This is probably due to insufficient recombination efficiency. It has been shown that a threshold number of Sertoli cells must be reached in order for testis cords to form (reviewed by Polanco and Koopman, 2006). It could be that CAGGSET-mediated recombination did not produce enough SOX9 expressing cells, therefore only a restricted number of cords could be formed. Alternatively, deletion of Sox9 mediated by CAGGSET at E8.5 displayed disorganized cord structures but not complete sex reversal, whereas XY Sox9nd mice obtained by conditional deletion using a
Ckl9:Cre are sex reversed (Barrionuevo et al., 2006). The lack of complete sex reversal using CAGGSET can perhaps be explained by reasons similar to that of Z/Sox9: if the formation of testis cord requires a certain threshold, the complete loss of cords probably also requires the number of Sertoli cells to fall beneath a threshold. Therefore the possible mosaicism of CAGGSET activity may not have reduced SOX9 positive cells to a number that can cause cord deformation.

Similarly, induction at E11.5, a stage when Sox9 is already upregulated in the testis, led to structural changes in testis cord structures in both misexpression and deletion studies. CAGGSET-mediated misexpression of Sox9 gave rise to the formation of testis cord-like structures in the XX gonad. In Sox9<sup>3/st</sup> XY gonads, cord structures were slightly disorganised, leaving more interstitial space in between testis cords. These observations revealed that either misexpression or deletion of Sox9 in a subset of cells is not enough to give rise to sex-reversal after sex determination has occurred.

**The expression of Sf1 in Sertoli cells is regulated by SOX9**

An Sf1 regulatory region has been shown to be activated in vitro by SOX9 (Shen and Ingraham, 2002). The results presented here (section 6.3.3), involving the use of β-actin-Cre to activate Z/Sox9, displayed ectopic SF1 in the XX gonad. Unlike SOX9, SF1 was not detected in the mesonephros, suggesting certain co-factor(s) which are only present in the gonad are required for Sf1 expression. On the other hand, CAGGSET-mediated deletion of Sox9 prior to sex determination extinguished SF1 only in Sertoli cells but not Leydig cells. Combining the in vitro data of Shen & Ingraham (2002) with this in vivo data makes it very likely that Sf1 is upregulated by SOX9. Conversely, unpublished data from the host lab
(Ryohei Sekido and Silvana Guioli) shows that Sox9 expression in the testis depends on SF1. This suggests the existence of an autoregulatory loop whereby both genes act to upregulate each other. However, this must depend on additional co-factors present within supporting cells or by co-repressors that block it within Leydig cells.

**Factors regulating AMH**

Several factors have been shown to bind and activate the 360 base pair minimal promoter of Amh, and to regulate its expression in Sertoli cells. These include SOX9, SOX8, WT1, SF1, DAX1 and GATA4 (Arrango *et al.*, 1999; Tremblay and Viger, 1999; Schepers *et al.*, 2003; Shen *et al.*, 1994; also reviewed by Rey *et al.*, 2003). Whether, and how, all of these factors are required for the regulation is still unknown. In the transgenic experiments I report here, AMH was detected ectopically in β-actin-Cre-mediated expression of Sox9 in the XX gonad, particularly in the coelomic epithelial cells, many of which are also positive for SOX9. A number of relevant transcription factors are expressed in the coelomic epithelium. These include WT1 and GATA4, but most importantly SF1 (de Santa Barbara *et al.*, 1998; Tremblay and Viger, 1999; Nachital *et al.*, 1998 Ingraham Cell). SF1, at least, was present in the XX gonads ectopically expressing SOX9, and this might be related to the upregulation of AMH confined within the coelomic epithelium. On the other hand, CAGGSET-mediated Sox9 misexpression in the XX gonad at E8.5 displayed low levels of AMH around SOX9-positive cells, but CAGGSET-mediated Sox9 misexpression after sex determination, or deletion of Sox9 at any stage tested, did not lead to significant changes in AMH levels, even though in the latter case SF1 is downregulated in Sertoli cells. These results suggest that SOX9 and SF1 might be required for the initial upregulation, but not the maintenance of AMH. After sex determination, WT1 and GATA4 are both expressed in Sertoli cells. WT1
has recently been shown to be important for AMH maintenance during testis differentiation. Conditional deletion of Wt1 in XY gonads of E14.5 mouse embryos led to downregulation of both SOX9 and AMH, giving rise to uterine structures in addition to normal male internal genitalia (Gao et al., 2006). On the other hand, a backup mechanism for AMH regulation has been proposed: SOX8 has been found able to bind and activate the Amh minimal promoter, partially compensating the function of SOX9 in the upregulation of Amh (Schepers et al., 2003; Chaboissier et al., 2004). It is possible that Sox8 has been upregulated in the CAGGSET-mediated SOX9da XY gonads, partially compensating for the loss of Sox9 in these mutant testes. Therefore the effects on AMH levels after altering SOX9 expression were not significant. Further analyses of marker expression will be required in these mutants.

