An investigation of the role of Notch signalling in germ cell development using mouse embryonic stem cells

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A thesis submitted for the degree of Doctor of Philosophy

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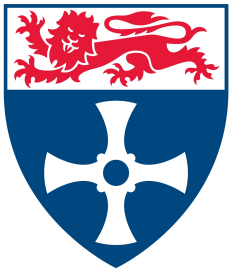

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Declaration

I, Vasileios Floros, declare that no portion of the work compiled in this thesis has been submitted in support of another degree or qualification at this or any other University or Institute of Learning. This thesis includes nothing which is the work of others, nor the outcomes of work done in collaboration, except where otherwise stated.

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Vasileios Floros

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Chapter 1

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AMH	Anti-Müllerian Hormone
ANK	Ankyrin repeats
BMP	Bone Morphogenetic Protein
CR	Cysteine Rich
C-terminus	Carboxy-terminus
DAB	diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phe nylglycine t-butyl ester
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxynucleotide Triphosphate
DMC1	Dosage Suppressor of Mck1
DNA	Deoxyribonucleic Acid
DSL	Delta, Serrate, LAG 2
DTC	Distal Tip Cell
ECD	Extracellular Domain
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
EGF	epidermal growth-factor
ES	Embryonic Stem
FACS	Fluorescence Activated Cell Sorting
FSC	Forward Light Scatter
FBS	Foetal Bovine Serum

GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GDNF	Glial cell line-Derived Neurotrophic Factor
GST	Glutathione-s-Transferase
HES1	Hairy and Enhancer of Split 1
HES3	Hairy and Enhancer of Split 3
HD	Heterodimer
HNRPG-T	Heterogenous Nuclear Ribonucleoprotein G-T
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
ICD	Intracellular Domain
ICM	Inner Cell Mass
IGM	Institute of Genetic Medicine
iPS	Induced Pluripotent Stem
IP	Immunoprecipitation
KCl	Potassium Chloride
kV	Kilo Volts
LB	Luria Bertani
LIF	Leukemia Inhibitory Factor
LNR	LIN Notch repeats
MEFs	Mouse Embryonic Fibroblasts
MIN	Minutes
mRNA	Messenger Ribonucleic Acid
MVH	Mouse Vasa Homologue
NEO	Neomycin Phosphotransferase II
NCBI	National Centre for Biotechnology Information
NLS	Nuclear Localization Signals
N-terminus	Amino-terminus

OCT4	Octamer-Binding Transcription Factor
PAC	Puromycin N-Acetyl-Transferase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P.C.	Post Coitum
PEST	Pro Glu Ser Thr
PFA	Paraformaldehyde
PGCs	Primordial Germ Cells
PGK2	Phosphoglycerate kinase 2
PND	Postnatal day
PRM1	Protamine 1
RNA	Ribonucleic Acid
RBMY	RNA-Binding Motif, Y chromosome
RPM	Revolutions Per Minute
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
SDS	Sodium Dodecyl Sulfate
SSC	Side light Scatter
STRA8	Stimulated by Retinoic Acid gene 8
SYCP1	Synaptonemal Complex Protein 1
SYCP3	Synaptonemal Complex Protein 3
TAE	Tris-acetate-EDTA buffer
TAD	Transcriptional Activation Domain
TP1	Transition protein 1
TE	Tris, pH 8.0 EDTA
VAL	Valine

Abstract

Understanding which genes and proteins are expressed of spermatogenesis and testing their roles by modelling germ cell development *in vitro* are possible routes to enable treatment of male infertility. Of the 15% of couples who experience difficulty in conceiving, approximately half involve some degree of male factor infertility and for 30-50% of these men no cause has been identified for the poor sperm characteristics. Although assisted reproductive technologies have dramatically improved the prospects for infertile couples, there are some types of male factor infertility that remain untreatable. There has previously been no robust cell-based model for examining germ cell development *in vitro*. All germ cell types analysed to date can be identified by marker analysis during ES cell differentiation. A report *where* the generation of post-meiotic germ cells from mouse embryonic stem (ES) cells was described presented the hope that directed differentiation of ES cells into germ-like cells can potentially provide a cell-based platform for investigating the molecular events that regulate germ cell development. Here, we exploit and further develop that protocol to establish stable transfected cell lines that harbour spermatogenic stage specific fluorescent markers that allow monitoring of germ cell development *in vitro* and therefore the investigation of molecular events underling this process. Having established the ES cell-based system we focussed on the investigation of the role Notch receptors and their ligands during germ cell development. Studies in *Drosophila* and *C. elegans* have been shown Notch components to regulate germ cell fate, however the exact function of Notch signalling in mammalian spermatogenic cells is not known. To better understand the function of Notch pathway, we initially identified members of the Notch receptors and ligands expressed in mouse germ cells during each developmental stage of spermatogenesis. Subsequently, using our *in vitro* model we investigated the possible role of each Notch component by achieving both blockage and activation of Notch signalling. We show that inactivation of Notch signalling does not impair germ cell development *in vitro*, but activation of Notch receptors by soluble ligands promotes meiotic progression of germ cells *in vitro*.

Chapter 1. Introduction

1.1 Germ cell development: an overview

Germ cells can be defined as the cells that will eventually mature into gametes (sperm and eggs). These cells represent a highly specialized cell population that is unique in its ability to carry the genetic information onto the next generation.

1.1.1 Germ cell specification

Studies in previous years have enormously advanced our understanding on the process of germ cell development. In mice, 3 days after fertilization a series of cleavages and cell proliferation give rise to a fully expanded blastocyst which is composed of around 64 cells and is comprised of two different tissues: the trophectoderm that will later in development will give rise to the placenta, and the inner cell mass (ICM) a few

pluripotential cells from which the epiblasts cells will arise and are these cells that later in development will constitute the embryo (Figure 1). Studies on the mouse model have revealed that until ~6 days post coitum (dpc) all epiblast cells retain their developmental potential and up until this stage all have the ability to become germ cells. Shortly before gastrulation, germ cell specification occurs in the proximal epiblast. Only the cells (in the proximal epiblast) that are adjacent to the extraembryonic ectoderm and so exposed to neighbouring signals from the extraembryonic ectoderm (secretion of bone morphogenetic protein (BMP) 4) are destined to become germ line cells (Fujiwara et al., 2001, Saitou et al., 2003). BMP4 signals are thought to increase the expression of the *Ifitm3* (*fragilis*) gene in the proximal epiblasts cells initiating by this way the germ line formation (Saitou et al., 2002).

After implantation of the embryo, some of the cells that express *fragilis* migrate to the extraembryonic ectoderm and begin to express the *Dpp3α* (*stella*) gene. These founder population of germ line cells, that are now called primordial germ cells (PGCs), are first identified at the base of the allantois around the onset of gastrulation embryonic day E7.25 (Figure 1) (Ginsburg et al., 1990, McLaren, 2003).

Another gene that is specifically expressed in cells of the germline is *Oct-4* also known as (*Pou5f1)* a transcription factor known to be associated with pluripotency. During these events, *Oct-4* expression is repressed in somatic cell lineages and is found to be expressed only in PGCs (Boiani and Scholer, 2005).

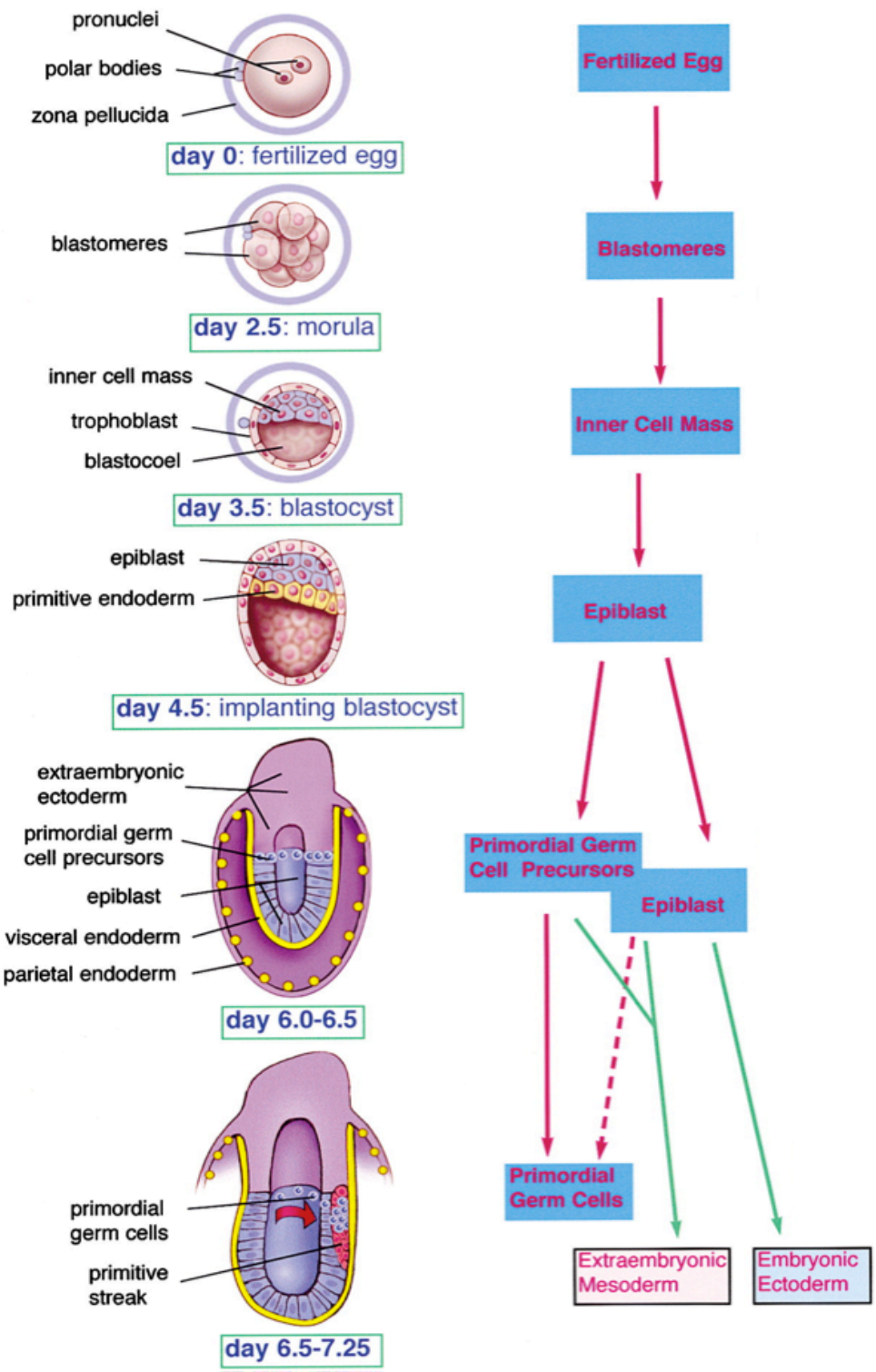

Figure 1. Specification of mouse embryonic primordial germ cells from fertilized egg to the time of PGC formation at day 7.25. Germ line specification starts around E6.0. PGCs appear first in the extraembryonic mesoderm by E7.0. PGC precursors in the proximal epiblast and PGCs in the primitive streak area are depicted as blue circles with darker smaller circles as nuclei. The appearance of different cell types during early development is shown on the right as boxes. Arrows are used to show differentiation. Cells considered totipotent or pluripotent are boxed in blue. These include the fertilized egg, blastomeres, cells in the inner cell mass (ICM), epiblast, PGC precursors, and PGCs. Red arrows are used to indicate the path from totipotency and pluripotency to PGCs. In addition to the PGC precursors, some epiblast cells have the potential to form PGCs *in vitro* (shown by a dashed red arrow). Green arrows are used to indicate differentiation of cells that do not have pluripotent character. Figure and legend adapted and modified from (Zhao and Garbers, 2002)

1.1.2 Germ cell migration

Within 24 hours (hrs) after the establishment of the mouse germ cell lineage; a downregulation of *fragilis* is observed and the cells begin to lose their cluster formation. Around E8.5 the endoderm invaginates and hindgut starts to form (McLaren, 2003). Upon extension of the hindgut the PGCs migrate from the base of the allantois through the mesentery towards the dorsal wall to finally arrive into the two nascent genital ridges (Figure 2). For many years, the activity of tissue non-specific alkaline phosphatase (TNAP) has been used to track PGCs and monitor their migration. During the migratory period, that takes place between E8.5 to E11.5 dpc in the mouse PGCs undergo active proliferation (McLaren, 2003).

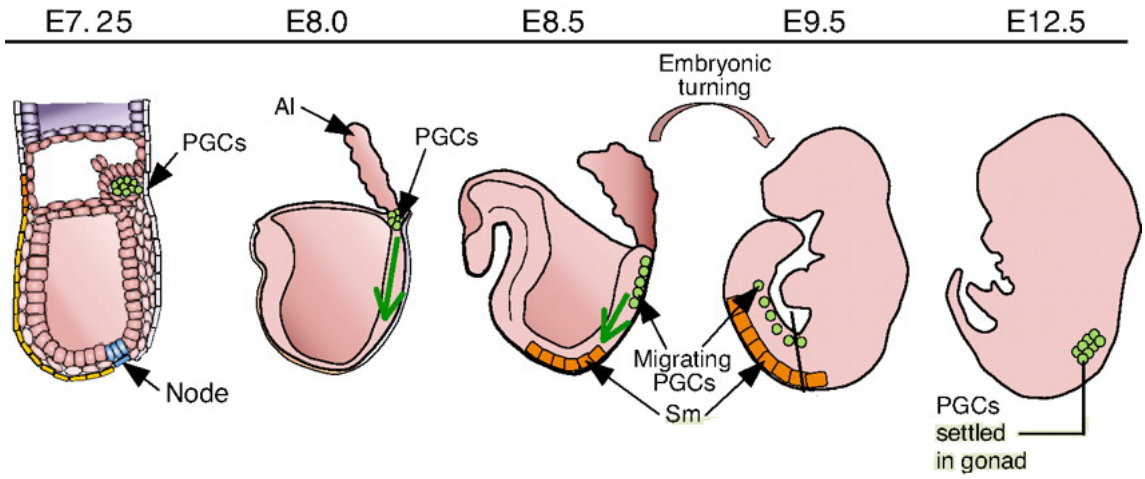

Figure 2. A schematic diagram of germ cell migration in developing mouse embryos. PGC are shown as green circles. Germ line migration starts around E8.0-E8.5. PGCs, while undergo proliferation, migrate from the hindgut though the mesentery and start reaching the genital ridges around E10.5. Adapted and modified from (Saitou et al., 2012).

1.1.3 Germ cells in the genital ridge

By 11.5 dpc, the PGCs complete migration, and upon their entry into the genital ridges, they loose their locomotory activity. From this stage on PGCs are usually referred as gonocytes. In the genital ridge, gonocytes start expressing a number of germ-cell-specific

genes including *Mvh* (also know as *Ddx4*) a highly conserved *mouse homolog* of *Drosophila vasa* (Fujiwara et al., 1994, Toyooka et al., 2000) which is widely used as marker gene for post migratory and spermatogenic germ cells (Noce et al., 2001a). Mutations in the *Mvh* result in defective proliferation and differentiation of PGCs.

Up to this stage, the sex of the organism cannot be determined by morphological means. However, by 13.5 dpc sex-specific development is initiated, and ovary and testis become morphologically distinguishable (Brennan and Capel, 2004). In the female genital ridge, gonocytes enter meiotic prophase; continue through leptotene, zygotene and pachytene stages and just before birth they become arrested in diplotene stage as oocytes (Hilscher et al., 1974). In the male genital ridge however, things are quite different. The primary testis cords start to form (which later will become seminiferous tubules) as the same time somatic-origin Sertoli precursor cells migrate inwards and envelope the gonocytes which are also surrounded by peritubular cells (Clermont and Perey, 1957, Sapsford, 1962). After a burst of mitotic activity, gonocytes stop dividing and enter a phase of mitotic arrest as G0/G1 pro-spermatogonia (Hilscher et al., 1974). Gonocytes will remain arrested at this stage until after birth (de Rooij, 1998).

1.2 Spermatogenesis

1.2.1 Phases of spermatogenesis

In adult male mammals million of sperms are produced every day. This dynamic cell producing process that starts right after birth in mice and during puberty in humans can be separated in three main phases.

Mitosis: Spermatogenesis is a continuing process and each spermatogenic wave relies on the active proliferation and differentiation of spermatogonial stem cells (SSCs). In rodents, a few days after birth, pro-spermatogonia, also known as gonocytes, resume proliferation while they move to the basal membrane of the seminiferous tubule (Figure 3), and develop into SSCs, also known as undifferentiated type-A spermatogonia (de Rooij, 2001). Undifferentiated spermatogonia are diploid germ cells (2n) that are usually

found in clusters of 3-5 cells close to the basement membrane. Upon mitotic division, SSCs can either give rise to two new progenitor cells or can remain connected by an intercellular bridge as a pair of so called Apr spermatogonia. One of the factors that have been found to control the genes essential for self-renewal and differentiation of the stem cells is the glial cell line-derived neurotrophic factor (GDNF) (Kubota et al., 2004). GDNF, which is secreted by Sertoli cells, binds to receptors expressed in SSCs. Apr formation initiates differentiation that leads to different types of spermatogonia which are recognised as type-A, intermediate and type-B spermatogonia.

Meiosis: The final division of B-spermatogonia leads to the production of preleptotene spermatocytes. As preleptotene spermatocytes enter meiosis and become leptotene and zygotene spermatocytes they start to migrate towards the inner part of the seminiferous tubules and move through the tight junctions between Sertoli cells (Figure 3). During the meiotic phase the chromosomes of the spermatocytes start to replicate and condense, and homologous chromosomes pair up and form synaptonemal complexes also known as synapses that allows them to recombine and exchange genetic material. Then homologue chromosomes align along the metaphase plate and each homologue, as the first meiotic division approached completion, ends up in the opposite cell. In the second division during anaphase the sister chromatids separate and beginning to move to opposite ends of the cell to form two haploid spermatids (Figure 3). Spermatocytes can easily be identified by microscopic analysis and can be found in all stages. This due to the fact that meiosis takes 14 days to complete in the mouse.

Spermiogenesis: Haploid spermatids (1n) then undergo a series of dramatic morphological changes. Their chromosomes become highly condensed and their shape becomes elongated. After further maturation they are released in the lumen as mature spermatozoa (Figure 3).

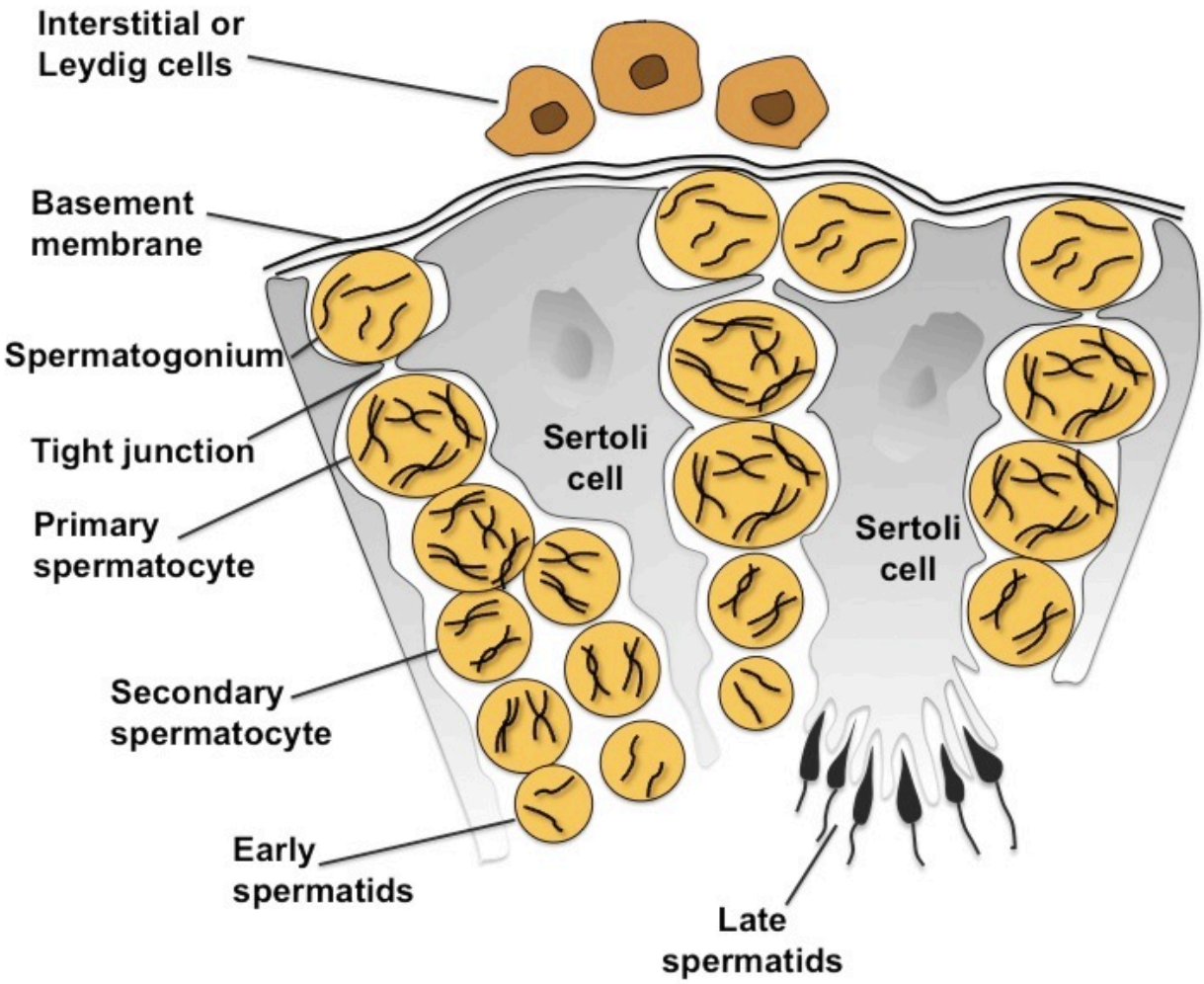

Figure 3. **Diagrammatic representation of the organization of the seminiferous tubule. Germ line cells of different developmental stages and somatic cells (Sertoli cells) constitute the stratified epithelium of the seminiferous tubules. As a spermatogonium differentiate it undergoes a series of morphological changes. Tight junctions formed by Sertoli cells act as checkpoints that regulate migration of germ cells towards the lumen where germ cells are released as haploid spermatids.**

1.2.2 Regulation of the spermatogenic cycle

Spermatogenesis takes place over a period of about 64 days in human testis and around 35 days in mouse (Wolgemuth et al., 1991, Adler, 1996). It is a highly regulated and complex developmental process that implies drastic changes in cellular morphology and series of dynamic variations at the molecular level. Many of the changes that occur during this time are regulated by Sertoli cells, the only somatic cells present in the seminiferous tubule.