The efficiency of CAGGSET-mediated recombination in Sertoli cells raises concerns for both the usefulness of the Cre-driver and for the interpretation of some of the results reported here. Mosaicism of transgene expression and accessibility of the inducer can both reduce the percentage of cells misexpressing or deleting Sox9. It is particularly difficult to guarantee that both alleles of Sox9 have been deleted in Sox9\textsuperscript{Flax/Flax} mice upon induction. Unlike humans, XY mice heterozygous for Sox9 deletion develop into normal males (Bi et al., 2001): disruption of testis differentiation requires both alleles to be inactivated. To ensure both Sox9 alleles can be successfully deleted at least in a subset of Sertoli cells, I am currently introducing a germline Cre into the CAGGSET\textsuperscript{Cre/Cr}e; Sox9\textsuperscript{Flax/Flax} background. Protamine-Cre (PrmCre; O’Gorman et al., 1997) and ZP3-Cre (Lewandoski et al., 1997b) are active only in the male and female germline, respectively. An embryo harbouring either of these in the CAGGSET\textsuperscript{Cre/Cr}e; Sox9\textsuperscript{Flax/Flax} background will have one allele of Sox9 deleted in the
germline before fertilization, leaving CAGGSET to delete the remaining allele upon tamoxifen induction.

An antagonistic relationship between Foxl2 and Sox9?

The presence of SOX9 has been shown in gonads of XX Foxl2+/− mouse embryos, despite the absence of testis cord formation (Ottolenghi et al., 2005). In contrast, Foxl2 transcripts were detected by real time RT-PCR in Sox9−/− XY gonads (Barrionuevo et al., 2006). Sox9 and Foxl2 have several things in common during sex determination in the mouse: (1) both are expressed in the supporting cell lineage in a sexually dimorphic manner (for review see Wilhelm and Koopman, 2006 and Yao, 2004), (2) both are maintained in the supporting cell lineage (although in different sexes) throughout life (for review see Wilhelm and Koopman, 2006 and Yao, 2004), (3) both are essential for gonadal function in terms of maintaining the germ cell niche and (4) both regulate the expression of supporting cell-specific hormone secretion (AMH for Sox9 and P450 aromatase for Foxl2; Arrango et al., 1999; Uhlenhaut and Treier, 2006). In the studies reported in this thesis, CAGGSET-mediated ectopic expression of Sox9 in the XX gonad or in adult granulosa cells led to a reduction in FOXL2 levels, whereas CAGGSET-mediated deletion of Sox9 led to ectopic expression of Foxl2 in XY gonads. These were consistent with and confirmed previous findings using Foxl2 mutants or RT-PCR in Sox9+/− mice (Ottolenghi et al., 2005; Barrionuevo et al., 2006). SOX9 and FOXL2 might mutually repress each other so as to reinforce correct sexual differentiation into either testis or ovary, therefore the deletion of either factor will remove the repression effect and lead to ectopic expression of the other. It therefore seems highly likely that Sox9 and Foxl2 have an antagonistic relationship during mammalian sex determination. These factors might mutually inhibit the function of each other by activating
certain paracrine factors, such as FGF9 and WNT4. In the XY gonad, \textit{Fgf9} is upregulated shortly after the onset of \textit{Sox9} upregulation, also coinciding with the downregulation of \textit{Wnt4} (for review see Kim and Capel, 2006). In the XX gonad, the absence of SRY (or more probably, SOX9), allows the continued expression of WNT4 (which is on in the early genital ridges of both sexes), and the downregulation of FGF9, a combination which might then lead to the activation of \textit{Foxl2} expression (see also Yao, 2004). Although early sex reversal is not seen in \textit{Foxl2} mutants, \textit{Sox9} and \textit{Foxl2} may both be required to reinforce cell fate decisions taken during sex determination, in part by repressing the opposite pathway.

In the granulosa cell culture system, the ectopic presence of SOX9 did not completely extinguish \textit{Foxl2} expression in all cells. Presumably, a cell will either express SOX9 or FOXL2. The co-existence (SOX9 as reflected by EGFP) of these proteins within the same cell could perhaps be explained by the nature of OHT- induction in cell culture. As this kind of control in gene expression is heterogeneous we cannot assure the time and duration of \textit{CAGGSET} activity. For example, \textit{CAGGSET} could become activated in a certain cell shortly before harvest, allowing insufficient time for FOXL2 to be downregulated. In this case, cells that have undergone recombination at the beginning of OHT treatment would have time to extinguish FOXL2, whereas those recombined at the end of the treatment would not. Further experiments will be required to address how these proteins and their mutual antagonism are regulated within this system.

\textit{Germ cells}

Examination of gonadal histology revealed differences in the morphology of testis cords following OHT-induction, of \textit{CAGGSET}^{Cre/WT}; \textit{Sox9}^{Ald} XY gonads at E14.5. There were
clear gaps within testis cords, suggesting possible germ cell loss. The germ cells did not express meiotic cell markers, at least at the stage of study. On the other hand, examination at E18.5 following induction at E11.5 revealed more interstitial space in the CAGGSET\textsuperscript{Cre}\textsuperscript{WT}; Sox9\textsuperscript{del} XY gonads. In wildtype testes, the cords were packed with germ cells, but in E18.5 CAGGSET\textsuperscript{Cre}\textsuperscript{WT}; Sox9\textsuperscript{del} XY gonads, gaps were observed inside several testis cords. Meiotic events were also not detected in these mutants. The possibility that germ cells in the XY mutant gonads might have entered meiosis can not be excluded, however, as they may be eliminated by other male factors, most likely AMH (Josso, 1990). Further investigations will include tunnel assays, or immunohistochemistry for activated Caspase3, which reports programmed cell death.

Apart from the observed gene expression changes, which included Sfl and Foxl2, several other genetic events must have happened following the alteration of Sox9. These events may not be obvious when looking at proteins if these are stable and persist. They will therefore require examination of transcripts. Real time reverse-transcription PCR provides an efficient and quantitative way to observe gene transcription levels in different cell types, and can therefore be employed to investigate whether the expression of genes involved in gonadal differentiation is affected due to the alteration of Sox9. It will be crucial to monitor the expression pattern of Sox9 following its alteration, and the genes that are affected by the controlled gain- or loss-of Sox9 expression. This will require collecting mutant embryos at different stages after induction, to observe gonadal morphology and gene expression at the cellular level. Sertoli cells can be labelled using Sox9:ECFP or its equivalent, and can therefore be separated from other gonadal cell types using fluorescent cell sorting for comparative examination between different cell lineages.
It will also be interesting to compare data derived from these experiments with induced Sox9 alteration with that from Sox9 heterozygous mutants (Sox9$^{WT/Δ}$). Sox9$^{WT/Δ}$ tissues can simply be obtained at different stages from embryos made by crossing mice carrying Flox-Sox9 with those carrying β-actin-Cre. These can serve as a control in all further experiments concerning changes in gene expression patterns, especially to address which genes are sensitive to the dosage of SOX9 in the developing mouse gonad.