The Sertoli cells are radially distributed around the perimeter of the seminiferous tubules. As spermatogonia, which are sited at the basement membrane, differentiate towards the lumen, they migrate in groups and can be separated in advanced and less advanced zones. This movement is mediated and closely controlled by tight junctions formed by adjacent Sertoli cells that act as a barrier and allow the coordination and controlling of the environment that surround the germ cells. The coordination of the spermatogenic process requires a system of distance originated signals, endocrine signals or hormones, and signals from a nearby cell called paracrine signals (Griswold, 1998).

Some of the known hormones that have been shown to regulate mammalian spermatogenesis are follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Walker and Cheng, 2005). When LH is produced, by gonadotroph cells in the anterior pituitary, it binds to receptors on the surface of Leydig cells (cells located between the seminiferous tubules) and stimulates the production of testosterone, in the testis. Testosterone is a steroid hormone that spreads into the seminiferous tubules and binds with high affinity to intracellular androgen receptors in Sertoli cells and stimulates gene transcription (Elliston et al., 1990). The route of FSH is less complicated, as upon secretion of FSH by gonadotroph cells the hormone travels to the testis via blood circulation and binds to G protein receptors that only Sertoli cells bear in the testis. FSH is thought to control the function of Sertoli cells by enhancing the production of androgen-binding protein and it has been found to be very important for the initiation of spermatogenesis (Alves et al., 2013).

The control of the progression of germ cells to spermatozoa also involves paracrine signals secreted by Sertoli cells. Some of the key substances are anti-Müllerian hormone (AMH) (Josso et al., 2006), which is secreted in early stages of fetal life, inhibin, which is secreted after puberty and it's thought to regulate FSH secretion (de Kretser et al., 2004) and glial cell line-derived neurotrophic factor (GDNF) which has been shown to be essential for the maintenance of the spermatogonial stem cell in the adult testis (Hofmann et al., 2005).

1.2.3 The role of retinoic acid in germ cell development

One of the molecules that has been shown to play a critical role in the initiation and progression of spermatogenesis is retinoic acid (RA) the biological active derivative of vitamin A (retinol). In fact, it has been shown that mice deficient for vitamin A are not fertile and spermatogonia differentiation is arrested. However, when these mice were treated with RA spermatogenesis was completly recovered (Hogarth and Griswold, 2010)

RA, is a small polar molecule that can easily diffuse into the cytoplasm and enter the nucleus where it acts by binding to nuclear RA receptors (Mark et al., 2006). In this manner, it can regulate the expression of a number RA-responsive gene. Various reports have shown evidence that the fate of postmigratory PGCs, whether they will follow the meiotic route or undergo mitotic arrest, is controlled by RA (Bowles et al., 2006, Koubova et al., 2006). In the male genital ridges degradation of RA by Cyp26b1 enzyme prevents progression of PGCs into meiosis. On the contrary, in the female genital ridges where no or limited expression of Cyp26b1 enzyme is detected, PGCs enter meiosis. Cyp26b1 expression has been linked with somatic cells such as Sertoli and interstitial cells (Menke and Page, 2002).

In spermatogenesis RA has been shown to be able to induce expression of *Stra8* gene a vertebrate-specific gene that encodes a cytoplasmic protein (Oulad-Abdelghani et al., 1996). In fact the expression of Stra8 gene is directly related to the amount of RA available in the environment. Studies have shown that *Stra8* is predominately expressed in pre-meiotic germ cells. Stimulation of *Stra8* by RA directly induces the transition of spermatogonia stem cells from an undifferentiated state to initiation of differentiation by activating *Kit* gene expression. Although *Stra8* activation has a smaller impact in differentiating spermatogonia the expression of *Stra8* gene is greater in the spermatogonia than the spermatogonia stem cells. It has been hypothesised that *Stra8* in differentiating spermatogonia is involved in spermatogonia maturation. RA is thought to stimulate the expression of genes with a role in metabolism, storage, signalling and transcription (Zhou et al., 2008, Koubova et al., 2006)

Although, recent results indicate that RA stimulates meiotic entry in the testis (Anderson et al., 2008) direct evidence for a role of endogenous RA in undifferentiated spermatogonia remain largely unknown. Interestingly, a recent study showed that

although RA triggers Sertoli cells to secrete paracrine signals, the presence of RA it self is sufficient to cell autonomously drive meiotic initiation in spermatocytes (Raverdeau et al., 2012)

1.2.4 Genes involved in germ cell development

A large number of genes are involved in the coordinated progression of spermatogenesis and these are expressed in different stages of germ cell development. Many of these genes are testis specific and some of them can be used to separate germ cells from somatic cells. In some other cases however, genes have been found to be specific for a spermatogenic stage. All the genes used in this study for the characterization of germ cells are summarized in Table 1.

Gene name	Stage	Gene function	References
Piwil2	Pre-meiotic	Central role during spermatogenesis by repressing transposable elements and prevent their mobilization, which is essential for the germline integrity. Essential role in meiotic differentiation of spermatocytes, germ cell differentiation and in self-renewal of spermatogonial stem cells.	(Kuramochi-Miyagawa et al., 2004, Lee et al., 2006)
Stella (Dpp3α)	Pre-meiotic	Participates in protection of DNA methylation and is involved in epigenetic reprogramming	(Sato et al., 2002)
Mvh (Ddx4)	Pre-meiotic	Involved in alteration of RNA secondary structure such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly.	(Noce et al., 2001b)
Oct-4 (Pou5f1)	Pre-meiotic	Transcription factor important in embryonic development and PGCs formation	(Kehler et al., 2004)
Stra8	Pre-meiotic	Involved in the regulation of meiotic initiation in both spermatogenesis and oogenesis	(Anderson et al., 2008)
Sycp1	Meiotic	Major component of the transverse filaments of synaptonemal complexes, formed between homologous chromosomes during meiotic prophase	(Costa et al., 2005)
Sycp3	Meiotic	Structural component of the synaptonemal complex also involved in synapsis, recombination and segregation of meiotic chromosomes.	(Miyamoto et al., 2003)
Dmc1	Meiotic	Essential for meiotic homologous recombination. Structurally and evolutionary related to the products of the yeast RAD51	(Yoshida et al., 1998)
hnRNPG-T	Meiotic	RNA binding protein involved in pre-mRNA processing which is expressed in spermatocytes through out meiosis	(Elliott et al., 2000)

Pgk2	Meiotic	Involved in glycolysis, energy pathway, and ATP generation	(McCarrey and Thomas, 1987)
Tp1	Post-meiotic	Spermatid-specific product of the haploid genome which replaces histone and is itself replaced in the mature sperm by the protamines	(Akama et al., 1994)
Prm1	Post-meiotic	Protamine 1 substitutes histones in the chromatin of sperm during the haploid phase of spermatogenesis	(Domenjoud et al., 1990)

Table 1. Genes used in characterization of ES-derived germ-like cells

1.3 Notch signalling pathway

1.3.1 Notch signalling pathway components

The Notch signalling pathway is an evolutionary conserved mechanism involved in the control of a wide spectrum of developmental and physiological processes. The role of this pathway in cell fate determination and pattern formation has extensively been described (Artavanis-Tsakonas and Rand, 1999). The Notch pathway orchestrates many developmental events by mediating cell-cell communication between neighbouring cells as both Notch receptors and ligands are single pass transmembrane proteins (Bray, 2006). It was first identified to function in *Drosophila melanogaster* in 1914 where flies with haploinsufficency of the *Notch* gene found to have a characteristic notched wing. Subsequent studies showed that Notch pathway is involved in many developmental processes in other invertebrates and vertebrates (Bate et al., 1993, Fre et al., 2005, Ohlstein and Spradling, 2006, van Es et al., 2005).

Four *Notch* receptor genes (*Notch1-4*) and five *Notch* ligands genes (*Delta-like1*, *Delta-like3*, *Delta-like4*, *Jagged1* and *Jagged2*) have been isolated in mammalian organisms (Figure 4). The Notch family proteins consist of large single-pass transmembrane proteins and share some common characteristic features. Notch receptors are found in the surface of the cells as heterodimers, as the extracellular domain was cleaved during the posttranslational modification and is now non-covalently attached with the intracellular domain. In Notch receptors the extracellular domain consists of epidermal growth-factor (EGF) like repeats and three LIN Notch repeats (LNR). Function of EGF-like repeats,

which are responsible for ligand binding, is influenced by glycosylation and is also shown to be Ca^{2+}-dependent (Rand et al., 2000, Bray, 2006). The intracellular domain consists four main regions: the Ankyrin (ANK) repeats and the RAM domain both involved in protein interaction, the nuclear localization signals (NLS) and the transcriptional activation domain (TAD). TAD is the region that interacts with members of the CSL (CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1) family of transcription factors. Notch3 and Notch4 contain no TAD domain. The C-terminal Pro Glu Ser Thr (PEST) sequence is required for degradation.

Notch ligands can be separated in two types Delta and Jagged. The extracellular domain of Jagged ligands contains more EGF-like repeats than Delta and a cysteine rich (CR) domain that Delta ligands lack. Both ligands however contain a cysteine rich N-terminal DSL (Delta, Serrate, LAG 2) domain. Although it has been shown that EGF repeats in Notch receptors can bind to EGF repeats in the ligands there is no established pattern that shows which ligands activate which receptors (Schweisguth, 2004).

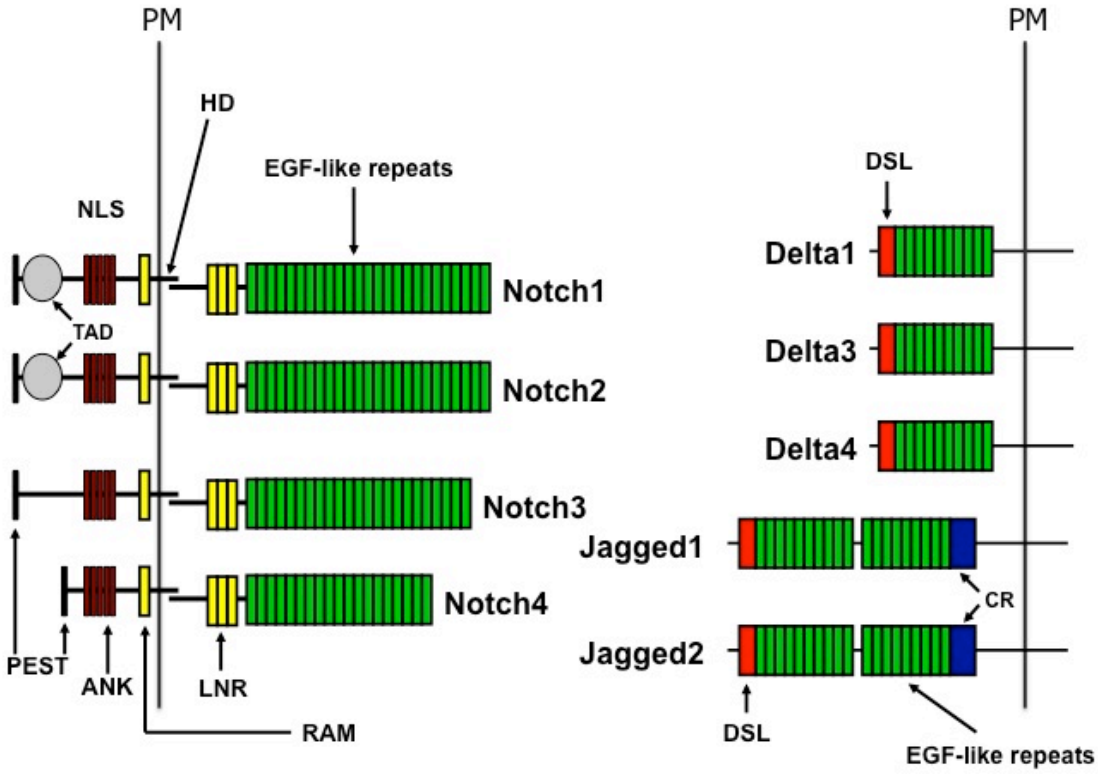

Figure 4. Structure of Notch receptors and ligands. Notch proteins are expressed in the plasma membrane (PM) of the cells. Notch receptors are found as heterodimers (HD) where the large extracellular domain is non-covalently linked to the intracellular domain. The extracellular domain

consists of epidermal growth-factor (EGF) like repeats and three LIN Notch repeats (LNR). The intracellular domain consists of Ankyrin (ANK) repeats and the RAM domain both involved in protein interaction. The nuclear localization signals (NLS) and the transcriptional activation domain (TAD) activate downstream events. Notch3 and Notch4 contain no TAD domain. The C-terminal Pro Glu Ser Thr (PEST) sequence is required for degradation. Notch ligands extracellular domain also consists of epidermal growth-factor (EGF) like repeats and in addition contain a cysteine rich N-terminal DSL (Delta, Serrate, LAG 2) domain. Jagged1, and Jagged2 contain an additional cysteine rich domain (CRD).

1.3.2 Notch signalling pathway in action

Interaction between the Notch ligand and the Notch-extracellular-domain ($Notch^{-ECD}$) triggers a proteolytic cleavage at the site where the $Notch^{-ECD}$ is non-covalently associated with the $Notch^{-ICD}$. This cleavage is processed by γ-secretase in the presenilin protein complex (for review see (Artavanis-Tsakonas et al., 1999) and dissociates the Notch-intracellular-domain ($Notch^{-ICD}$) for the rest of the receptor. As a result the $Notch^{-ICD}$ is released in the cytoplasm and then translocates to the nucleus where it bounds to other transcription factors of the CSL family and together they form a complex that activates the expression of target genes (Figure 5).

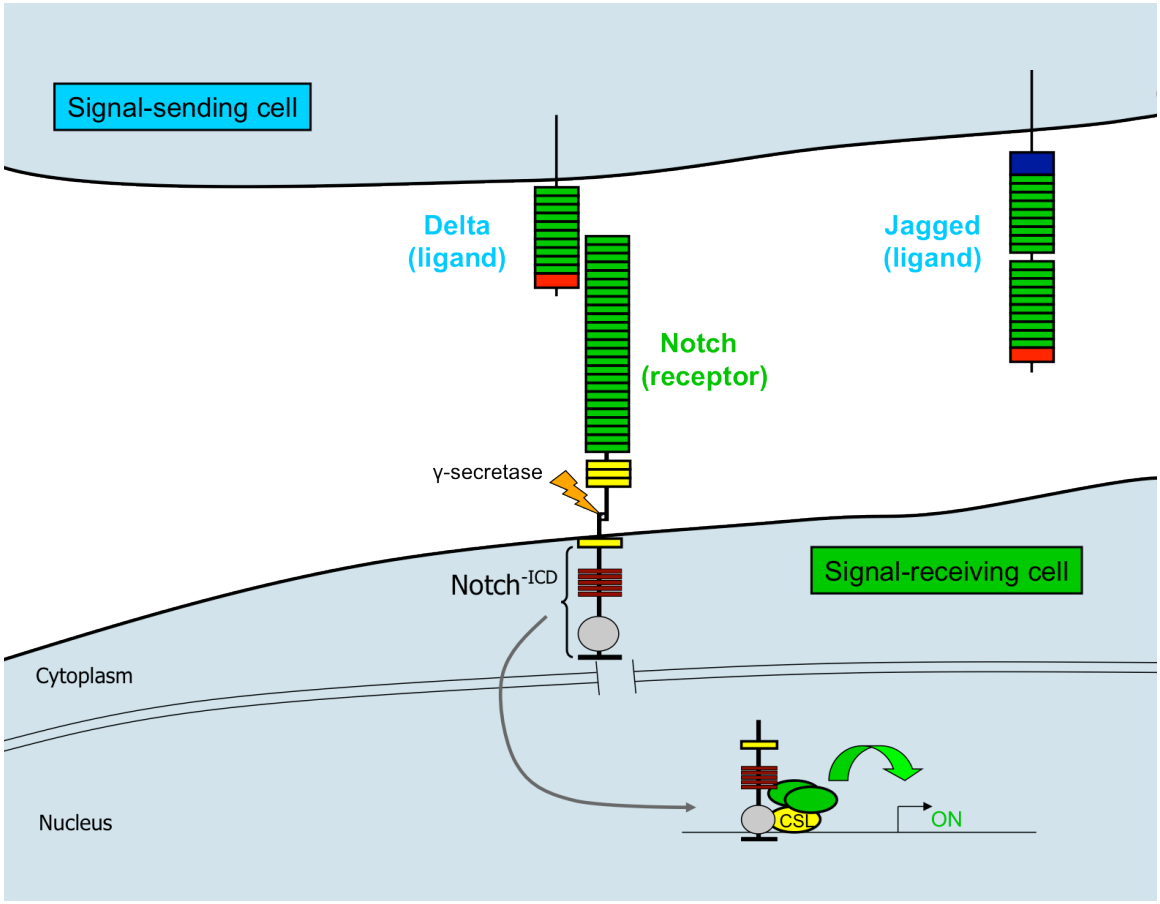

Figure 5. The canonical Notch signalling pathway. Binding of the ligand to the Notch receptor triggers a proteolytic cleavage of the transmembrane domain by the γ-secretase complex and the release of Notch intracellular domain (Notch^{-ICD}) from the membrane. The Notch^{-ICD} then translocates to the nucleus where it forms a transcriptional complex with CSL and activates the transcription of target genes.

Several studies have shown that Notch signalling between adjacent cells plays a critical role in cell fate decisions. Although different developmental processes requires different cell-fate decisions the way Notch mediates these decisions can be classified based on their result in three distinct types (Figure 6). In the first one, and the most recognized, called lateral inhibition cells within the same group that share the same developmental potential can acquire different fates by signal surrounding cells through the Notch pathway to inhibit their ability to acquire the same fate (Figure 6 A). Activation of Notch receptor in a cell regulates the expression of Notch ligands in its surface. So as the level of Notch activation is proportional to the amount of ligands in its adjacent cell and vice-versa this results in a feedback mechanism that determines the fate of neighbouring cells. In the case of lateral inhibition activation of Notch inhibits the production of ligands. As

a result, a cell with more ligands signals the adjacent cells to reduce their ligand production. Many genetic studies in flies and worms have elucidated the mechanism of this process (for review see (Chitnis, 1995, Fortini, 2009). The second type know as inductive signalling and responsible for the formation of tissue layers in a developing organism is when one group of cells signals through the Notch pathway to a distinct neighbouring group of cells to induce a new cell fate along the interface between them (Figure 6 B). And the third type called lineage divisions in which at each cell division, Notch is asymmetrically activated in only one daughter cell but not in the other cell. This results in the Notch activated cells to adopt distinct cell fates (Figure 6).

In addition to these main models of signalling, Notch ligands part from their regular function to activate Notch receptors in adjacent cells, they have also been implicated in inhibiting Notch signalling within the same cell by cis-interaction with the Notch receptors in its own cells (del Alamo et al., 2011). Moreover, Notch pathway has been shown to be regulated at numerous levels, such as receptor and ligand modification during posttranslational processing, degradation and auto regulation (Bray, 2006).