Some of the additional in vivo studies suggested above are underway, but require extensive breeding to achieve the correct combinations of transgenic and mutant alleles, and this will take time.
 CHAPTER 7

GENERAL DISCUSSION

This thesis describes the creation of several new tools that rely on conditional strategies to study gene function in mice. These include the improved-codon Cre-recombinase (iCre) driven by different regulatory elements, which we call “Cre-drivers”, and “responder” transgenes that allow conditional overexpression of Sry or Sox9.

The Cre/loxP conditional system

The Cre/LoxP system was chosen for these studies. This is the most commonly used system to allow conditional gene alteration in mice and therefore, the new Cre-drivers will be compatible to many other already established conditional alleles. Another reason for favouring Cre/loxP instead of, for example, Flp/Frt, concerns the details of inducible-conditional systems. Cre-ERT is the most commonly available fusion recombinase that allows induction control, and therefore seems an obvious choice for study. The recent availability of the improved versions, iCre and iCreERT2, at the time I began the work, meant that even controls should provide some new information (Casanova et al., 2002). Several Cre-drivers described in this thesis were inducible by tamoxifen. Although the alternative tetracycline-on/off (Tet-O) system may provide better efficiencies and theoretically allows reversible reaction, it requires continuous administration of the inducer doxycycline, and also a much longer time to terminate a reaction due to the long half-life of doxycycline. The more complicated genetic build-up required in order to generate the mice
and to achieve permanent changes in vivo, was also taken into consideration. While tamoxifen-inducible Cre requires only two elements to activate the system, namely the Cre-ERT-driver and a floxed allele, Tet-inducible Cre requires three elements: a Tet-effector, a Tet-responder coupled to Cre, and the flox allele (for review see Lewandoski, 2000; Bockamp et al., 2002). This complexity requires at least four subsequent crosses of mice carrying different elements to obtain double homozygotes. Taking time and the administration protocols into concern, the tamoxifen-inducible Cre system was favoured.

**The new tamoxifen-inducible Cre-drivers**

In this thesis, four elements were used to drive iCre expression. With the exception of a 3.2kb Sox9 enhancer element, which was used to drive non-inducible iCre, Dax1, Sfl and a ubiquitous CMV enhancer/chicken β-actin promoter were used to drive ERT2iCreERT2. The estrogen receptor-like domain for tamoxifen-binding (ERT) has been modified, such that ERT2 is made more sensitive to 4-hydroxytamoxifen (OHT), the active form of tamoxifen, and was used in this project.

At least one mouse founder from each Cre-driver construct demonstrated recombination activity in vivo, namely Line V for Sox9:iCre, Line M for DaxCSET, Line P for SflET, and Line B for CAGGSET. SflET Line P is lost. It is not known why all the progeny stopped transmitting the transgene. Therefore, additional transgenic mouse lines have to be created and characterized to establish the Sfl-inducible Cre-driver. The only founder mouse obtained for AngelBLUE-Sry is not an expressor, therefore more transgenic lines have to be established with the aim of conditionally misexpressing Sry in the XX gonad. Different methods were used to create Z/Sox9 mice. The imported Line 10F was already known to
express the *LacZ* transgene at high levels (Cheah, unpublished). Lines C and M created by pronuclear injection, also expressed the *LacZ* transgene at high levels. Unfortunately, Line M was lost due to problems in breeding and possibly to embryonic lethality.

**Strong expressors and weak expressors: position effects and genetic background**

Different founders carrying the same transgene displayed different expression levels. Also, mosaic transgene expression has been noticed in several lines (e.g. *Sox9:iCre* and *Z/Sox9* Lines B and J). These are commonly seen in transgenic mice generated by random integration, and usually reflect position effects (Kioussis and Festenstein, 1997, also discussed in Chapter 4). The 5 different *CAGGSET* mouse lines generated are good examples of this effect: the injected embryos carried the same transgene but displayed different transcriptional activities. This is almost certainly due to different sites of chromosomal integration (Kioussis and Festenstein, 1997).

To avoid these effects, transgenes can be targeted into identified loci that are not affected by DNA remodelling and that are ubiquitously expressed. An example of this is the *Rosa26* locus on the mouse chromosome 6 (Friedrich and Soriano, 1991). Many reporter alleles have been targeted into *Rosa26* locus, aiming to reflect Cre-mediated activities (Soriano, 1999; Srinivas *et al.*, 2001). These mice have been extensively used by different research groups and have provided consistent, reliable results. They were also selected for this study to act as a reporter for Cre-mediated activity (Chapters 3, 4, and 6). Particularly, *R26R EYFP* (Srinivas *et al.*, 2001) provided 100% reporter protein expression under the mediation of *β-actin-Cre* mice (Lewandoski *et al.*, 1997a; data not shown), and has served as controls for all relevant experiments described in this thesis. To further improve transgene expression,
attempts could be made to target a gene of interest driven a strong ubiquitous promoter into
*Rosa26* or similar loci.

Position effects are also likely to be affected by different genetic backgrounds, which are
different in chromatin structures and DNA remodelling, as well as in sets of genes showing
polymorphism between different strains (Silver, 1995). Therefore, activities or expression
profiles of the same transgene can differ according to the genetic background in which the
animals were generated or subsequently bred into. Several genes involved in mammalian sex
determination displayed different phenotypes in different genetic backgrounds. For example,
*Dax1* was reported to be important in male development, but only when the conditional allele
was bred onto C57BL/6 (Meeks *et al.*, 2003; Bouma *et al.*, 2005). Some other mutants were
bred onto C57BL/6 in order to achieve steadier phenotypes, for example, *Fgf9* (Schmahl *et
al.*, 2004). In our study, attempts were made to backcross all functional mouse lines to
C57BL/6. However, some of these lines displayed (possible) lethality under the influence of
this genetic background. The most obvious examples were the *Z/Sox9* mouse lines. Line 10F
was generated in a 129Sv/J; C57BL/6 background, and was backcrossed to C57BL/6 for
maintenance. A significant reduction in litter size has been observed by the third generation
of backcrossing. On the other hand, *Z/Sox9* Line C was the only line directly generated onto
C57BL/6, and has so far produced very small litter sizes (n=3-5 per litter). To test if the small
litter sizes of these lines are related to the C57BL/6 genetic background, Lines 10F and C are
currently kept on a mixed 129Sv/J and C57BL/6 background. *Flox-Sox9* mice have been
backcrossed to C57BL/6, and the third generation of homozygotes were recently established.
Subsequent crosses with *CAGGSET* mice will be required to generate a conditional null
allele for Sox9. In future studies, comparisons will be made between Flox-Sox9 mice on both the CBA/BL10 mixed and C57BL/6 backgrounds.

The future of gonad-specific Cre-drivers

Attempts were made by our lab and others to create Cre-drivers that act only in the gonad (e.g. Sfl-Cre, as used in Chaboissier et al., 2004; Amh-Cre, as used in Gao et al., 2006; Amh type2 receptor-Cre, as used in Jeyasuria et al., 2004; Sry/Ce as described in Ito et al., 2006). Three common problems have been encountered with these gonad “specific” Cre-drivers. (i) The regulatory elements chosen may also give expression in the very early embryo, e.g. Sry, Dax1 and Sox9 (as reported here). (ii) The expression level or recombination level is not high enough (Chaboissier et al., 2004; Ito et al., 2006), and/or (iii) the time of expression is delayed compared to endogenous gene expression patterns (Chaboissier et al., 2004; Gao et al., 2006; Jeyasuria et al., 2004). Described in this thesis is a novel Sox9:iCre mouse. One line generated with this construct (Line V) displayed a Sertoli cell expression pattern, although its recombination efficiency is does not reach 100% until E14.5 (Figure 3.10). Nevertheless, we have already started crossing this Cre-driver with Flox-Sox9 to try to obtain sex reversal phenotype. Future attempts to make gonad-specific Cre-driver transgenes will consider targeting Cre or its derivatives, including inducible versions, into gonad-specific loci. Gonad-specific regulatory regions of Sox9 have been identified, and proven to drive strong transgene expression in vivo (Sekido and Lovell-Badge, unpublished). Targeting iCre or ERT2iCreERT2 into the genomic locus of this region will in theory allow robust, continuous expression of Cre in a Sertoli cell-specific manner. It would also be important to be able to drive Cre activity in either genetic sex very early, and Lhx9 ought, at least in
theory, be able to provide appropriate regulatory elements to do this. To alter gene expression in genetic females, the genomic regulatory region of Foxl2 could be considered.