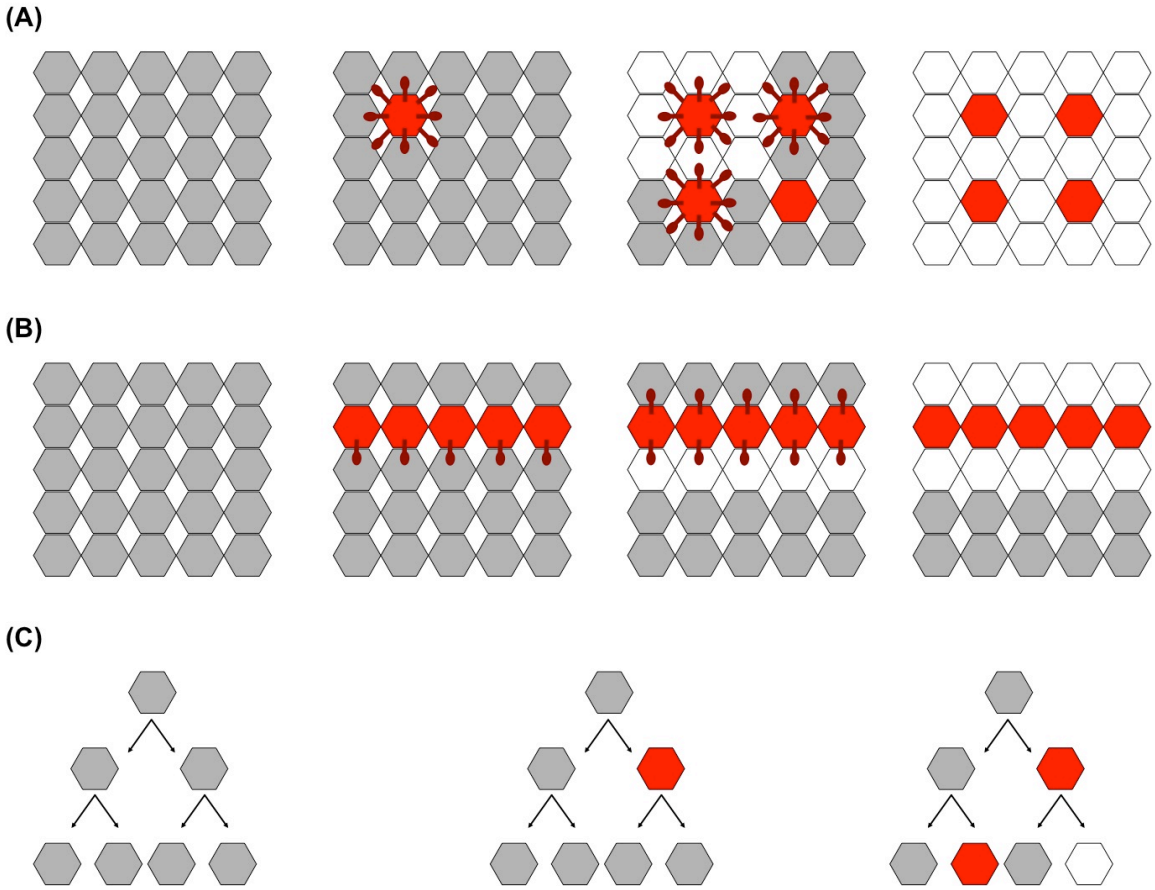

Figure 6. Examples of Notch pathway regulatory functions. (A) Lateral inhibition, (B) Inductive signalling and (C) Lineage decisions. Figure adapted and modified from (Mazur, 2013)

Even though several studies have shown that Notch signalling plays a critical role in regulating cell fate in many different specialized local environments only a small number of target genes have been discovered. One of the targets that have been considerably studied is the *Hairy and Enhancer of split* (*Hes*) family. Although the family consists of seven genes *(Hes1-7)* only *Hes1* and Hes3 are induced by activation of Notch receptors. The products of these genes function as transcriptional repressors and are very important in development as knockout mice die before birth (Iso et al., 2003).

1.4 Research objectives

Spermatogenesis is a highly dynamic biological process that requires the coordination of renewal, proliferation, differentiation and maturation of a variety of cells. One key mechanism in controlling cell fate decisions and cell-cell communication is the Notch signalling pathway. The Notch signalling pathway is an evolutionary conserved mechanism involved in the control of a wide spectrum of developmental and physiological processes. The role of this pathway in cell fate determination and pattern formation has extensively been described. Biochemical and genetic analyses have shown that importance of the Notch pathway in developmental processes.

Previous studies have demonstrated the Notch signalling pathway activity to be also essential for gametogenesis in invertebrate and vertebrate organisms. Although, a few sporadic studies have shown the expression of Notch pathway components in mammalian spermatogenesis the function of Notch signalling pathway in this process remains obscured.

Therefore, the aim of this study was to shed light to the physiological role of Notch signalling system in the development of germ cells. Given the discrepancy of previous studies in the expression of Notch proteins an extensive postnatal and adult expression analysis was performed to identify all the components present in the seminiferous tubule. The numerous Notch receptors and ligands of the pathway make the genetic study of all these components almost impossible. For this reason an *in vitro* system where the development of ES-derived germ-like cells had been shown was adapted. The *in vitro* system was further developed to allow the investigation of Notch pathway in both pre-meiotic and meiotic stages. After the *in vitro* platform was established manipulation of Notch activity and various Notch proteins was performed examine their function.

Chapter 2. Materials and Methods

2.1 Standard molecular biology techniques

2.1.1 Polymerase Chain Reaction (PCR)

The reagents used in a typical 50µl reaction mixture are listed in Table 2.

Reagent	Quantity for a 50µl reaction
Deionised H_2O	39.1µl
10X PCR Buffer (500mM KCl, 200mM Tris-HCl pH8.4)	5µl
50mM $MgCl_2$	1.5µl
10mM dNTP Mix	1µl
10µM Forward primer	1µl
10µM Reverse primer	1µl
Template DNA (~100ng)	1µl
Taq DNA polymerase	0.4µl

Table 2. PCR recipe.

10X PCR buffer and Taq DNA polymerase were purchased from Invitrogen. Deoxyribonucleotide triphosphates (dNTPs) were purchased from Promega and primers were purchased from Eurogentec. Primers used in this project are listed in appendix A.

Typical PCR conditions consisted of an initial denaturation step at 92ºC for 1 minute followed by 30 cycles of denaturation at 92ºC for 30 seconds, annealing at 55-60ºC for 30 seconds (depending on the primers), extension at 72ºC for 1 minute with a final extension at 72ºC for 5 minutes. PCR amplification was carried out using a Mastercycler gradient (Eppendorf).

2.1.2 RNA extraction from tissue or cultured cells

Total cellular RNA was extracted from cultured cells or tissue using Trizol Reagent (Invitrogen) following manufacturer's instructions. Briefly, 1ml of Trizol reagent was added to samples in centrifuge tubes and incubated at RT for 5 minutes. 200ml of chloroform was added and samples were shaken by hand (15 sec) to mix. After a further incubation for 3 minutes at RT (RT), the samples were centrifuged at 12,000 × g for 15 minutes at 4ºC. The upper aqueous layer was transferred to a new tube and an equal volume of isopropanol was added. The samples were incubated at RT for 10 minutes and

then centrifuged again at 12,000 × g for 10 minutes at 4ºC. The supernatant was removed and the RNA pellet was washed with 1ml of 75% ethanol. The RNA pellet was air dried for 10 min and then resuspended in nuclease free dH_2O. RNA concentration was determined using a NanoDrop Spectrophotometer (NanoDrop Technologies).

2.1.3 Reverse transcriptase PCR (RT-PCR)

cDNA was synthesised from total RNA using SuperScript® III reverse transcriptase (Invitrogen) and then amplified using the Taq DNA polymerase (Invitrogen) following manufacturer's instructions. Briefly, 20µl reactions were set up as indicated in Table 3.

Reagent	Quantity for a 20µl reaction
oligo$(dT)_{20}$ (50µM)	1µl
total RNA (10pg-5µg)	1µl
dNTP Mix (10mM each)	1µl
Deionised H_2O	13µl
Incubate at 65^{o}C for 5 min and on ice for 1min	
5x First-Strand Buffer	4µl
0.1 M DTT	1µl
RNaseOUT™ (RNase inhibitor)	1µl
Superscript™ III Reverse Transcriptase	1µl
Incubate at 50^{o}C for 60 min	
Incubate at 70^{o}C for 15 min	

Table 3. First-Strand cDNA synthesis. (adapted from the Invitrogen protocol)

The cDNA was then used as a template for PCR amplification in PCR. Briefly, 50µl reactions were set up as indicated in Table 4. Reactions were incubated at 94^{o}C for 2 minutes, followed by 30 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 56^{o}C for 30 seconds, extension at 72^{o}C for 1 minute with a final extension at 72^{o}C for 10 minutes. Reactions were carried out using a Mastercycler gradient (Eppendorf). Primers used for RT-PCR are listed in Table 5.

Reagent	Quantity for a 50µl reaction
10x PCR Buffer (500mM KCl, 200mM Tris-HCl pH8.4)	5µl
50mM $MgCl_2$	1.5µl
10mM dNTPs Mix	1µl
10µM Forward primer	1µl
10µM Reverse primer	1µl
Taq DNA polymerase	0.4µl
cDNA (from first-strand reaction)	2µl
Deionised H_2O	38.1µl

Table 4. PCR amplification of cDNA (adapted from the Invitrogen protocol).

Primer name	Sequence (5′-3′)	Purchased from
Hprt1-F	CCTGCTGGATTACATTAAAGCACTG	Eurogentec
Hprt1-R	GTCAAGGGCATATCCAACAACAAAC	Eurogentec
Piwil2-F	TTGGCCTCAAGCTCCTAGAC	Eurogentec
Piwil2-R	CATGCCACGGAACATGGAC	Eurogentec
Stella-F	GACCCAATGAAGGACCCTGAA	Eurogentec
Stella-R	GCTTGACACCGGGGTTTAG	Eurogentec
Mvh-F	CACCGGCAATTTTGACTTTT	Eurogentec
Mvh-R	GTTTGAGCACAAGCCATCAA	Eurogentec
Oct4-F	CACCATCTGTCGCTTCGAGG	Eurogentec
Oct4-R	AGGGTCTCCGATTTGCATATCT	Eurogentec
Sycp1-F	TGAAAAGAAGGATCATTTAACATCAG	Eurogentec
Sycp1-R	TGTTGAGTTCTTCCATTTGAGC	Eurogentec
Sycp3-F	TGCCAAGAGGAAAAGAATAGAAA	Eurogentec
Sycp3-R	CACTGCTGCAACACATTCAT	Eurogentec
Dmc1-F	ACCGCTTCAACGTAGACCAT	Eurogentec
Dmc1-R	CCACTCGAAAAAGTGCCATT	Eurogentec
Pgk2-F	GGTCGGCCTGATGGTATCC	Eurogentec
Pgk2-R	GCAGGGTCAGCACTAATCTTTT	Eurogentec
Tp1-F	AAGAACCGAGCTCCTCACAA	Eurogentec
Tp1-R	GGGGAGAAACAGCCAACATA	Eurogentec
Prm1-F	ATGGCCAGATACCGATGCT	Eurogentec
Prm1-R	CAGCATCTTCGCCTCCTC	Eurogentec
Hes1-F	TCAACACGACACCGGACAAAC	Eurogentec
Hes1-R	ATGCCGGGAGCTATCTTTCTT	Eurogentec
Hes3-F	GCACGCATCAACGTGTCAC	Eurogentec
Hes3-R	TGAGTTCTGGAGGCTTCTCAT	Eurogentec
Notch1-F	AGGGTGGTCAGGAAAATCAT	Eurogentec
Notch1-R	CGATAGGAGCCGATCTCATT	Eurogentec
Notch2-F	AGTGTGCCACAGGTTTCACT	Eurogentec
Notch2-R	TTGGCAGTTGCACTGGTAAC	Eurogentec
Notch3-F	CCGTGTGGCCTCTTTCTACT	Eurogentec
Notch3-R	CAATCGAGCACTCATCCACA	Eurogentec
Notch4-F	AGTGTCTCCCAGGCTTTGA	Eurogentec
Notch4-R	GTGTTCCTGACCTTGGCATT	Eurogentec
Delta1-F	GGTTTGTGTGTGACGAGCAC	Eurogentec
Delta1-R	CTCCCCTGGTTTGTCACAGT	Eurogentec
Delta3-F	CCGGTCTATACGGAGCACC	Eurogentec
Delta3-R	CAGGTTTCAATGACGAGGGAG	Eurogentec
Delta4-F	ACCTGCGGCCAGAGACTTC	Eurogentec
Delta4-R	CATCTGGCTGGCACTCATAA	Eurogentec
Jagged1-F	TGTCGGGATTTGGTTAATGG	Eurogentec
Jagged1-R	CTCGCAGTAATCGATGTCCA	Eurogentec
Jagged2-F	ATGCAAAGAAGCCGTGTGTA	Eurogentec
Jagged2-R	TGGCTGCCACAGTAGTTCAG	Eurogentec

Table 5. Primers used for RT-PCR.

2.1.4 Restriction digests

For each reaction approximately 500ng of plasmid DNA or PCR product was added together with 5-10 units of the appropriate restriction enzyme with the suitable buffer (New England Biolabs or Fermentas) in a total volume of 50µl. Samples were incubated at 37ºC for 1-3 hrs or 16 hrs.

2.1.5 Purification of DNA after PCR or restriction digest

To purify samples from enzymes, primers and dNTPs after PCR or restriction digestion reactions the QIAquick PCR purification kit (Qiagen) was used according to the manufacturer's instructions.

2.1.6 Ligations

DNA insert and plasmid vector were individually digested to yield complementary ends. Then DNA insert was added to the vector at approximately the same molarity to minimize tandem inserts in 20µl volume reaction. Reactions consisted of 200 units of T4 DNA ligase and T4 DNA ligase buffer (New England Biolabs). Ligations were incubated at 16ºC overnight in a Mastercycler gradient (Eppendorf).

2.1.7 Dialysis

Dialysis of 50µl ligation/digest reaction was carried out on mixed cellulose esters dialysis discs (Millipore) to remove residual salt from the ligation/digest buffers. Samples were dialyzed against dH_2O for 1 hour at RT.

2.1.8 Transformation of electrocompetent bacterial cells

Plasmids were transformed into *E. coli* DH5α by electroporation using a method derived from Dower et al. (1988). In brief, plasmid DNA was mixed with electro-competent cells on ice and then transferred to a pre-cooled 1mm electroporation cuvette (Molecular BioProducts). Electro-competent DH5α frozen stocks were prepared by Caroline Dalgliesh (IGM, Newcastle University, UK). Cells were electroporated at 1.5kV, 25µF capacitance, and 200Ω resistance using a Gene Pulser (Bio-Rad). Electroporated cells were immediately incubated in 1ml of LB broth (10g/l Sodium Chloride, 10g/l Tryptone, 5g/l Yeast extract) for 1 hour at 37ºC, allowing them to recover. Cells were plated out on

LB agar plates containing the appropriate selective antibiotic (LB Broth + 15g/l Agar + 50µg/ml Ampicillin, 50µg/ml Kanamycin or Puromycin 125µg/ml). Plates were incubated overnight at 37ºC.

2.1.9 Transformation of heat-shock competent bacterial cells

Plasmid DNA was added with a 30µl aliquot of JM109 *E. coli* cells. Cells and DNA were then placed on a water bath (heat-shocked) at 42°C for exactly 45 sec and immediately transferred to ice. After about 1 minute on ice, 1ml of pre-warmed LB medium was added and transformants were allowed to recover for 1 hour at 37°C. Cells were pelleted by centrifugation and supernatant medium was discarded. Cell pellets were re-suspended in the remaining LB and then plated out on LB-agar containing the appropriate selective antibiotic. Plates were incubated overnight at 37ºC and checked the next day for single colonies.

2.1.10 Purification of plasmid DNA

Single "healthy looking" colonies of transformed bacteria were picked with sterile tip and inoculated into 5ml of LB broth containing the selective antibiotic (50µg/ml Ampicillin, 30 or 50µg/ml Kanamycin or Puromycin 125µg/ml) and were incubated in a 37ºC shaker overnight. Cultures were centrifuged at 2500rpm at 15ºC for 10 minutes and plasmid DNA was prepared from the pelleted bacteria using a QIAprep Miniprep Kit or EndoFree Plasmid Maxi Kit (Qiagen) following the manufacturer's instructions. The DNA concentration was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies).

2.1.11 Agarose gel electrophoresis

PCR DNA samples were prepared by adding an appropriate amount of 5X Orange G loading dye (20% ficoll, 1mM EDTA and 2µg/µl Orange G) and loaded on1-2% agarose gels containing 1X Tris-acetate-EDTA buffer (TAE) (0.04M Tris Base, 0.04M Acetate and 0.001M EDTA) and ethidium bromide (final concentration of 0.5µg/ml). Samples were electrophoresed at 60-100 volts in TAE buffer for 40 to 60 min. DNA bands were visualized under UV light about 300ms exposure using a Gene Genius Bioimaging System (Syngene) and sizes were estimated using either 1kb Ladder Ready-to-use (Fermentas) or 100bp plus ladder (New England Biolabs).

2.1.12 Multiplex-PCR - Quantification of PCR products

For Multiplex PCR the same procedure as described for normal PCR was followed, with the only difference that two pairs of primers that lead to amplification of unique regions of DNA were added in the same reaction with the other PCR reagents (Figure 7).

PCR products were processed using the **QIAxcel System** following the manufacturer's instructions. DNA bands were quantified using the instrument's software (Figure 8).

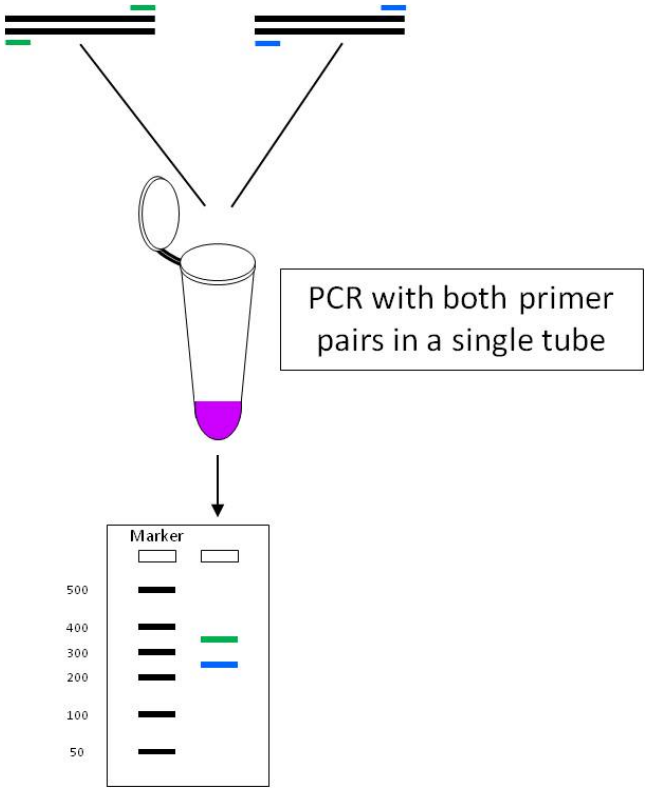

Figure 7. Schematic explanation of Multiplex-PCR, two or more primer pairs can be used to amplify multiple target sequences.

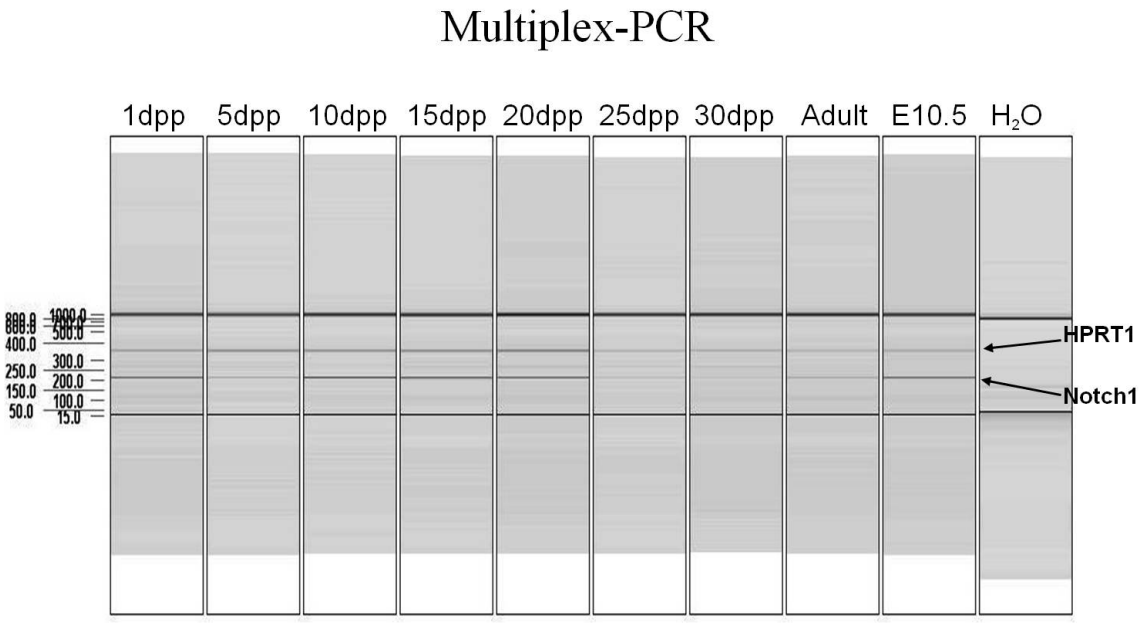

Figure 8. Quantification of PCR products using QIAxcel system for automated gel electrophoresis and accurate analysis of DNA fragments.