**Sox9 and mammalian sex determination – new findings**

The biological aim of this project was to ask what happens if Sox9 expression in the gonad is altered after sex determination has already taken place. Using β-actin-Cre mice, Sox9 was successfully overexpressed in the XX genital ridge, leading to the formation of cord-like structures (Figure 6.2). Unfortunately, mice constitutively overexpressing Sox9 die by E12.5, rendering further examination of gonadal development only feasible by either organ culture experiments or conditional mutagenesis.

Nevertheless, our studies on misexpression or deletion of Sox9 led to two new findings: (1) Sox9 regulates Sfl expression in Sertoli cells, but not Leydig cells in the XY genital ridge, and (2) Sox9 and Foxl2 repress each other, so as to repress the opposite sexual development pathway (Figure 7.1).

SF1 was detected in XX gonads overexpressing Sox9, and it co-localized with EGFP, being used in this case as a reporter for Sox9 expression (Figure 6.4). Moreover, CAGGSET mediated Sox9 deletion led to the downregulation of SF1 in Sertoli cells (Figure 6.15). Expression of Sfl in Leydig cells was not affected by the partial loss of Sox9 (Figure 6.15). In vitro experiments have demonstrated the importance of SOX9 in activating Sfl (Shen and Ingraham, 2002). Our data therefore provide the first evidence in vivo to conclude that Sox9 is not only essential to upregulate Sfl expression in Sertoli cells, but is also required for maintaining the expression of Sfl in this gonadal cell lineage.
Bipotential gonad | Sex determination | Sexual differentiation
---|---|---
\[Sry\] | \[Sox9\] | \[Amh + others\]
\[Fgf9\] | \[Sf1\] | \[\(Wnt4\)\]
\[Foxl2\] | \[\_\_] | \[?\]

Figure 7.1 Schematic diagram of the current genetic pathway involved during sex determination in the mouse. Newly identified genetic regulations were highlighted in red lines. Genes in the male pathway are in blue; whereas those in the female pathway are in pink.

Foxl2 is a proposed antagonist of Sox9 during mammalian sex determination (Ottolenghi et al., 2005; Barrionuevo et al., 2006). Foxl2 transcripts have been detected in XY gonads depleted of Sox9 (Barrionuevo et al., 2006). Using immunohistochemistry, we located FOXL2 positive cells in Sox9\textsuperscript{null} XY gonads to testis cords, indicating that these FOXL2 positive cells were, or rather had been Sertoli cells. Foxl2 was also downregulated in embryonic XX gonads misexpressing Sox9, (Figure 6.11) and in adult granulosa cells that were misexpressing Sox9 (Figure 6.13), further strengthening the belief that Sox9 and Foxl2 antagonize each other during gonadal development in mice.

Attempts have been made to delete Sox9 in vivo by CAGGSET. Results showed that testis cord structures were moderately disorganized. Surprisingly, Amh, a direct downstream target of SOX9 in Sertoli cells (Arrango et al., 1999), did not seem affected. Several hypotheses could explain this: (i) The maintenance of Amh expression in Sertoli cells is independent of the levels of SOX9 and perhaps SF1. In other words, SOX9 may be required to initiate
expression but other factors can maintain it (e.g. GATA4, WT1, etc). (ii) The AMH protein is very stable in the absence of SOX9 (and SF1). (iii) SOX9 protein is itself stable (in the absence of new transcripts). Further investigations will be required to draw a conclusion.