2.1.13 DNA extraction from tissue or cultured cells

10-20 mg of tissue was harvested and snapped frozen in liquid nitrogen. Then, tissue was homogenised using a tissue homogeniser for 3-5 min without allowing the specimen to thaw and DNA extraction buffer with freshly added Proteinase K was added and incubated for 16 hrs at 60ºC. In the case of cell culture, cells were harvested mechanically using a cell scraper and washed twice in 10 ml of $Ca2^{+}$, $Mg2^{+}$-free PBS. Then were centrifuged at 3000 rpm for 3 min at RT. Supernatant was removed and cell pellet was re-suspended in DNA extraction buffer (1M Tris-HCl; pH 8.0, 0.5M EDTA, 10% (w:v) SDS, 5M NaCl, 60% dH_2O and 10mg/ml Proteinase K) and incubated for 16 hrs at 60ºC. After this point both tissue and cultured cells follow the same steps. Equal volume of phenol was added to the cell lysate to remove proteins and other contaminants followed by a brief vortex. Samples were centrifuged at 13,000 rpm for 10 min at 4ºC. Supernatant (aqueous phase) was transferred into a new tube and an equal volume of phenol, chloroform/isoamylalcohol (25:24:1) was added to increase the efficiency of protein removal. Samples were briefly vortexed and centrifuged at 8,000 rpm for 10 min at 4ºC. Then, supernatant (aqueous phase) was transferred into a new tube and 1/10 volume of

3M sodium acetate (NaOAc) (pH 5.2) and 2 x the volume of 100% ice cold ethanol; were added, followed by incubation at -80ºC for 1hour. The precipitated DNA was pelleted by centrifugation at 13,00rpm for 10 min and supernatant was removed and samples were let to air dry at RT for 10 min. DNA pellet was re-suspended in deionised water and samples were quantified using NanoDrop ND-1000 Spectrophotometer.

2.1.14 DNA sequencing

Samples of purified plasmid DNA or PCR products at a minimum concentration of 100ng/µl were sent along with 32pmol of the appropriate primer to Geneservice (Cambridge, UK) were sequencing reactions were performed.

2.1.15 Site directed mutagenesis

Mutagenic oligonucleotide primers were designed according to site-directed mutagenesis Stratagene's kit protocol. Primers had a melting temperature of at least 78°C and center the desired point mutations in the middle of the primer with 10-15 bases of correct plasmid sequence either side. They were designed to be between 25 and 45 bases in length and with a minimum GC content of 40%, and to terminate in one or more C or G bases on either end. For the introduction of two identical unique restriction sites (for Kpn21 restriction enzyme) flanking the *Neomycin* cassette, four oligonucleotides were designed which anneal to the same sequence on opposite strands on either site of the *Neomycin* cassette of the *Stra8*-eGFP1-*Neo* plasmid. The generation of two unique restriction sites flanking the *Neomycin* cassette by site-directed mutagenesis allowed the excision of the cassette and insertion of the Puromycin antibiotic gene in *Stra8*-eGFP1 plasmid.

Primers used for mutagenesis are listed in Table 6.

Primer name	Target	Sequence (5′-3′)	Mutation
D-MutUpstATG-F	upstream	AGACAGGATGAGGATCGTTCCGGATGATTGAACAAGATGGA	A➔C, C➔G
D-MutUpstATG-R	upstream	TCCATCTTGTTCAATCATCCGGAACGATCCTCATCCTGTCT	A➔C, C➔G
D-MutDwnstTGA-F	downstream	CTTCTTGACGAGTTCTTCTGATCCGGACTCTGGGG	A➔C, C➔G
D-MutDwnstTGA-R	downstream	CCCCAGAGTCCGGATCAGAAGAACTCGTCAAGAAG	A➔C, C➔G

Table 6. Primers used for site directed mutagenesis. Primers were purchased from Eurofins MWG. Mutated bases were introduced are highlighted in green.

Point mutations were generated following the Stratagene QuickChange mutagenesis protocol. Reactions were set up as indicated in Table 7.

Reagent	Quantity for a 25µl reaction
dH_2O	19.02µl
10x Pfu Buffer (Stratagene)	2.5µl
dNTPs (100mM total)	0.5µl
Primer mix (100ng each)	1.5µl
Template plasmid DNA (10ng/µl)	1.5µl
PfuTurbo DNA polymerase (Stratagene) 100U	0.5µl

Table 7. Site-directed mutagenesis PCR recipe (adapted from the Stratagene QuickChange mutagenesis protocol).

Mutant plasmids were generated by temperature cycling using a Mastercycler gradient (Eppendorf). The template plasmid was denatured by an initial denaturation step at 95ºC for 2 minutes followed by 18 cycles of denaturation at 95ºC for 30 seconds, annealing of oligonucleotide primers containing desired mutation at 55ºC for 1 minute, PfuTurbo DNA polymerase extension at 68ºC for 10 minutes followed by a final extension at 68ºC for 10 minutes.

1µl of the *Dpn*I restriction enzyme (20 U/µl) was added directly to each sample and reactions were incubated at 37°C for 3 hrs to digest the parental (i.e., the non-mutated) DNA. The plasmid DNA was then purified using the Qiagen PCR purification kit following manufacturer's protocol and transformed into heat-shock competent *dam*$^-$/*dcm*$^-$ competent *E. coli* cells. Plasmid DNA was purified from bacteria colonies and mutations were verified by DNA sequencing.

2.1.16 Western blot

Tissue or cell samples were lysed in SDS lysing buffer (100mM Tris-HCL pH6.8, 200mM DTT, 4% SDS, 20% Glycerol, 0.2% Bromophenol Blue) and intact DNA was disrupted using an ultrasonicator. Samples were boiled for 5 minutes at 95ºC, centrifuged briefly and loaded onto a discontinuous gel. Gels were made with a 10% acrylamide resolving gel (375mM Tris pH8.8, 0.1% SDS, 0.1% ammonium persulphate, 0.2% TEMED and 10% acrylamide) and a 4% stacking gel (0.125mM Tris pH6.8, 0.1% SDS, 0.1% ammonium persulphate, 0.2% TEMED and 4% acrylamide). Samples were electrophoresed in 1X SDS-PAGE running buffer (30mM Tris, 188mM Glycine, 0.1% SDS) at 150V for 45 minutes. Protein sizes were determined using Pre-stained Protein Marker (New England Biolabs). PAGE gels were transferred to a pre-equilibrated Hybond-C Extra nitrocellulose membrane (Amersham Biosciences) in Western transfer

buffer (39mM Glycine, 58mM Tris, 0.04% SDS, 20% MeOH) using a Semi-Dry Trans-Blot apparatus (Bio-Rad) for 45 minutes at 15V. Filters were stained with Ponceau solution 0.1 % (w/v) (Sigma) to visualise blotting transfer efficiency and then rinsed twice in TBS-T (8.75g/l NaCl, 6.5g/l Tris-HCL, 2ml/l Tween). Filter membranes were first blocked with TBS-T containing 5% non-fat dried milk and 5% horse serum for 1 hour at RT to block unspecific binding. The membrane was then probed for 1 hour with primary antibody at appropriate dilution (**Table 8**) and incubated at 4°C for 16 hrs on a rotating wheel. Membranes were then rinsed three times in TBS-T and Horseradish peroxidise (HRP) conjugated secondary antibody was added at dilution 1:1000 in block solution and incubated at RT for 1 hour on rotating wheel. Membranes were then washed twice in TBS-T to remove residual secondary antibody. The signals were visualized by enhanced chemiluminescence (ECL). For this, equal volumes of ECLI (1% Luminol, 0.44% Coumaric acid, and 100mM Tris pH 8.5) and ECLII (0.02% hydrogen peroxide in100mM Tris pH 8.5) were added to the membrane and left for 1 minute at RT. Excess ECL was then removed and filters were exposed on photographic film (Kodak). Films were developed in a Compact X4 developer (Xograph Imaging Systems).

2.2 Histological techniques

2.2.1 Fixation, paraffin wax embedding and sectioning of murine testis

Mouse testis were dissected out of adult animals and transferred immediately into 4% PFA in PBS (Sigma) and kept at 4°C for 36 hrs. After fixation the testis were dehydrated through a series of 2 hrs incubation in 70%, 95% and 100% and then left O/N in 100% ethanol on a shaking board. The next day, the testis were washed for 10 min in a 1:1 histoclear/100% ethanol mixture and then twice for 20 min in histoclear alone (National Diagnostics), and were then placed in a 1:1 mix of paraffin wax (National Diagnostics) and histoclear for 30 min at 65°C. They were given 1 incubation in 1:3 mix of histoclear/paraffin wax at 65°C, and the 2 incubations, one hour each, in wax at 65°C. Finally, the testis were orientated in plastic moulds and left uninterrupted to allow for the wax to set.

Embedded testis were sectioned using a Leica RM2135 rotary microtome at a thickness of 8µm. Wax sections were cut and placed on SuperFrost® slides (Thermo) with distilled water heated to 37°C. The water was removed and the slides dried overnight at 37°C.

2.2.2 Immunohistochemistry (Fluorescent and DAB peroxidase staining)

Paraffin wax-embedded testis sections were de-waxed by double incubation in HistoClear for 12 min. Sections were then hydrated by serial incubations in ethanol dilutions (2x 5 min washes in 100% ethanol, followed by a 5 min wash in 70% ethanol and finally a 5 min wash in 50% ethanol). To inhibit endogenous peroxidase activity sections were incubated in a methanol peroxide solution (3% H2O2 in MeOH) for 30 min and then rinsed in water for 2 min. Antigen retrieval step was performed by microwaving the sections at 900W for 20 minutes in 0.01M sodium citrate buffer pH 6.0. The sections were allowed to cool for 20 min and they were then incubated in blocking solution (10% horse serum in TBS) for 20 minutes at RT in a humidified chamber to prevent unspecific binding of the antibody. The sections were then incubated with the primary antibody diluted in blocking solution overnight at 4°C in a humidified chamber (for dilutions see **Table 8**). Sections were then washed 3X with TBS before addition of the appropriate biotinylated secondary antibody (Dako ARK) which was diluted in blocking solution (for dilutions see **Table 8**). Sections were incubated with the secondary antibody at RT for 30 minutes followed by another 3 washes in TBS. Biotinylated secondary antibody was visualized using the avidin-biotin-horseradish peroxidise system (ABC-HRP) and diaminobenzidine (Sigma) following manufacturer's protocol.

The nuclei of the cells in the testis sections were counter stained with Harris's haematoxylin for 10 seconds and the rinsed with running tap water for 2-3 min. The sections were dehydrated in a graded series of ethanol (5 minute washes in 50% ethanol, 5 minute washes in 70% ethanol, followed by 2x 5 minute washes in 100% ethanol) and HistoClear (2x 5 minute wash), before being mounted in Histomount (National Diagnostics) and cover-slip and let to dry O/N. The next day edges of the cover slip were sealed by nail varnish and images were captured/analysed using a Zeiss Axioplan 2 light microscope.

In cases were a fluorescent secondary antibody was used; the inhibition step for endogenous peroxidase activity was omitted. After incubation with the fluorescent secondary antibody the sections were washed 4x 5min with TBS and mounted with Vectashield mounting medium with DAPI (Vector Labs) and images were captured/analysed using a Zeiss Axioimager fluorescent microscope.

Antibody name	Source	Mono/ Polyclonal	Species	Dilution for IHC/IF	Dilution for WB
α-NOTCH1-ICD	Rockland	Polyclonal	Rabbit	1:100	1:500
α-NOTCH2-ICD	Abcam	Polyclonal	Rabbit	1:100	
α-NOTCH3-ICD	Santa Cruz	Polyclonal	Rabbit	1:250	-
α-NOTCH4-ICD	Dr Keith Brennan (Manchester University)	Polyclonal	Goat	1:100	-
α-DELTA1	Dr Keith Brennan (Manchester University)	Polyclonal	Goat	1:500	-
α-DELTA3	Dr Keith Brennan (Manchester University)	Polyclonal	Rabbit	1:200	-
α-DELTA4	Dr Keith Brennan (Manchester University)	Polyclonal	Goat	1:100	-
α-JAGGED1	Abcam	Polyclonal	Rabbit	1:100	-
α-JAGGED2	Abcam	Polyclonal	Rabbit	1:50	-
α-PIWIL2	Abcam	Polyclonal	Rabbit	1:300	-
α-DMC1	Santa Cruz	Polyclonal	Goat	1:200	-
α-STRA8	Santa Cruz	Polyclonal	Rabbit	1:400	-
α-hnRNP G-T	(Ehrmann et al., 2008)	Polyclonal	Sheep	1:500	-
α-HES1	Abcam	Polyclonal	Rabbit	-	1:500
α-HES3	Santa Cruz	Polyclonal	Goat	-	1:500
α-Tp1	Santa Cruz	Polyclonal	Rabbit	1:200	1:500
α-β Actin	Sigma	Polyclonal	Rabbit	-	1:1000
α-mouse IgG(HRP)	Amersham	Polyclonal	Sheep	1:500	1:1000
α-goat IgG(HRP)	DAKO	Polyclonal	Rabbit	1:1000	1:1000
α-rabbit IgG(HRP)	Jackson Lab	Polyclonal	Goat	1:1000	1:1000
α-sheep IgG(HRP)	DAKO	Polyclonal	Rabbit	1:1000	1:1000
α-rabbit IgG(488)	Molecular Probes	Polyclonal	Donkey	1:400	-
α-sheep IgG(594)	Molecular Probes	Polyclonal	Donkey	1:400	-
α-mouse IgG(594)	Molecular Probes	Polyclonal	Donkey	1:400	-
α-rabbit IgG(Biotin)	DAKO	Polyclonal	Goat	1:300	-

Table 8. Antibodies used for immunohistochemistry (IHC), immunofluorescence (IF) and Western blotting (WB).

2.2.3 Immunocytochemistry

ES cells were grown on coverslips (in 6-well plates) in the appropriate growth medium until 70-75% confluent. Cells were washed in 1X PBS pH 7.4 and fixed in 4% PFA for

15 min at RT. The PFA was aspirated and cells were washed three times (5 min each wash) with 1X PBS for 15 min

To permabilize the cells, samples were incubated for 10 min with PBS containing 1% Triton X-100 at RT. Permeabilized cells were then washed three times (5 min each wash) with 1X PBS for 15 min to remove Triton X-100. To block unspecific binding of the antibodies samples were incubated in blocking solution (5% horse serum, 5% milk powder in 1X PBS) for 30 min at RT and then incubated in the appropriate dilyted primary antibody in blocking solution O/N at 4°C (for antibodies and dilutions see **Table 8**). Next day, the solution was aspirated and samples were washed three times in PBS, 5 min each wash. The cells were then incubated with the appropriate fluorescent conjugated secondary antibody diluted in blocking solution for 1 hour at RT and washed with 1X PBS for 5 min each in the dark. The coverslips were then mounted in VectaShield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) and images were captured using either a Zeiss Axioimager fluorescent microscope or a Nikon A1 confocal laser scanning microscope.

2.3 Animal techniques

2.3.1 Isolation of murine embryonic fibroblasts (MEFs)

For isolation of primary murine embryonic fibroblasts (MEFs) a pregnant C57Bl6 female mouse was sacrificed at day 13p.c by cervical dislocation. Using sterile dissecting instruments (fine scissors, tweezers, razor blades) the uterine horns were dissected out and place it into a petri dish with sterile PBS. The embryos were then isolated from the uterus and released from the embryonic sacs and were transferred to a second petri dish with sterile PBS. The embryo heads and limps were cut away and dark red organs (liver, intestines and heart) were also removed with a pair of forceps. Using a minimal amount of PBS, razor blades and scissors the embryos were finely mince until they could be pipetted. Then, each 3 embryo carcasses were transferred to a 15ml sterile tube and washed once with PBS pH 7.4 and after 5 min centrifugation at 400xg the supernatant was removed. Embryonic cells/tissue were suspended in 2 ml of 2.5% trypsin-EDTA

(Gibco) and incubate with gentle shaking at 37°C for 15min. The trypsin was neutralize with 10 ml MEF culture medium (Dulbecco's MEM (DMEM) with glutamax-1 medium supplemented with 10% Foetal Bovine Serum (FBS) and 1% penicillin-streptomycin (all Invitrogen)), and the content was evenly add (4ml in each) to three T75 culture flasks containing 20 ml culture medium. The flasks were left for 3 hrs at 37°C 5%CO_2 to allow the fibroblasts to attach and fresh media was added.

2.3.2 Isolation of murine testis

For isolation of murine testis newborn C57Bl6 mice at different postnatal stages were sacrificed by cervical dislocation. Using sterile dissecting instruments (fine scissors, tweezers, razor blades) the testis were dissected out and placed into a petri dish with sterile PBS. After two washes with PBS the testis were either transferred to 4% PFA solution or were snap frozen in liquid nitrogen and chopped into very small pieces before transferred to Trizol solution for RNA extraction.

2.4 Cell culture

2.4.1 Expansion, freezing, thawing and inactivation of murine embryonic fibroblasts

After dissociation and plating of primary MEFs into T75 culture flasks (at 37°C 5%CO_2) the cells attached and started to divide within the first 1-3 days. The culture medium (look 2.3.1) was changed every 2 days and usually in 3-4 days the flasks were confluent enough to either freeze down the cells or passage them to other flasks. To obtain single cell suspension, 3 ml of 0.05% trypsin-EDTA (Gibco) was added to each T75 flask and incubated at 37°C 5%CO_2 for 5 min. Then 12ml of culture medium was added to neutralise the trypsin and cells were transferred to a 15 ml centrifuge tube. Cells were centrifuged for 5min at 300xg and supernatant was removed. The cell pellet was resuspended with 6 ml fresh media and cells were splitted in 3 or 4 new T75 flasks (max. split ratio: 1:4). Alternatively, the cell pellet was resuspended with 3 ml fresh media, cell number was determined by ViCell (look 2.4.4) and $2x10^6$ cells were transferred to a cryo-vial (labelled P0) where 0.5ml freezing media (10% DMSO in culture medium) was

added. Vials were placed into MrFrosty (Nalgene) container or a pre-cooled styrofoam box and transferred to -80°C freezer for 24-48 hrs and then to liquid nitrogen for long term storage.

Thawing and replating of MEFs was done by quickly thaw frozen MEFs vial in a warm waterbath, then transfer the fibroblasts to a few ml of warm MEF medium and pellet by centrifugation (5 min, 300xg). MEFs were resuspended in MEF medium and plate out in T75 flasks. When MEFs reached passage 3 or 4 and grew up to 70% confluency were mitotically inactivated by mitomycin C (Sigma) treatment. Briefly, cells were washed twice with PBS and MEF medium with 10μg/ml mitomycin C was added. MEFs were incubated for 2.5 hrs at 37°C, 5% CO2 and washed three times with PBS, trypsinized and frozen down as described above in vials containing $3x10^6$ cells/vial.

2.4.2 Culture of R1 ES cell line

A low passage number (11) of R1 ES cells line was provided by Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Canada). Cells were grown on a MEF feeder layer at 37ºC in 5% CO_2 in Dulbecco's MEM (DMEM) with glutamax-1 medium (Gibco) supplemented with 10% Foetal Bovine Serum (FBS) (PAA) and 1% penicillin-streptomycin (GIBCO) and with sodium pyruvate (1 mM) (Gibco). Cells were seeded as a single cell suspension at a rate of approximately $1.5x10^5$ - $4x10^5$ cells per cm^2 and were passaged every 2-3 days or when 70-75% confluency was reached. To obtain single cell suspension, 3 ml of 0.05% trypsin-EDTA (Gibco) was added to each T75 flask and incubated at 37°C 5%CO2 for 5 min. Then 12ml of culture medium was added to neutralise the trypsin and cells were transferred to a 15 ml centrifuge tube. Cells were centrifuged for 5min at 300xg and supernatant was removed. The cell pellet was resuspended with 6 ml fresh media and cells were splitted in 4 or 5 new T75 flasks (max. split ratio: 1:5). Alternatively, the cell pellet was resuspended with 3 ml fresh media, cell number was determined by ViCell (look 2.4.4) and $2x10^6$ cells were transferred to a cryo-vial where 0.5ml freezing media (10% DMSO in culture medium) was added. Vials were placed into MrFrosty (Nalgene) container or a pre-cooled styrofoam box and transferred to -80°C freezer for 24-48 hrs and then to liquid nitrogen for long term storage.

2.4.3 R1 ES cells transfection

For transfection ES cells were cultured with no antibiotics and the day of transfection were approximately 65% confluent. They were transfected using the Amaxa™ Nucleofector™ (Lonza) as per the manufacturer's instructions using 4 μg of each construct DNA previously linearised with a unique restriction enzyme. 24 hrs after transfection the appropriate antibiotic was added to the media and changed daily. After 8-9 days for neomycin and 5-6 days for puromycin antibiotic round colonies were collected and transferred to 96-well plate for analysis and expansion.

2.4.4 Cell counting, size, shape and viability assessment

Cell number, size and viability was determined using Vi-CELL (Beckman Coulter) instrument as per the manufacturer's instructions.