Despite the limited efficiency of CAGGSET-mediated recombination in Sertoli cells, CAGGSET is still useful to study the regulation of genetic events in Sertoli cells (or other gonadal lineages). For example, the downregulation of Sfl in Sertoli cells in CAGGSET-mediated Sox9^{del} XY gonads suggests further experiments to look at possible changes in other markers. This can be done by immunohistochemistry on protein levels, in situ hybridization on transcript levels, and more importantly, using real time RT-PCR to quantify differences in gene expression levels between mutant and wildtype gonads. Alternatively, it would be possible to make use of cDNA microarrays. The results of these experiments will highlight the genes regulated by SOX9 in Sertoli cells.


Nagy, A. Cre Transgenic Database. [http://www.mshri.on.ca/nagy/default.htm](http://www.mshri.on.ca/nagy/default.htm).


**Appendix A: Preparation of electro-competent cells**

1. Inoculate a single colony of bacteria of interest in 5 ml LB and incubate at 37°C overnight shaking at 250 rpm.
2. Transfer 1 ml of overnight culture of 1L of LB and incubate with shaking at 37°C until OD=0.5-0.6.
3. Centrifuge at 3000 rpm for 5 mins.
4. Discard supernatant. Resuspend pellet in 40 ml of ice-cold sterile ddwater.
5. Sit on ice for 30 mins.
6. Centrifuge at 3000 rpm for 5 mins.
7. Resuspend gently in 20 ml of cold ddwater with 15% glycerol.
8. Aliquot 50µl, freeze immediately with N₂(l).
9. Store at -80°C.

**Appendix B: Tail lysis buffer (for phenol extraction)**

Buffer consists of (per 100mL):
- 1M Tris Ph 7.5 5mL
- 0.5M EDTA 100µL
- 20% SDS 2.5mL
- Water to 50mL

Per sample use 95µL buffer + 5µL proteinase K (20mg/mL)

**Appendix C: β-galactosidase (X-gal) staining**

1. Wash embryos/tissues in 1X PBS—CMF.
2. Fix in fresh cold fixative.
   - Fixative: 0.2% Glutaraldehyde (stock 25% at -20°C), 2% Formaldehyde.
   - 80µL 25% glutaraldehyde + 540µL 37% Formaldehyde / 10mL L.
   - For cells, fix for 10' r.t. (NO MORE THAN 20')
   - Whole embryos up to 8.5dpc, fix for 5', add 5' for each day onwards, 4°C with rocking.
3. Wash trice, 5' with Buffer L0.
4. Add fresh-filtered LacZ staining solution.
   - Staining base: 1mL 500mM K₃Fe(CN)₆ + 1mL 500mM K₄Fe(CN)₆·3H₂O (both 4°C) per 50mL Buffer L.
   - X-gal stock 50mg/mL in Dimethyl Formamide at -20°C.
   - Fresh staining mix: 1/50 dilution of X-gal in staining base, mix well and filter before use.
   - 37°C or 25°C, time varies with strength, from few hours to O/N. (TRY TO MONITOR EVERY 30')
5. Wash trice 5' PBS-A.
6. Embryos/tissues can either be stored in PBS or refixed with 4% paraformaldehyde.

**Buffers involved:**

**Buffer L (Cells and early embryos):**
- Phosphate buffer (PB), pH7.2 100mM
- MgCl₂ 2mM
- EGTA (stock 0.5M) 5mM
- NP40 (stock 4%) 0.02% (0.2% for adults)
- Sodium-Deoxycholate (stock 1%) 0.01% (0.1% for adults)

**Buffer L0:**
- Phosphate buffer (PB), pH7.2, 100mM; MgCl₂, 2mM; EGTA, 5mM

**Phosphate buffer (PB), pH7.2:**
- Per 500mL of 0.1m sodium phosphate stock (water based), add
  - 34.2mL Na₂HPO₄, 1M stock, pH7.2; 15.8mL NaH₂PO₄, 1M stock, pH7.2. Filter before use.
### Appendix D: Genotype abbreviations used to describe mouse genotype in this thesis.