2.4.5 Differentiation of cells-Retinoic Acid induction

Cells were cultured on feeder layer approximately $4x10^5$ - $6x10^5$ cells per cm^2 with media supplemented with 10^{-5}M of Retinoic acid. For differentiation to obtain germ-like cells, cells were plated without feeder layer with 10^{-5}M of Retinoic acid.

For differentiation with Notch ligands plates were coated with 20μg/ml goat polyclonal anti-human IgG for 30 min at 37°C, washed with PBS, blocked with 2% BSA-5%NGS for 1hr RT and then coated with either 10μg/ml human IgG or any of the Notch ligands.

2.4.6 Preparation of cells for Fluorescence Activated Cell Sorting (FACS)

Cells were trypsinised as described in section 2.4.2 and collected by centrifugation at 300 x g for 5 min at 4°C. 5 ml of PBS were added to the cells, gently resuspended and collected by centrifugation at 300 x g for 5 min at 4°C before processed in FACS II (BD Biosciences) for cell analysis of FACS Aria II (BD Biosciences) for cell sorting.

2.4.7 Preparation of cells for FACS DNA content analysis

For DNA content analysis cells were harvested as described in section 2.4.2 and collected by centrifugation at 300 x g for 5 min at 4°C. After that cells were gently resuspened in

extraction buffer of CyStain® DNA 2 step kit (Partec) and the manufacturer's instructions were followed. FACS analysis was done in FACS Canto II (BD Biosciences). For DNA ploidy analysis ModFit LT (Verity Software House) software was used.

2.5 Computational analysis

2.5.1 Sequence alignments

Sequence alignments were carried out using the ClustalW program available online at http://www.ebi.ac.uk/clustalw/.

2.5.2 BLAST searches

Nucleotide similarity searches were carried out using the NCBI Basic Local Alignment Search Tool programme available online at http://www.ncbi.nlm.nih.gov/BLAST/.

2.5.3 Primers design

Mouse DNA sequences were found online at http://www.ensembl.org and Primer3 software available online http://frodo.wi.mit.edu/primer3/ was used to design the primers.

Chapter 3. Analysis of Notch signalling components in spermatogenesis

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3.1 Introduction to the chapter

3.1.1 Notch and its role in spermatogenesis

Previous studies have shown that members of the Notch pathway family have an essential role in germ line development and gametogenesis in both invertebrate and vertebrate species. It has been demonstrated that Notch receptors and their homologues are necessary in the adult germ line of *C. elegans* (Crittenden et al., 1994, Kimble and Simpson, 1997, Crittenden et al., 2003), and they have been found to promote germ cell proliferation in *Drosophila* (Xu et al., 1992). Notch signalling genes have also been found to be expressed in mammalian testis. Several laboratories have presented evidence of Notch pathway components expressed in rodent (Hayashi et al., 2001, Dirami et al., 2001) and human (Hayashi et al., 2004b) testis suggesting a role of Notch signalling in germ cell differentiation. However, the possible role of Notch in spermatogenesis and the *in vivo* cellular-type distribution of Notch components have not been investigated in great detail. In an attempt to better understand how the Notch signalling pathway may mediate the fate of the male germ line cells, the expression, localization and activation of Notch receptors and ligands in murine testis was examined.

3.1.2 Aim of the current study

The initial objective was to determine what components of the Notch pathway are transcribed during postnatal testicular development and adult testis. Having identified the expressed Notch components, the next step was to determine the presence and localization of Notch signalling proteins. To achieve this, Notch proteins were investigated using immunohistochemical analysis of fixed testicular sections. This examination not only identified the cellular localization of Notch proteins within the seminiferous tubules, but also, using antibodies against specifically activated Notch receptors, it could determine whether or not the receptor was activated by a notch ligand.

By identifying the localization and activation of Notch proteins within the seminiferous tubules we could then determine 1st: what cell types express these proteins, 2nd: the time

during the seminiferous epithelial cycle that these proteins are expressed and 3rd: how these interactions may affect germ cell differentiation during spermatogenesis.

3.2 Expression analysis of Notch signalling transcripts in murine testis

Spermatogenesis in mammals depends on the continuous differentiation of progenitor cells known as the undifferentiated spermatogonia. In the mature seminiferous tubule undifferentiated spermatogonia are able to self-renew themselves and also give rise to differentiating spermatogonia. More mature germ cells developing from these differentiating spermatogonia then gradually migrate from the basal membrane across the epithelium towards the lumen, during which time they proliferate, undergo meiosis and a series of morphological changes to be finally released as mature haploid spermatozoa (see Introduction section 1.2.1).

As each group of germ cells moves towards the tubule lumen a successive batch of germ cells commences a new spermatogenic cycle. Consequently, at a given time several layers of germ cells are present in the seminiferous epithelium. The different layers however, do not develop in a random manner but they seem to be governed by a spatio-temporal program, discovered many decades ago (Leblond and Clermont, 1952). The total length of a spermatogenic cycle in mouse is 35 days (Oakberg, 1956b) and according to the cellular associations observed in cross sections of the seminiferous tubule (Oakberg, 1956a), it has been separated in twelve different cytological stages (I-XII) (Figure 9). This dynamic pattern progresses in a highly-coordinated manner in adjacent regions of the epithelium throughout the tubule, and results in spatial and temporal sequence of stages which ensures a continuous production of sperm. This precise temporally and topographically organized process is known as the spermatogenic wave (Perey et al., 1961, Leblond and Clermont, 1952, Russell et al., 1993).

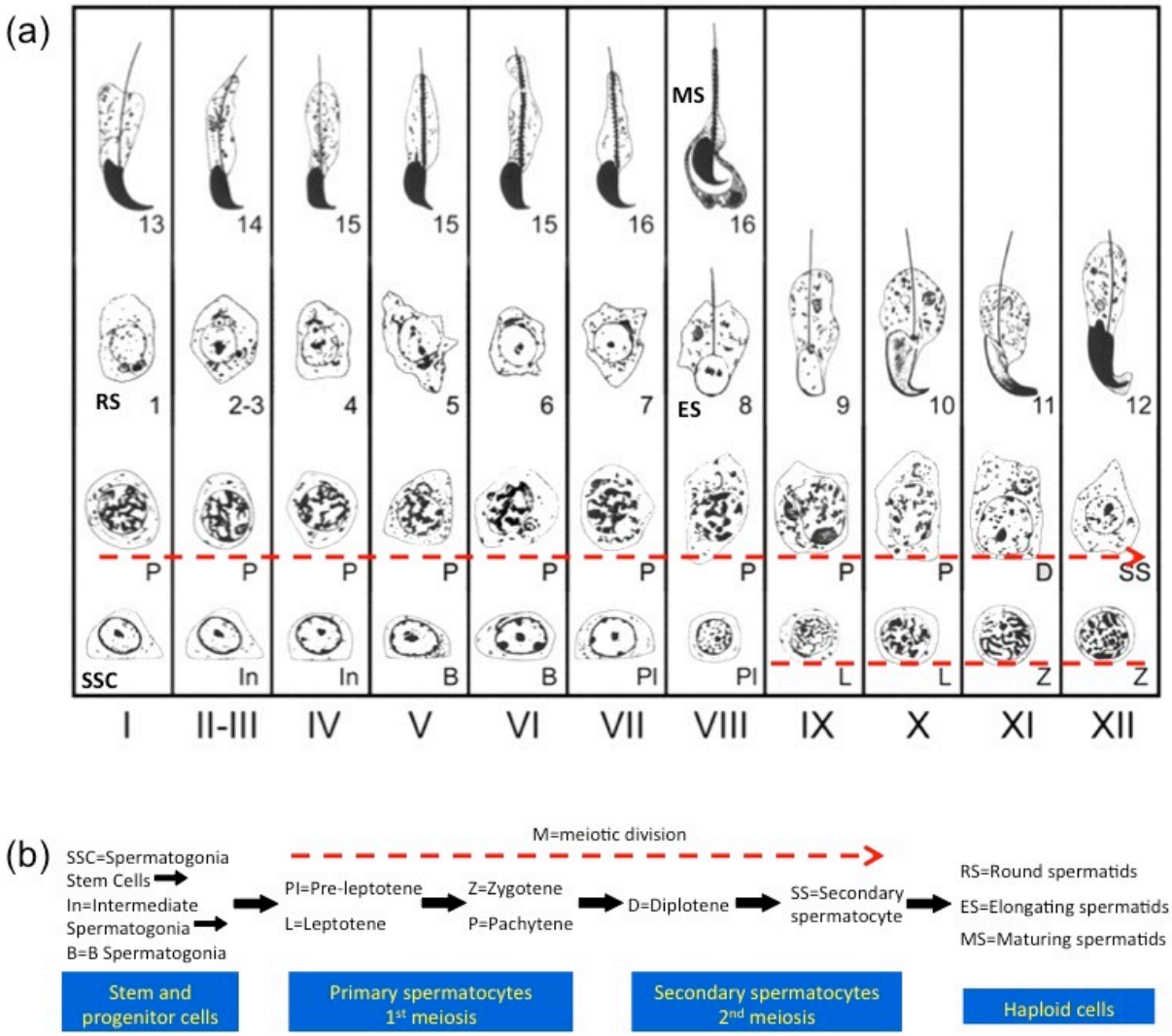


Figure 9. Schematic representation of the murine seminiferous epithelial cycle. (a) The germ cellular associations within each layer of the seminiferous epithelium described above have been used to identify the sequence of stages (I-XII) of the spermatogenic waves in the seminiferous tubules. The stages occur in sequence along the epithelium in a successive order, Stage I is followed by II, followed by III and so on. The red dotted line marks and follows the initiation, progression and completion of meiosis. The Roman numbers represent the stages of the seminiferous epithelial cycle. Arabic numbers represent the steps of spermiogenesis. (b) Diagrammatic sequence following the progression of germ stem cells to haploid sperm. Spermatogonia stem cells (SSC) differentiate to intermediate spermatogonia (In) to B spermatogonia (B) to pre-leptotene (Pl) spermatocytes to leptotene (L) spermatocytes to zygotene (Z) spermatocytes to pachytene (P) spermatocytes to diplotene (D) spermatocytes to secondary spermatocytes (SS) to round spermatids (RS) to elongating spermatids (ES) and finally to maturing spermatids (MS). Figure modified from (Russell et al., 1993).

In mice, the first wave of spermatogenesis is initiated in the first week after birth and 30 days later the first mature sperm can be observed in the testis. Immediately after birth, only a small number of quiescent gonocytes and Sertoli cells are present in seminiferous epithelium (Vergouwen et al., 1993), but in the next few weeks the number and types of germ cells increase dramatically. The previously arrested gonocytes at G_0/G_1 resume their

cell cycle and start moving away from their original central position towards the basal membrane of the seminiferous tubule.

While this migration happens, around postnatal day (PND) 3 to PND 6, the gonocytes develop morphological characteristics comparable to type A spermatogonia in adults (Yoshida et al., 2006). A number of these cells stop proliferation and start to undergo differentiation that gives rise to type A-, intermediate and type B-spermatogonia at PND 8. The type B spermatogonia divide to form early spermatocytes, which mark the initiation of the meiotic phase at PND 10. During the progression of the meiotic period, which last for ten to twelve days, early and late pachytene germ cells make their appearance by PND 14 and 18 respectively, and secondary spermatocytes between PND 18 and PND 20. The latter cells quickly enter the second reduction division by PND 21 yielding the postmeiotic haploid round spermatids, which indicate the initiation of spermiogenesis. Nine to ten days later the first mature sperm are present in the testis (Bellve et al., 1977). The Sertoli cell population shows a high proliferative activity at birth to finally decrease and reach adult levels around PND 17 (Table 9).

Cell type	Day 1	Day 5	Day 10	Day 15	Day 23	Day 31
Sertoli cells	86.9	88.5	75.8	40.9	21.9	12.0
A spermatogonia		8.0	19.0	12.7	5.7	2.2
Int/B spermatogonia			3.8	11.6	7.3	6.0
Early spermatocytes (preleptotene to zygotene)			1.4	19.3	17.5	14.7
Late spermatocytes (pachytene + secondary)				15.5	33.5	24.8
Haploid spermatids					14.1	30.4

Table 9. Cellular proportions as a percentage of total cell number in the seminiferous epithelium at various postnatal days. Data from (Ellis et al., 2004)

This highly controlled spatiotemporal progression of the first wave of spermatogenesis offers a unique opportunity for a dynamic analysis of gene expression that can be correlated with the presence of defined populations of germ cells at each stage. We used this approach to follow the Notch signalling gene expression pattern for each differentiation step, from undifferentiated spermatogonia to mature sperm.

3.2.1 Assaying the expression of Notch signalling receptors transcripts in postnatal and adult testis

Notch receptors form a family of four single-pass trans-membrane proteins. They consist of a large functional extracellular domain, a transmembrane domain, and a small intracellular domain. Even though different Notch receptors share similar structures and ligands they have only partially overlapping functions. These Notch components are involved in mediating cell-cell communication and transmit signals between adjacent cells by direct contact. This interaction between neighbouring cells regulates a diverse array of cell fate decisions in different tissues and developmental processes (Artavanis-Tsakonas et al., 1999) (see Introduction section 1.3.1-1.3.2). In the canonical Notch signalling pathway, binding of a ligand to the extracellular portion of the Notch receptor on the signal-receiving cell, initiates a proteolytic cleavage of the intracellular portion of the protein (domain) N^{ICD} from the transmembrane domain. Once cleaved this intracellular domain is then free to move to the nucleus where it forms, together with members of the CSL (CBF1/RBP-Jκ/Suppressor of Hairless/LAG-1) family of transcription factors, a transcription factor complex which results in the transcriptional activation of specific target genes.

In vertebrates, four *Notch* genes (*Notch1*, *Notch2*, *Notch3*, and *Notch* 4) have been described (Andersson et al., 2011). Previous studies have investigated the expression of the same four genes in mammalian testis but these findings are not consistent (Dirami et al., 2001, Mori et al., 2003). Furthermore, there have been no reports that have examined these Notch transcripts in a quantitative way in postnatal stages and adult testis. Thus, in an attempt to shed more light on the outcome of Notch signalling in mouse spermatogenesis, the stage dependent activation of the Notch receptor transcripts was investigated using a semi-quantitative method known as Multiplex polymerase chain reaction (Multiplex PCR).

Multiplex PCR uses the same amplification principle as PCR with the difference that more than one sequence is targeted for amplification (see Materials and Methods section 2.1.12). The advantage that this technique provides is that the amount of a particular template can be assessed. Here, the amount of different Notch receptor transcripts was assessed relative to the amount of the transcript of a known housekeeping gene *Hypoxanthine phosphoribosyltransferase 1* (*Hprt1*).

To examine the expression pattern of each of the Notch receptors (*Notch1-4*) during the postnatal period, seven different time points during early postnatal development (PND 1,

PND 5, PND 10, PND 15, PND 20, PND 25 and PND 30) were selected, each one marking a more advanced stage in the progression of the maturation of germ cells and their associated morphological changes during the first spermatogenic cycle. For comparison, the adult stage (3 months old testis) was also included in the investigation.

Mouse testes from different postnatal and adult mice were isolated and total RNA was purified. This was followed by cDNA synthesis and Multiplex PCR for each of the Notch receptors genes with the housekeeping gene *Hprt1*. The PCR products were loaded on the QIAxcel System that can automatically separate DNA fragments by gel electrophoresis, and quantify and provide the actual DNA concentration present. The ratio of the DNA concentration between the Notch receptor and *Hprt1* transcripts for each stage was calculated and plotted on bar charts.

The *Notch1* gene expression profile showed transcripts to be present throughout the postnatal time points tested and also in adult stage (Figure 10). In particular, *Notch1* RNA is present at the day of birth (PND 1) where about 87% of the cells, that constitute the seminiferous tubule, are Sertoli and only a small percentage are gonocytes (Table 9). *Notch1* mRNA expression falls about 2.9 fold change at PND 5. At PND 5 gonocytes have finished their migration towards the basal membrane and appear to be comparable to type A spermatogonia in adults (Yoshida et al., 2006). The number of A spermatogonia at PND 5 now represents 8% of the cell population, with Sertoli cells comprising the other 88.5%. At the time where the first meiotic cells start emerging (PND 10) *Notch1* expression increases and stays at this higher level as early spermatocytes progress through meiosis (PND 15) towards the completion of the second meiotic division. Around days PND 19-21, when the first post-meiotic haploid cells start to appear, *Notch1* expression begins to decline, and as more cells continue to complete meiosis and more spermatids emerge (PND 23-25) the expression is even lower. *Notch1* transcripts reach their lowest level in the postnatal period around PND 30 when most the previously meiotic cells have become haploid sperm. The expression of *Notch1* in the adult testis is comparable with the levels seen during the "meiotic progression" postnatal period (PND 10-20). Total RNA from E10.5 whole embryos was used as positive control knowing that *Notch1* transcripts are present at this stage.

The *Notch2* gene follows a slightly different expression pattern in the postnatal period (Figure 10). A high level of transcripts is present right after birth (PND 1) followed by a

decline at PND 5, right after the initial phase of spermatogonial proliferation that occurs in the basal compartment of the seminiferous epithelium and results in what are known as type A spermatogonia. *Notch2* expression does not seem to change much during the formation of Intermediate and B spermatogonia (PND 10. Similar *Notch2* levels are observed even when early spermatocytes are present and late spermatocytes have progressed through meiosis (PND 15). However, as spermatocytes begin to complete meiosis and the first post-meiotic cells start to appear *Notch2* expression declines to more than half was previously observed. As more spermatids are formed (PND 30) the level of expression remains low. However, in adult testis much higher levels of *Notch2* transcripts are present, with levels much higher from all the observed postnatal levels of expression. As in the case of *Notch1* transcripts also for *Notch2* total RNA from E10.5 whole embryos was used as positive control.

As it was mentioned previously, the seminiferous epithelium of a newborn mouse testis contains two distinct cell types, gonocytes and Sertoli cells. When testes from newborn mice were examined for expression from the *Notch3* gene, levels of expression right after birth were much higher compared to the subsequent days tested (Figure 10). In particular a 6.3 fold down regulation appears after 4 days, when primitive type A spermatogonia comprise 16% of the cells present in the seminiferous epithelium (PND 5). The expression continues at this low level when primary spermatocytes at the pre-leptotene and leptotene stages of meiotic prophase are present (PND 10), drops further as late spermatocytes emerge (PND 15) and haploid spermatids appear (PND 20) and reaches almost undetectable levels in PND 25 and 30 testis when post-meiotic spermatids appear in increasing numbers. In adult testis, very low expression of *Notch3* transcripts was detected.

Examination for the transcripts of the last of the *Notch* receptors showed that in contrast to the other 3 receptors, the expression for *Notch4* was below the level of detection both in postnatal and adult stages. Total RNA from embryonic tissue also resulted in no detectable *Notch4* expression therefore, brain and lung total RNA were used as positive controls.

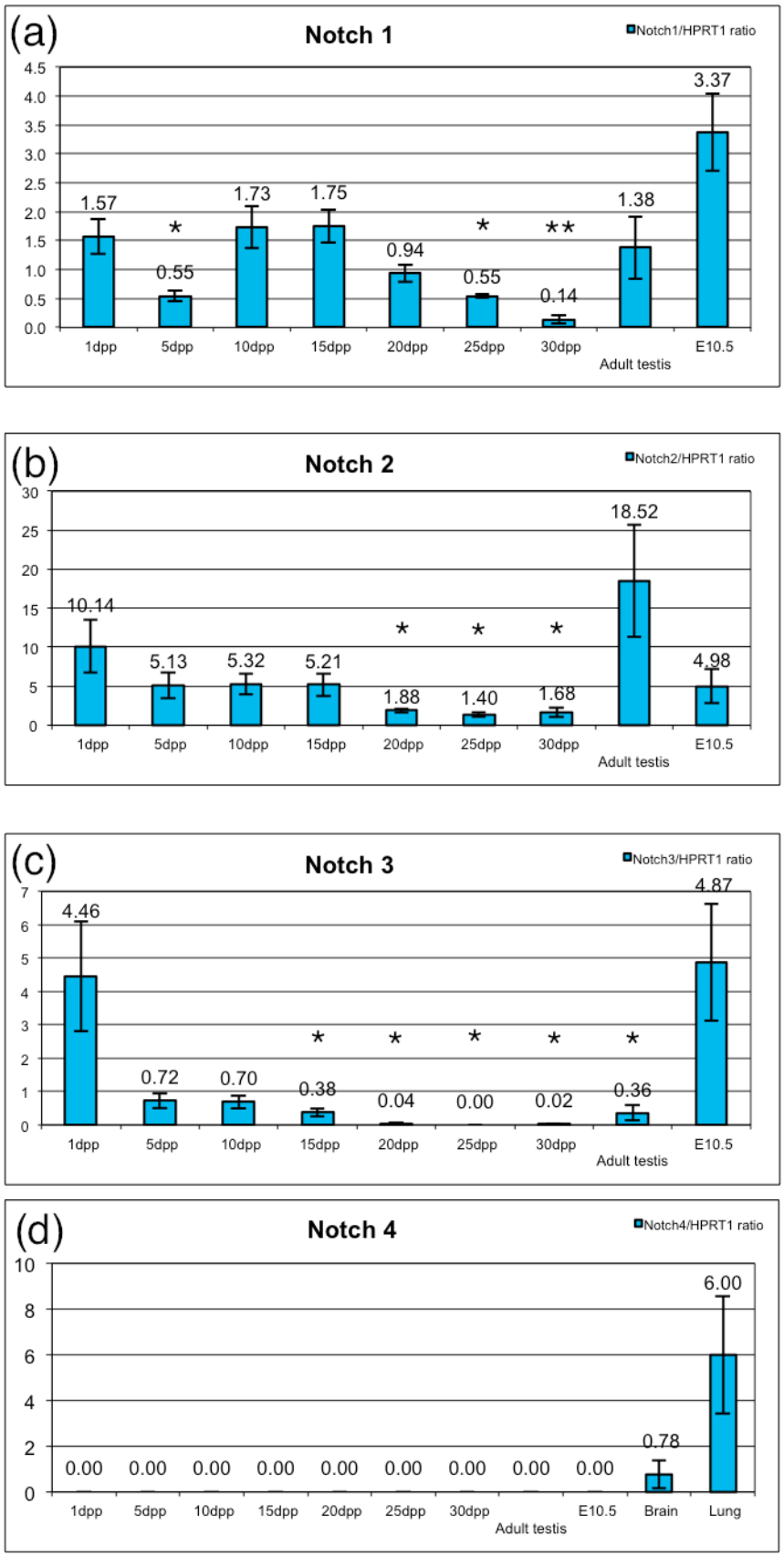


Figure 10. Semi-quantitative expression analysis of Notch receptors transcripts in various stages of spermatogenesis. (a) *Notch1* transcripts, (b) *Notch2* transcripts, (c) *Notch3* transcripts and (d) *Notch4* transcripts. RNA from E10.5 whole embryo, brain and lung adult mouse tissue was used as a positive control. Error bars indicate standard error of the mean (three biological replicates and a technical

triplicate for each biological sample). Stars represent P-values relative to the expression level of 1 day post partum (dpp). No star P>0.05, * P≤0.05, ** P≤0.01.

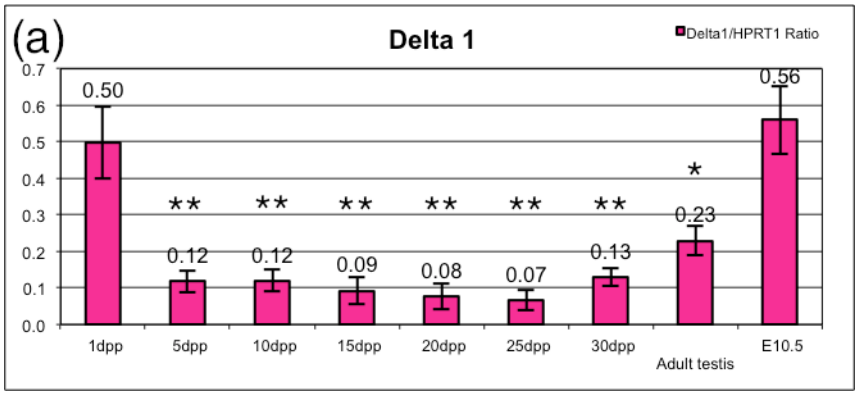
3.2.2 Assaying the expression of Notch signalling ligand transcripts in postnatal and adult testis

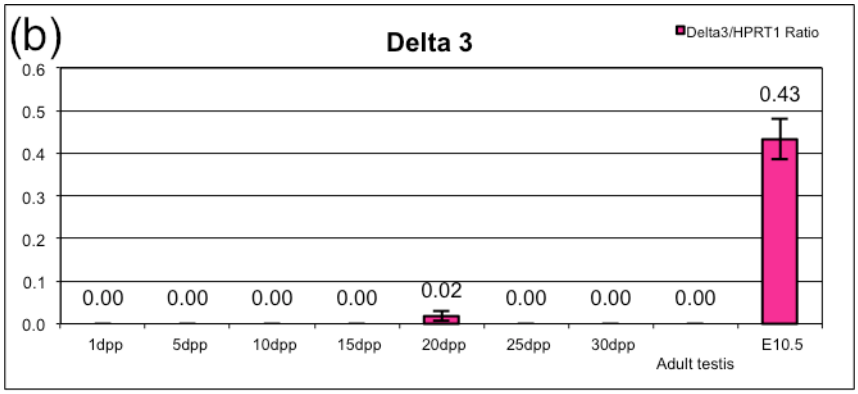
Notch has been extensively reported to dictate multiple developmental events and to induce the formation of multiple cell types even from a developmentally homogeneous population of cells (Andersson et al., 2011). Notch signalling requires intercellular communication through ligand-receptor interactions. Initially, Notch was discovered as a single gene involved in neural development in *Drosophila.* However, later studies revealed that together with the four Notch receptors examined above, five Notch ligands (Delta1, Delta3, Delta4, Jagged1 and Jagged2) have evolved in mammals depicting the complexity involved in the regulation of multiple developmental events and the multifaceted role of Notch (Weinmaster and Kintner, 2003). Even though the Delta and Jagged families consist of single-pass transmembrane proteins that contain a conserved DSL (Delta/Serrate/Lag-2) motif, which is involved in the binding of the ligand to the receptor, the general size of their extracellular domains varies significantly. Jagged ligands contain primarily more small cysteine rich motifs called EGF-like repeats compared with Delta ligands (Egan et al., 1998). Both Delta and Jagged ligand families are thought to result in the activation of CBF1/RBPJ transcriptions factors and the expression of target genes upon binding to Notch receptors (Bray, 2006). Nevertheless, each of the ligand-receptor combination has been extensively studied and there is evidence that signals transmitted through either Delta or Jagged ligands can differentially affect the fate of Notch receptor-bearing cell. The extent of their functional redundancy and their interplay in various differentiation and lineage specification events remain to be determined.

The expression of *Notch* receptors transcripts in postnatal testis implied that the Notch pathway might be functionally important for spermatogenesis and also that *Notch* ligands should be involved too. Therefore, the pattern of expression for each of the five Notch ligands (*Delta1-2-4* and *Jagged1-2*) was investigated at the same seven different postnatal time points and in adult testis using Multiplex-PCR. The ratio between the *Notch* ligand and *Hprt1* transcript for each stage was calculated and plotted on bar charts.

Analysis of *Delta1* expression in postnatal and adult testis revealed that transcripts of the ligand are present throughout the postnatal period and also in mature stage (Figure 11 a). In particular, the analysis showed high levels of *Delta1* transcripts to be present right after birth (PND 1) where mainly gonocytes (pre-spermatogonia) and Sertoli cells can be found in the seminiferous tubule. In the next few days the expression level drops almost 4/5 at the time corresponding to the appearance of A spermatogonia (PND 5). As Intermediate and B spermatogonia appear at the beginning of second week after birth, the transcripts continued to stay at low levels and remained like this almost throughout the postnatal period, with just a small increase at PND 30 as more spermatocytes complete the second meiotic division and more haploid spermatids appear.

A different situation was observed for the transcripts of *Delta3* and *Delta4* genes, which had very low level or no expression at the points examined relative to *Hprt1* (Figure 11 b and c). A similar situation was observed in adult testis matching the finding in postnatal period.





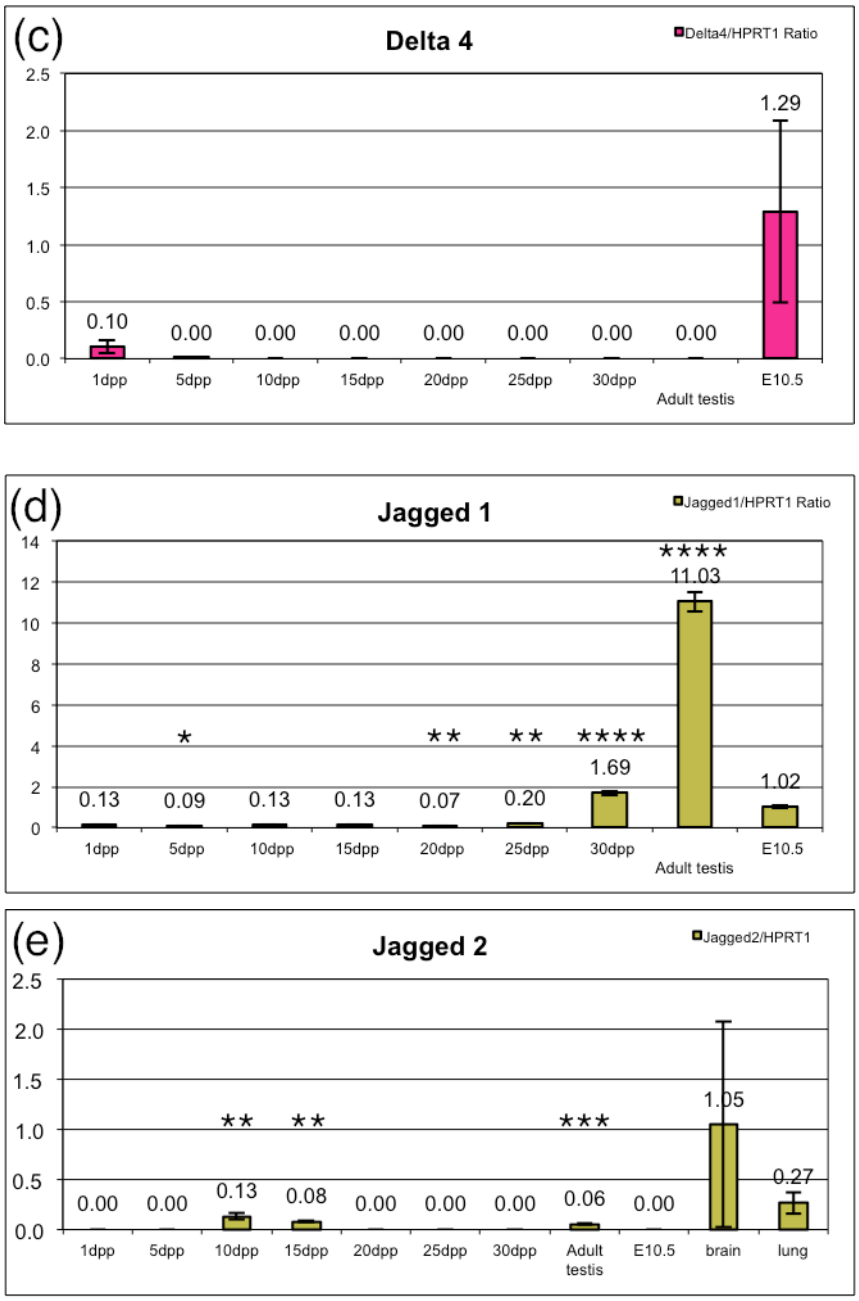


Figure 11. Semi-quantitative expression analysis of Notch ligands transcripts in various stages of spermatogenesis. (a) *Delta1* transcripts, (b) *Delta3* transcripts, (c) *Delta4* transcripts, (d) *Jagged1* and (e) *Jagged2* transcripts. RNA from E10.5 whole embryo, brain and lung adult mouse tissue was used as a positive control. Error bars indicate standard error of the mean (three biological replicates and a technical triplicate for each biological sample). Stars represent P-values relative to the expression level of 1 day post partum (dpp). No star P>0.05, * P≤0.05, ** P≤0.01, *** P≤0.001, **** P≤0.0001.

Conversely to the *Delta3 and Delta4* genes, the other ligand family *Jagged1* and *Jagged2* genes displayed two interesting differential expression patterns. The level of *Jagged1* transcripts commenced with very low expression that was maintained throughout the first 25 postnatal days. Intriguingly, the expression showed to increase at PND 30, the time

where the first spermatogenic wave approaches completion and 1/3 of the cells present in the seminiferous tubule are haploid spermatids. What is even more fascinating is that the level of *Jagged1* ligand transcripts increased almost 7 fold in the mature testis compared to PND 30 transcripts, implying that Jagged1 plays an important role in influencing the germ cell differentiation-maturation during spermatogenesis (Figure 11 d).

A different temporal expression pattern was detected for *Jagged2* transcripts. The analysis showed that for most of the first 30 days after birth no transcripts were detected, however this pattern was disrupted in some postnatal stages where *Jagged2* seemed to be expressed temporarily. In particular, expression of *Jagged2* transcripts was observed at PND 10 and PND 15 (Figure 11 e), stages that coincide with the progression of A spermatogonia to intermediate and B spermatogonia and to pre-leptotene spermatocytes respectively (Table 9). Expression of *Jagged2* was also observed in adult testis implying that it may be required for the same function in mature testis (Figure 11 e).

3.3 Expression analysis of Notch signalling proteins in murine testis

The expression of Notch signalling transcripts in postnatal and adult stage testis suggested a potential role of the pathway in spermatogenesis. To further elucidate and dissect the functional importance of Notch signalling, the expression pattern and cellular distribution of the Notch protein components were investigated in adult testis.

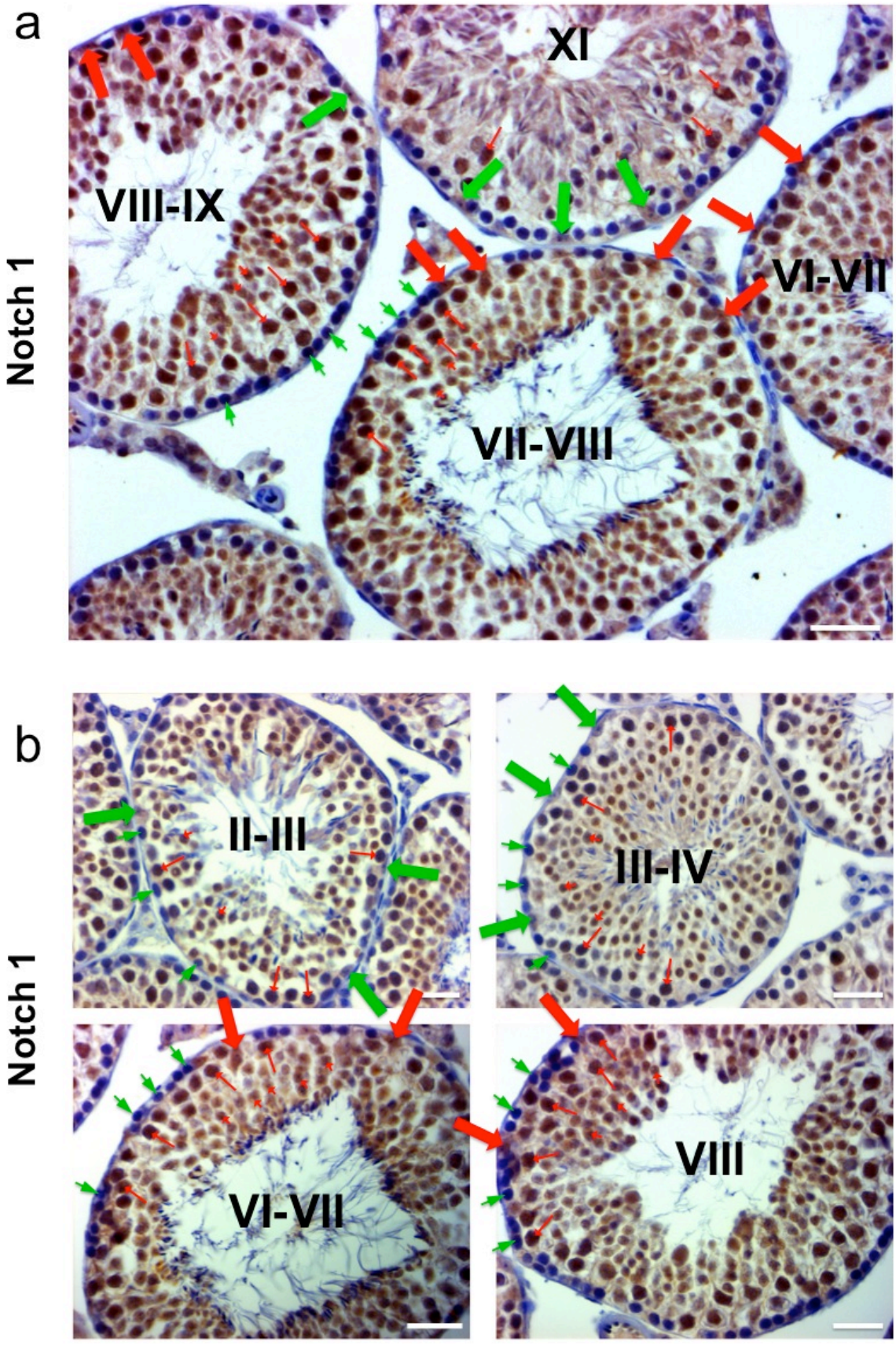
3.3.1 Assaying the expression of Notch signalling proteins receptors in adult testis

Expression of various Notch proteins in mammalian testes has been previously reported by various groups but there is a huge inconsistency within different findings (Dirami et al., 2001, Hayashi et al., 2001, Weinmaster and Kintner, 2003, von Schonfeldt et al., 2004, Hahn et al., 2005, Tang et al., 2008, Andersson et al., 2011, Batista et al., 2012). Furthermore there has been only one report that has examined the expression of Notch proteins in relation to the seminiferous epithelial cycle (Andersson et al., 2011). Thus, the

implication of Notch signalling in mouse spermatogenesis and the similarities or differences with the function of Notch in invertebrates remains unclear.

As explained previously (Introduction section 1.3.1) Notch proteins are large cell-surface receptors that interact with membrane-bound ligands on adjacent cells. This interaction leads to the activation of the receptor resulting in the cleavage of the N^{-ICD} by γ-secretase and its translocation to the nucleus, where together with other transcription factors it activates target genes. In an attempt to examine, not only the cellular distribution of Notch receptors within the seminiferous tubules, but also to identify whether the receptor has been activated by a ligand, antibodies that would recognised the N^{-ICD} in its "active form" (only after cleavage the epitope is unmasked) were used for the immunohistochemical analysis. This would provide insight on the temporal activation of the Notch receptor during the spermatogenic cycle.

Expression analysis of $NOTCH1^{-ICD}$ receptor revealed a cyclic activation of the receptor in Sertoli cells during stages VI to VIII (Figure 12 a, b). During these epithelial stages the intermediate spermatogonia progress to B spermatogonia and subsequently to pre-leptotene spermatocytes that eventually enter meiosis at the immediate epithelial stage IX (Figure 19). This finding is also consistent with an *in-situ* and immunohistochemical study performed by Hasegawa K. and colleagues (2012) where they found that $NOTCH1^{-ICD}$ is cyclically activated in Sertoli cells during stages VII to VIII and regulates stage-dependent expression of downstream Notch target genes. "Active" forms of $NOTCH1^{-ICD}$ were also detected in Pachytene spermatocytes and round spermatids implying a polyphasic role of NOTCH1 (Figure 12 a, b, and c). Partially in line with this is a similar study which identified $NOTCH1^{-ICD}$ in spermatocytes and round spermatids but failed to detect it in Sertoli cells (Mori et al., 2003). It's worth also mentioning however that Mori and colleagues (2003) did not examined their seminiferous tubules sections according to their epithelial stage, so this could account for the failure to detect $NOTCH1^{-ICD}$ in Sertoli cells.