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<th>Genotype abbreviation</th>
<th>Referring to</th>
<th>As in publication</th>
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<td>Presence of transgene</td>
<td>CAGGSET^Cre/Cre</td>
<td>Homozygous for iCre</td>
<td>unpublished</td>
</tr>
<tr>
<td>Presence of transgene</td>
<td>CAGGSET^Cre/WT</td>
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<td>Homozygous for Cre</td>
<td>1β-actin-Cre homozygotes</td>
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<td>Sox9^flox/flox</td>
<td>Both alleles of Sox9 floxed</td>
<td>2Sox9^flox/flox</td>
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<tr>
<td>Floxed allele</td>
<td>Sox9^flox/WT</td>
<td>One allele of Sox9 floxed</td>
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<td>Floxed or deleted allele</td>
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</tr>
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<td>Deleted allele (assumed)</td>
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<td>Both alleles of Sox9 (partially) deleted</td>
<td>2Sox9^ΔΔ</td>
</tr>
</tbody>
</table>

*also refers to the presence of iCre in all ERT2iCreERT2 or iCre transgenic lines

1Lewandoski et al., 1997a
2Chaboissier et al., 2004

Genes presented in superscript refers to the identification of the presence of a transgene by PCR. WT: locus not altered.
LIST OF PRESENTATIONS

Conferences attended with poster presentation:

15th ISDB Congress, 3-7 September 2005, Sydney, NSW, Australia

Poster title:
An efficient, inducible Cre-recombination system for conditional gene expression in the mouse and chick

Angela Tye*, Christophe Galichet, Martin Cheung, James Briscoe, François Guillemot and Robin Lovell-Badge

BSDB & BSCB Spring Joint Meeting, 20-23 March 2006, York, England

Poster title:
An efficient, inducible Cre-recombination system for conditional gene expression in the mouse and chick

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GRC Reproductive Tract Biology, 18-23 June 2006, Connecticut, CT, USA

Poster title:
Conditional strategies to study gene functions during mammalian sex determination and gametogenesis.

Tye, AJ*, Wynn SL, Cheah KSE, Lovell-Badge, R

BSDB & BSCB Spring Joint Meeting, 29 March – 1 April 2007, Edinburgh, Scotland

Poster title:
Conditional strategies to study gene functions during mammalian development.

Angela Tye*, Christophe Galichet, Martin Cheung, Sarah Wynn, James Briscoe, Kathy Cheah and Robin Lovell-Badge
Conditional strategies to study gene function during mammalian development

Angela Lee, Christophe Galièche, Marina Cheung, Sarah Wynn, James Brisac, Kathy Cheah, Robin Lovell-Badge

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Introduction

Previous studies have shown that the development of the gonad is a complex process involving interactions between genetic and environmental factors. To study these processes, researchers have used a variety of experimental techniques, including the use of transgenic and knock-out mice. In this study, we aim to understand the role of different genes in the development of the gonad by using conditional strategies to study gene function during mammalian development.

Methods

We used CAGGSET, a transgene that is inducible by tamoxifen (OHT) or 4-hydroxytamoxifen (OHT), to study the effects of gene deletion or misexpression in different mouse strains. The transgene is driven by a chicken beta-actin promoter, which is expressed ubiquitously in all tissues. The transgene contains a floxed loxP site, which can be removed by Cre recombinase, allowing for gene deletion or misexpression in a tissue-specific manner.

Results

We observed changes in gene expression in different tissues and cell types, including the developing gonad. We observed changes in gene expression in different tissues and cell types, including the developing gonad. We observed changes in gene expression in different tissues and cell types, including the developing gonad. We observed changes in gene expression in different tissues and cell types, including the developing gonad.

Discussion

Our findings suggest that different genes have different roles in the development of the gonad. Further studies are needed to understand the exact role of each gene in the development of the gonad.

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References