a
Notch 1
XI
VIII-IX
VI-VII
VII-VIII
b
Notch 1
II-III
III-IV
VI-VII
VIII

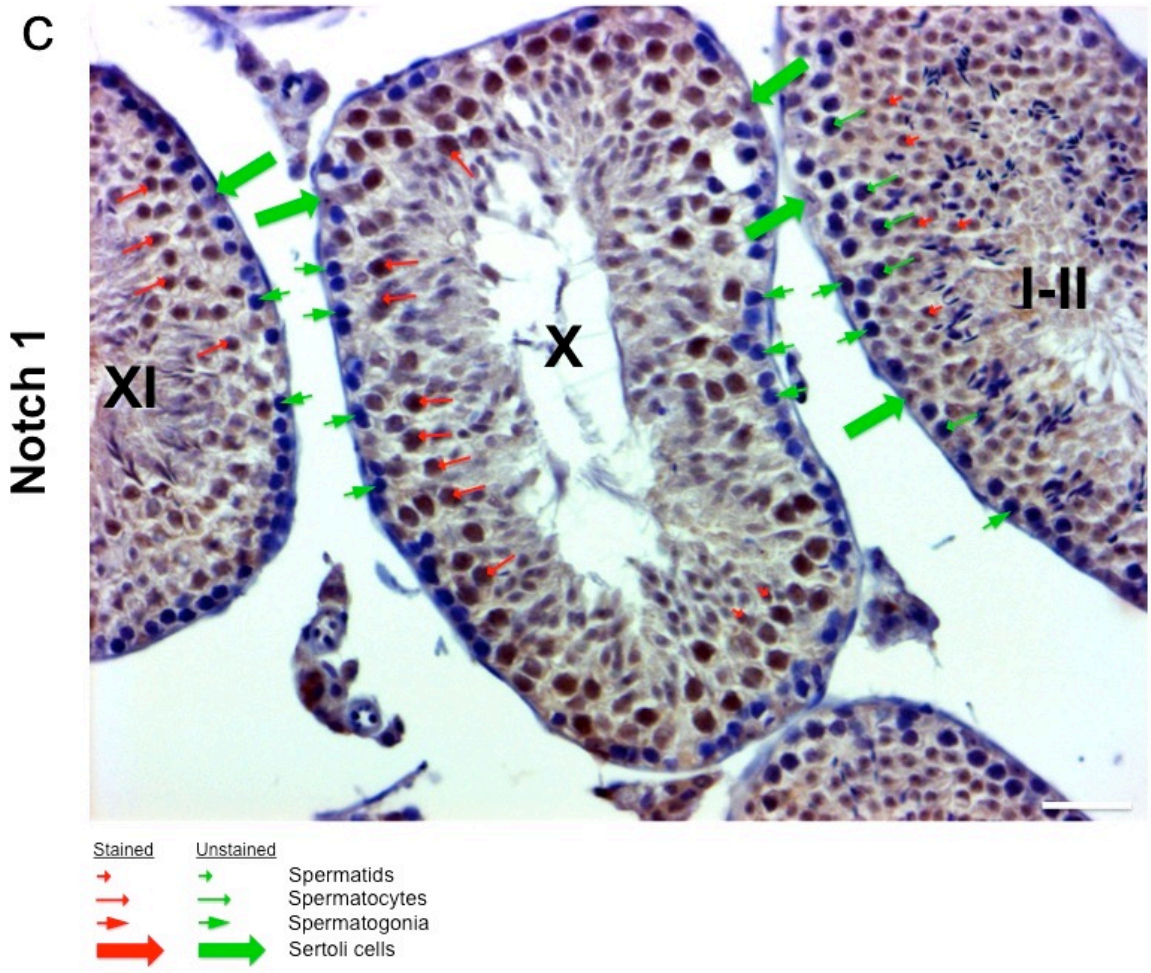
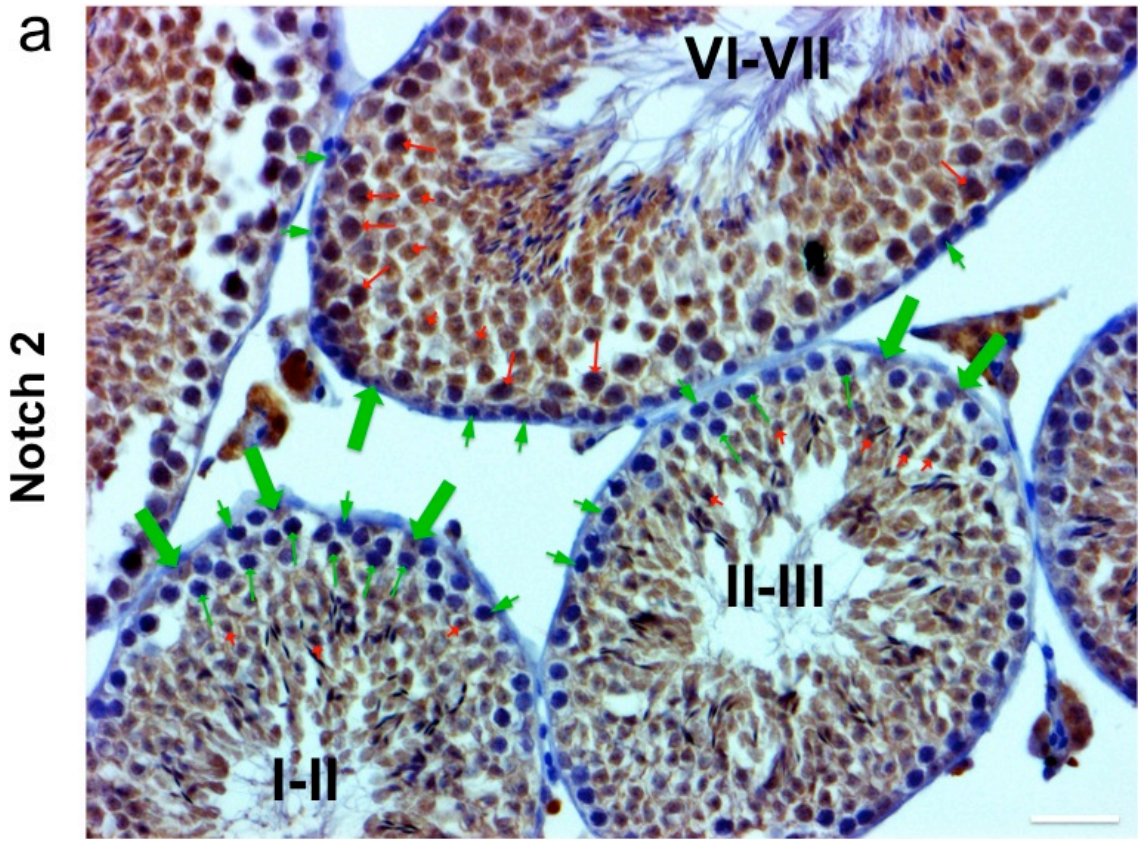


Figure 12. Immunohistochemical localization of NOTCH1 "activated" receptor in cross sections of seminiferous tubules. Intracellular domain N^{ICD} epitopes of NOTCH1 were found (brown staining) in Sertoli cells during epithelial stages VI-VIII (a) and (b), but not in all the other stages (a), (b) and (c). $NOTCH1^{ICD}$ epitopes were also distributed in spermatocytes and round spermatids (a), (b) and (c). Bars, 50 µm.

Antibody against $NOTCH2^{-ICD}$ was used next to determine the cellular distribution and temporal expression of this receptor. The immunohistochemical analysis revealed staining of all pachytene spermatocytes after epithelial stages III-IV, a slightly later onset than the expression pattern observed for $NOTCH1^{-ICD}$. The staining was maintained throughout meiosis (diplotene and secondary spermatocytes) and continued in round spermatids. Unlike the expression of $NOTCH1^{-ICD}$, $NOTCH2^{-ICD}$ was not found to be present in Sertoli cells at all epithelial stages (Figure 13).

A slightly different expression pattern was presented by Mori S. and colleagues (Weinmaster and Kintner, 2003) where NOTCH2 was similarly distributed in spermatocytes and round spermatids but it was also reported to be localized in spermatogonia.


a
Notch 2
VI-VII
II-III
I-II

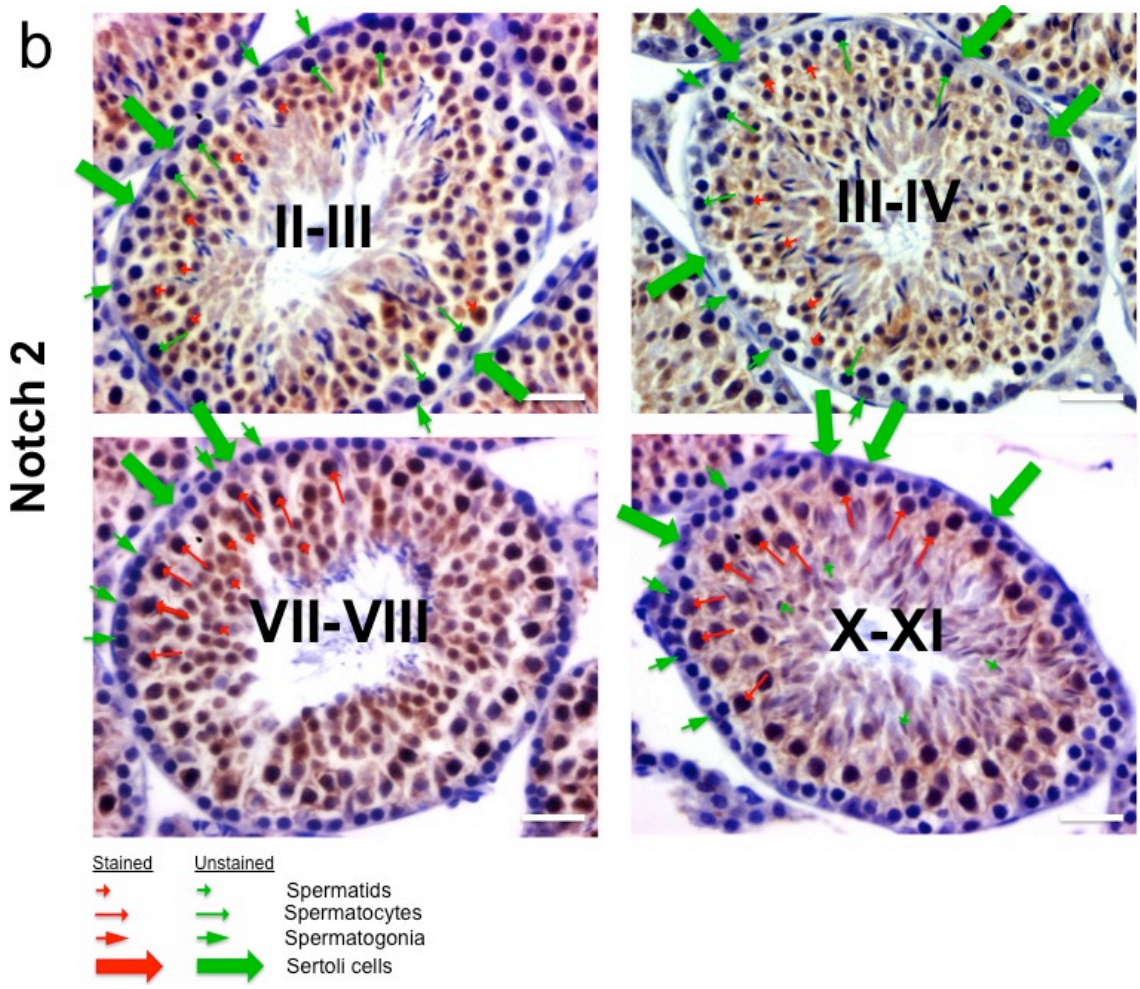
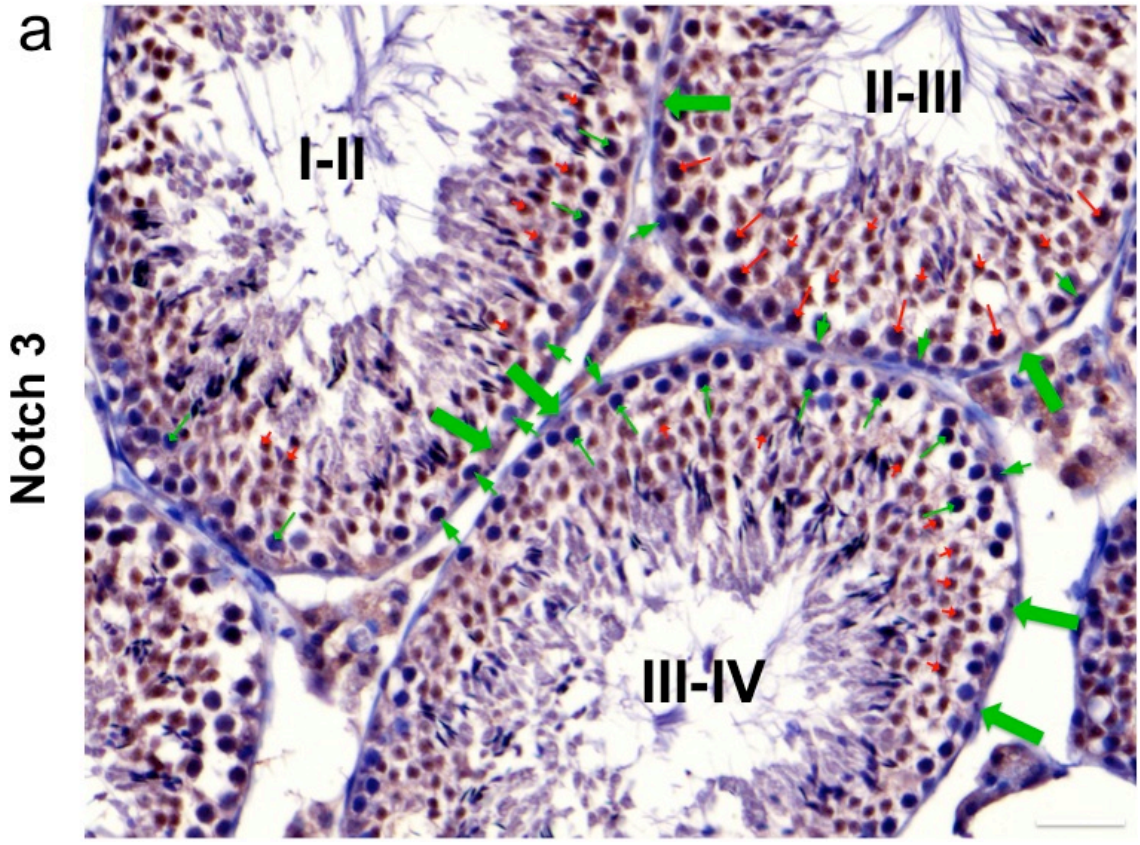


Figure 13. Immunohistochemical localization of NOTCH2 "activated" receptor in cross sections of seminiferous tubules. Intracellular domain NICD epitopes of NOTCH2 were found (brown staining) in pachytene and diplotene spermatocytes during epithelial stages IV-XII and round spermatids (a) and (b), but not in spermatogonia and Sertoli cells (a) and (b). Bars, 50 µm.

To further investigate the distribution and the function of other Notch receptors, the localization of NOTCH3^{-ICD} was examined in the seminiferous tubules of adult testis. The NOTCH3^{-ICD} expression pattern was found to mimic the distribution observed for the NOTCH2^{-ICD} receptor that is, the staining in pachytene spermatocytes, ddiplotene and secondary spermatocytes and round spermatids (Figure 14). However, NOTCH3^{-ICD} exhibited a slightly earlier expression onset (after epithelial stage II), compared to the onset shown by NOTCH2^{-ICD} receptor (after stage IV) and didn't persist after the elongation phase in spermatids as for the NOTCH2 receptor.

Only one study has shown NOTCH3^{-ICD} to make its appearance in the seminiferous tubules but this was not in progressed meiotic cells but in spermatogonia and Sertoli cells (Mori et al., 2003).


a
Notch 3
I-II
II-III
III-IV

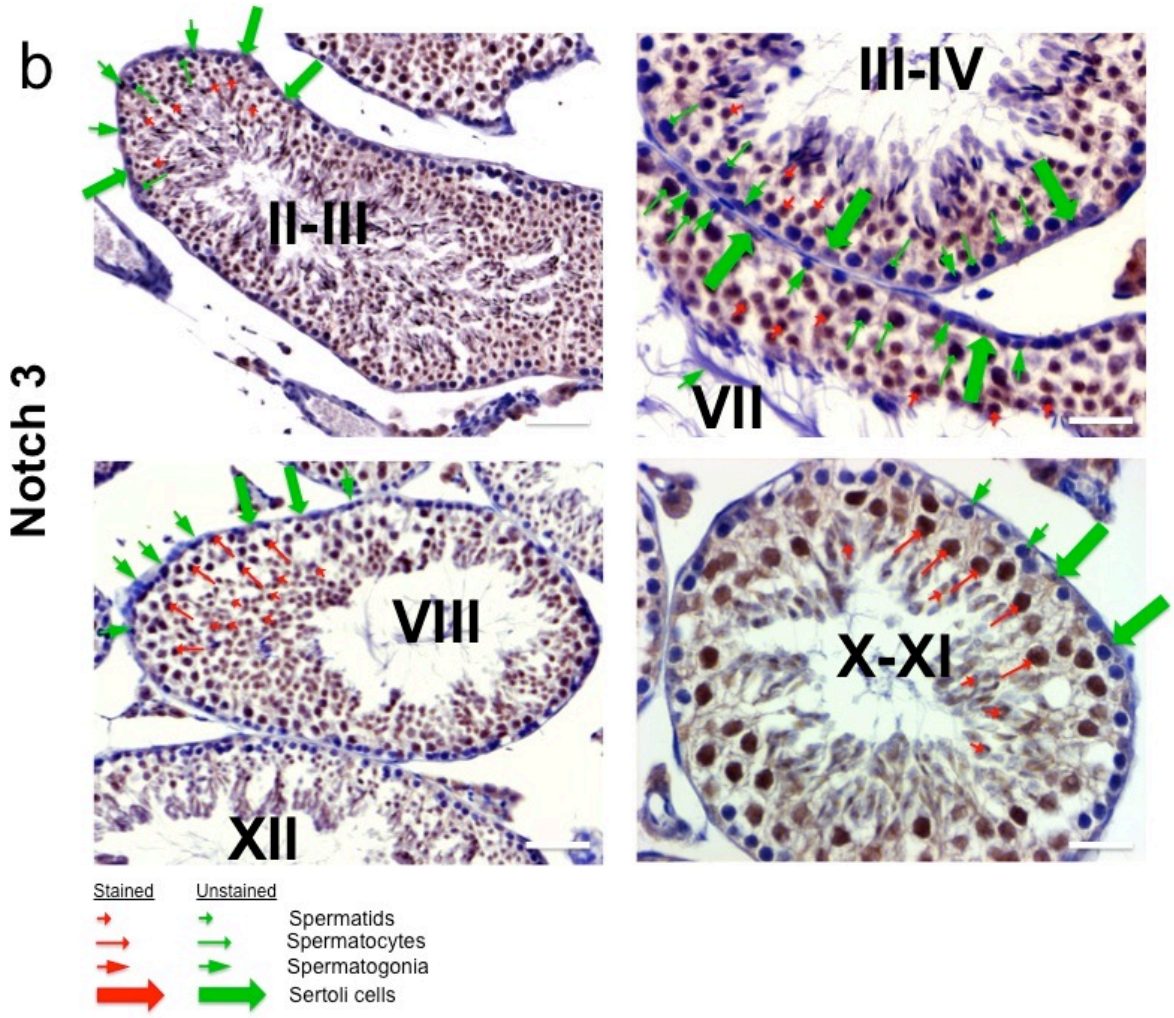


Figure 14. Immunohistochemical localization of NOTCH3 "activated" receptor in cross sections of seminiferous tubules. Intracellular domain N-ICD epitopes of NOTCH3 were found (brown staining) in pachytene and diplotene spermatocytes during epithelial stages II-XII and round spermatids (a) and (b), but not in spermatogonia and Sertoli cells (a) and (b). Bars, 50 µm.

The last receptor of the Notch family was also investigated. But as predicted by the Multiplex PCR analysis (Figure 10), no signal was detected, when an antibody against the NOTCH4^{-ICD} was used to stain cross sections of healthy fertile adult mouse testis (Figure 15).

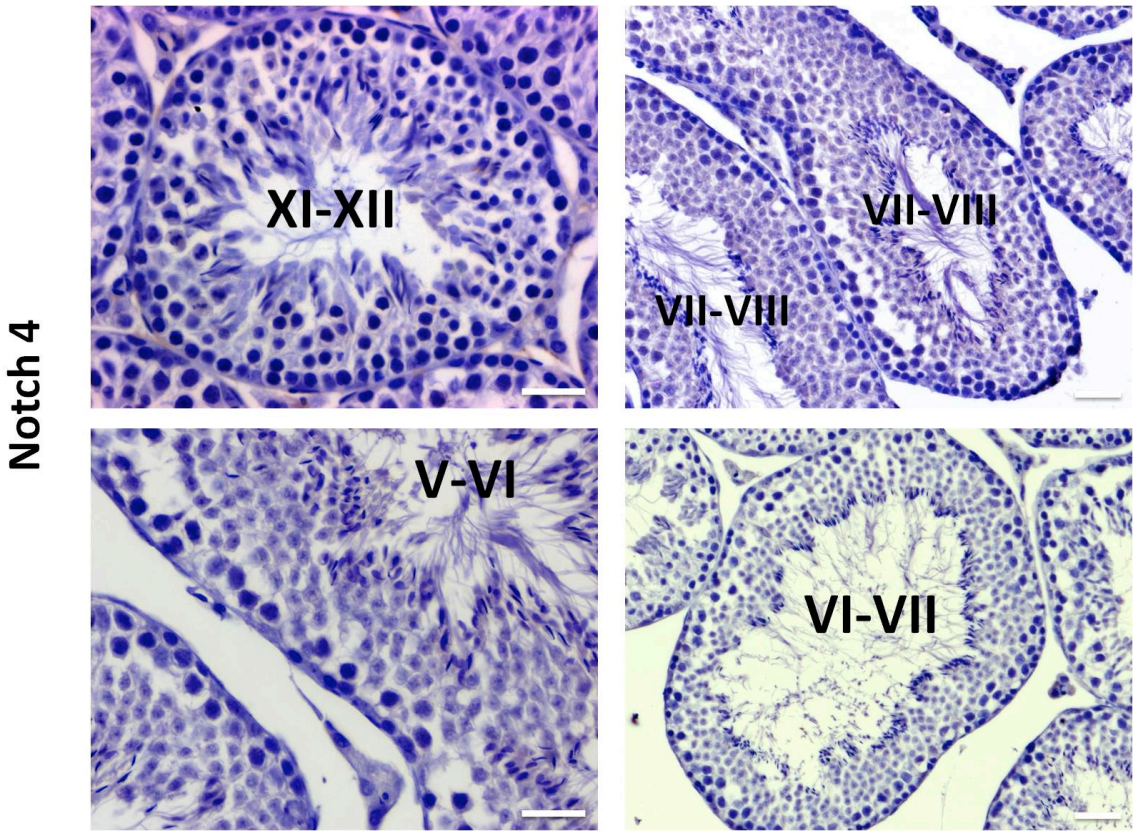


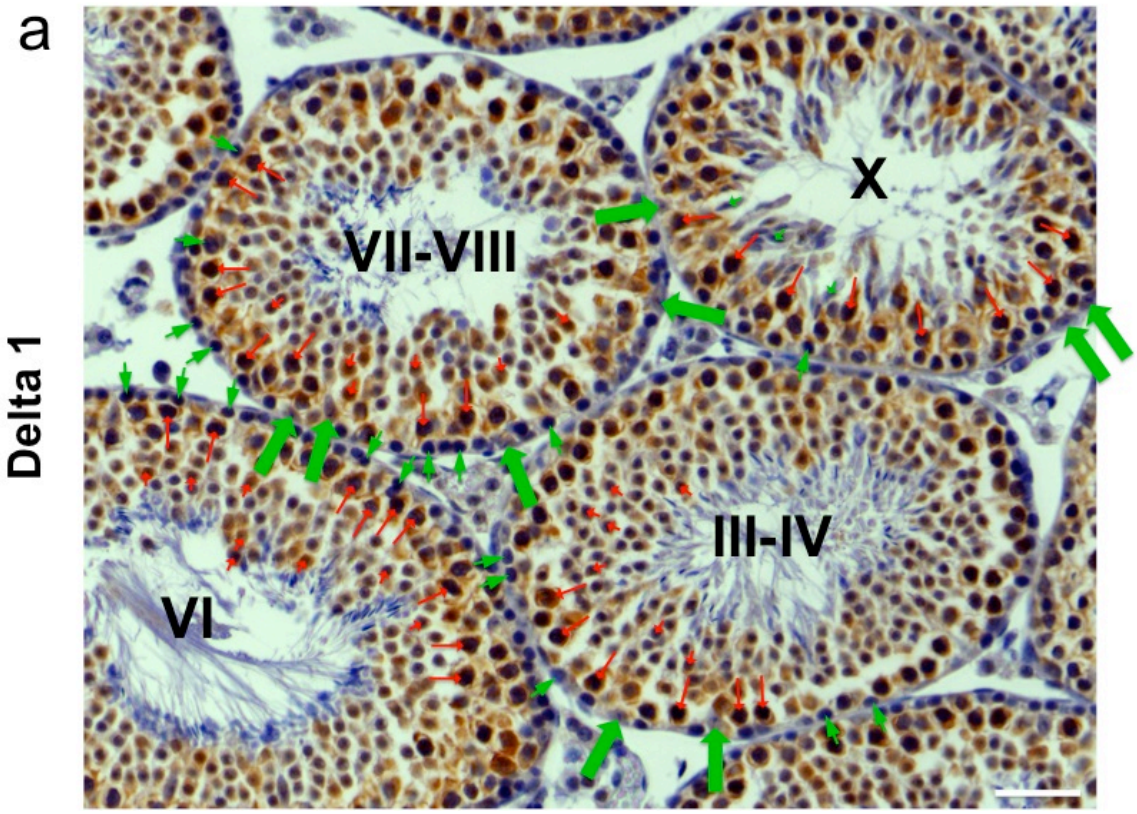
Figure 15. Immunohistochemical staining against the NOTCH-ICD epitope showed no evidence of NOTCH4 activated intracellular domain in cross sections of adult mouse seminiferous tubules in all the sequence of seminiferous epithelial stages examined. Bars, 50 µm.

3.3.2 Assaying the expression of Notch signalling proteins ligands in adult testis

The immunohistochemical investigation showed that most of Notch receptor proteins are present in the mouse testes, and their expression patterns suggest a major function in spermatogenesis and imply a potential role in the meiotic progression of the germ cells. Furthermore, the detection of "activated" Notch intracellular domains receptors suggests the presence of Notch ligands in adjacent cells. Therefore, to examine the localization and temporal expression of Notch ligands in relation to the epithelial stages, cross sections of adult mouse testis were stained with α-DELTA1, α-JAGGED1 and α-

JAGGED2 antibodies, which where found to be expressed in postnatal and adult stage from the Multiplex PCR analysis (Figure 11).

The first ligand to be investigated was DELTA1. Immunohistochemical analysis showed that DELTA1 is absent in pre-meiotic germ cells, A Spermatogonia, Intermediate Spermatogonia and B Spermatogonia, and was only detected after meiotic initiation had occurred. A strong DELTA1 signal was initially detected in Leptotene spermatocytes, continued throughout meiosis and was also present in round spermatids, but this signal was switched off in elongated spermatids (Figure 16). This result fits well with the localization signal of NOTCH1-3 receptors in meiotic and post-meiotic germ cells and suggests a potential function of DELTA1 in the meiotic progression and/or survival of germ cells.



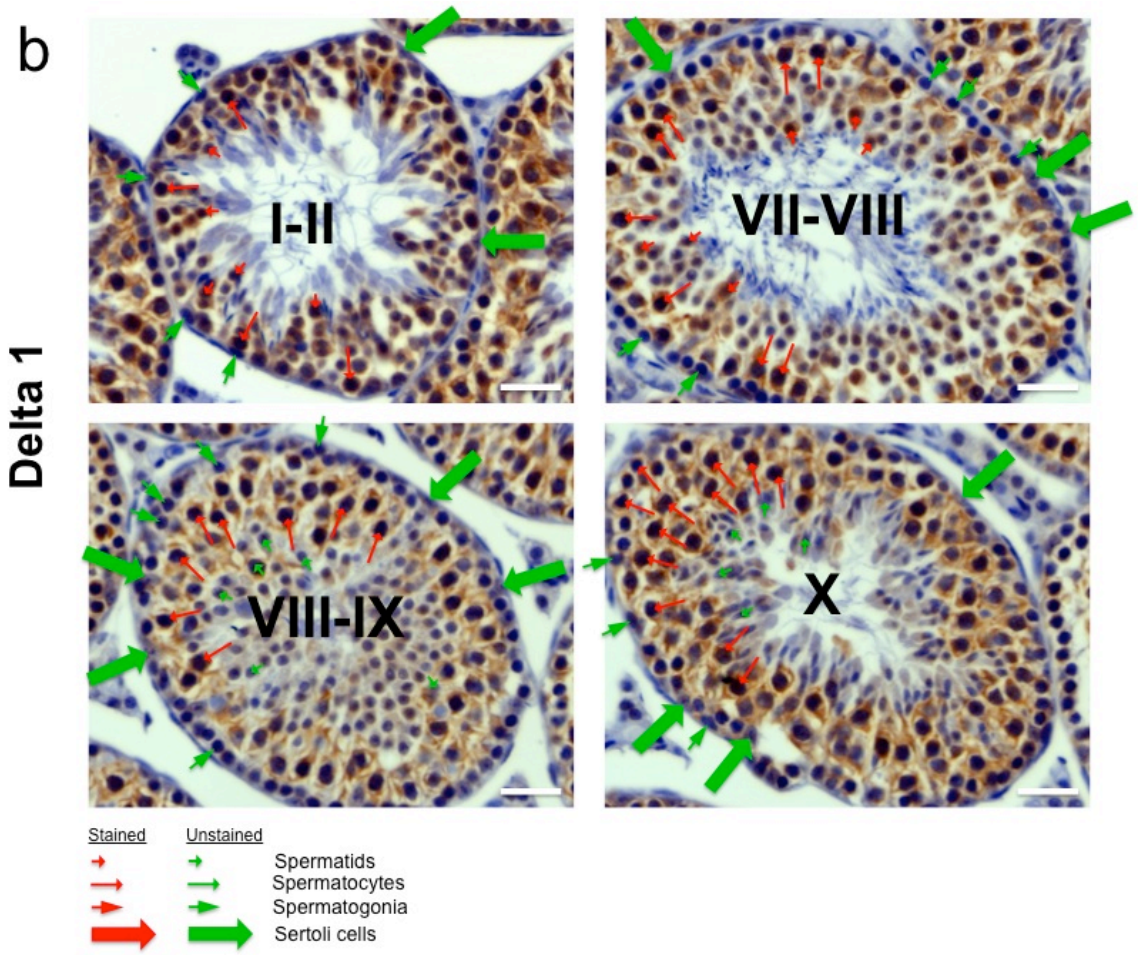


Figure 16. Immunohistochemical localization of DELTA1 ligand in cross sections of seminiferous tubules. DELTA1 epitopes were found (brown staining) in leptotene, pachytene and diplotene spermatocytes during epithelial stages I-XII and round spermatids (a) and (b), but not in spermatogonia, Sertoli cells and elongated spermatids (a) and (b). Bars, 50 µm.

The second Notch ligand examined was JAGGED1. Signal from the α-JAGGED1 antibody was found to share a quite similar expression pattern to the α-DELTA1 antibody. No signal was detected in any of the pre-meiotic germ cells but only after the germ cells had entered meiosis (Leptotene stage) a strong signal was clearly visible. In a similar again manner to DELTA1 the immunohistochemical analysis also showed the signal from JAGGED1 to be detectable in all meiotic phases and also in round spermatids, but unlike DELTA1 the signal could also be found in elongated spermatids (Figure 17).

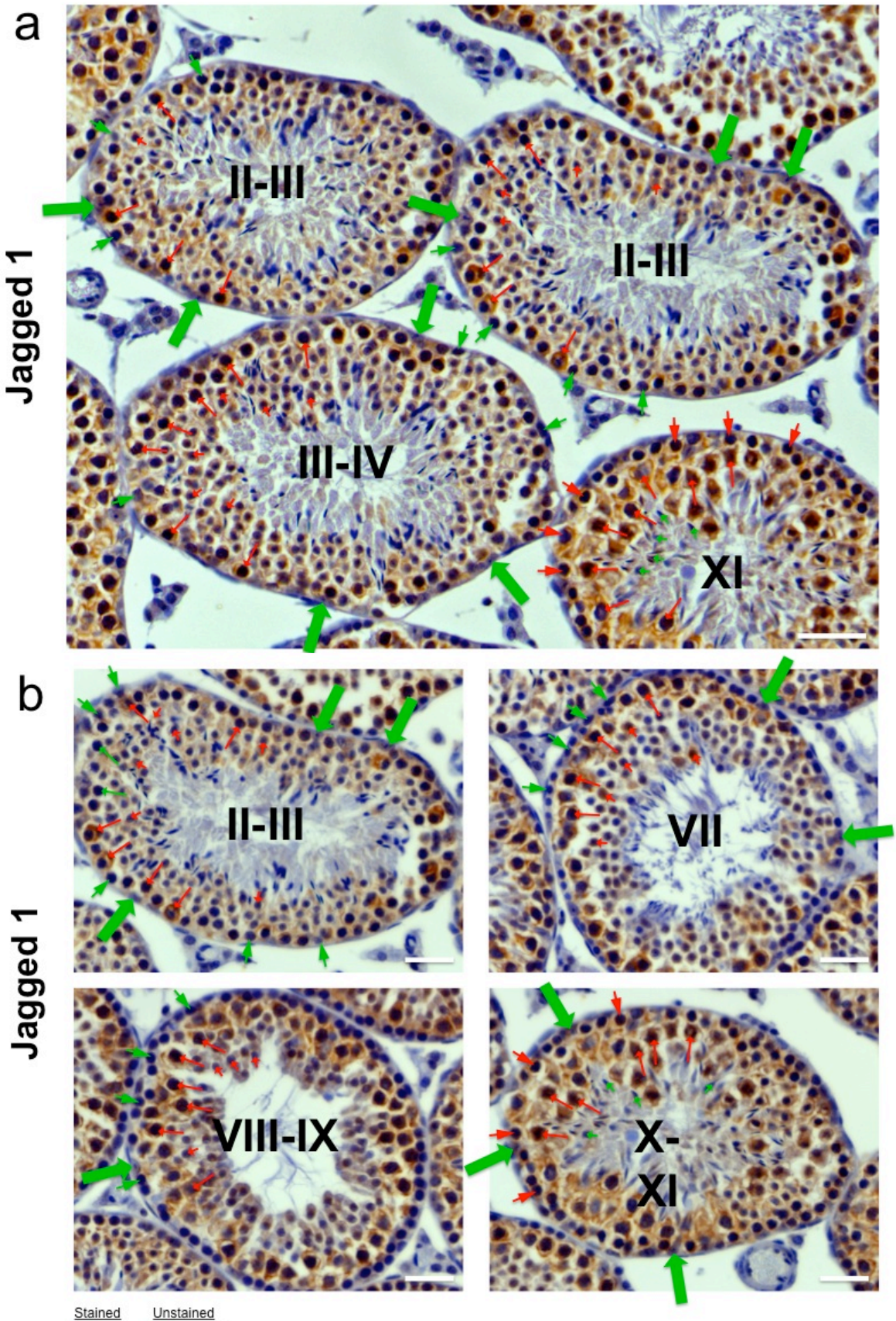

a
Jagged 1
II-III
II-III
III-IV
XI
b
Jagged 1
II-III
VII
VIII-IX
X-
XI
Stained
Unstained
Spermatids
Spermatocytes
Spermatogonia
Sertoli cells

Figure 17. Immunohistochemical localization of JAGGED1 ligand in cross sections of seminiferous tubules. JAGGED1 epitopes were found (brown staining) in leptotene, pachytene and diplotene spermatocytes during epithelial stages I-XII and round spermatids (a) and (b), but not in spermatogonia and Sertoli cells (a) and (b). Bars, 50 µm.

To elucidate the role of JAGGED2 in spermatogenesis the expression pattern was investigated in adult testis sections. Due to the fact that α-JAGGED2 antibody was found to work better with the immunofluorescent staining protocol it was the preferred method for this case. Unlike the other two Notch ligands DELTA1 and JAGGED1, the localization of JAGGED2 was not found in meiotic cells (pachytene, diplotene or secondary spermatocytes) but only in spermatogonia germ cells. Furthermore, examination of various seminiferous tubules revealed that the expression is temporally regulated and is restricted in the epithelial stages V to VIII (Figure 17). This finding is in agreement with another study where *in situ* hybridization showed JAGGED2 transcripts present in spermatogonia in the same epithelial stages (V to VIII) (Andersson et al., 2011). Interestingly, these stages correspond to the progression of intermediate spermatogonia to B spermatogonia and subsequently to pre-leptotene spermatocytes (Figure 9) suggesting a potential function of JAGGED2 in the differentiation of spermatogonia, and matches the expression pattern observed for NOTCH1 receptor in Sertoli cells in identical epithelial stages (Figure 12 a and b).

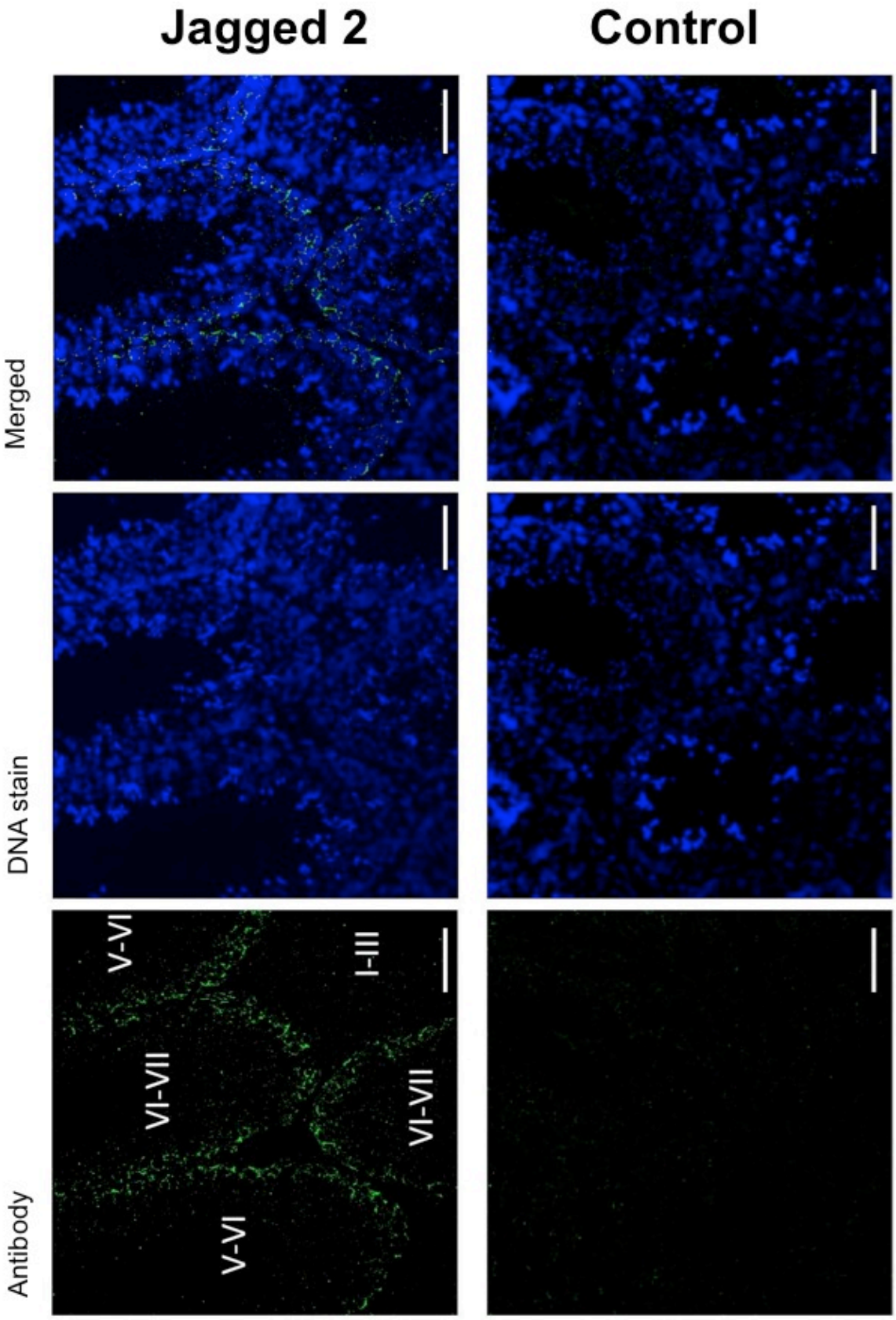


Figure 18. Immunofluorescent localization of JAGGED2 ligand in cross sections of seminiferous tubules. JAGGED2 epitopes were found (green staining) in spermatogonia cells during epithelial stages VI-VII, but not in spermatocytes and Sertoli cells. Bars, 100 µm.

3.4 Summary and discussion

The established essential role of Notch receptors and ligands in invertebrate and vertebrate organisms and the demonstrated presence of Notch signalling proteins in rat and mouse testis led us to hypothesise a possible role of the Notch signalling pathway in spermatogenesis. Thus, in order to better understand how the Notch pathway may mediate the differentiation and maturation of the male germ line the following aims were set for this chapter (a) to investigate the expression of transcripts encoding Notch receptors and ligands in postnatal and adult testicular tissue, (b) to establish the cellular-type localization of Notch receptors and ligands by immunohistochemical analysis in cross-sections of seminiferous tubules of adult testis and (c) to characterise the spatial and temporal activation of Notch receptors during an adult seminiferous epithelial cycle using antibodies raised specifically against "activated" Notch receptors.

The expression pattern analysis of the Notch receptors transcripts during the postnatal and adult period revealed that all (*Notch1-3*) but one (*Notch4*) of the Notch receptors were expressed in the examined phases. There was no difference in the receptor transcripts that were present or absent between postnatal and adult testis. The level of transcripts present showed an apparent fluctuation in the seven different postnatal time points and an evident variation at the adult testicular stage. The transcript levels of the *Notch2* and *Notch3* receptors followed a steady drop after postnatal day 1. *Notch1* transcript levels, after a short decline at PND 5, increased for the next 5 to 10 postnatal days. These days correspond to when the progression of Intermediate spermatogonia to B spermatogonia and subsequently to early spermatocytes occurs, implying a potential role in the transition of spermatogonia to spermatocytes, and possibly providing signals that allow entrance into meiosis.

Examination of the expression of Notch ligands genes during the postnatal period and also in the adult stage, revealed the presence of *Delta1*, *Jagged1* and *Jagged2* and the absence of *Delta3* and *Delta4* transcripts. Similar to the *Notch* receptors, the general transcription pattern of the *Delta1* transcripts appeared to decline after postnatal day1 and kept a fairly steady level after that, although in the adult stage the expression was found to be higher. Interestingly, transcripts of *Jagged1* ligand appeared to have a minimum

level of expression through out the postnatal period but a much higher level of transcripts in the adult stage. A more striking difference in expression pattern was observed in *Jagged2* transcripts. The analysis revealed a temporal expression during the postnatal period and specifically in postnatal days 10-15, which as explained above relates with the progression-transition from mitosis to meiosis. Intriguingly, this is the same postnatal period where expression of *Notch1* transcripts was found to be upregulated.

It's worth also to note the different level of transcripts in postnatal day1, during the postnatal period and in adult stage testis, for almost all the expressed Notch transcripts and for some Notch ligands. This can be attributed to many things, a) due to the fact that initiation of spermatogenesis starts right after birth at postnatal day1 where gonocytes resume mitosis and begin their migration and transformation into spermatogonia (Bellve et al., 1977), b) due to that fact that the first round of spermatogenesis lacks important progression stages such as the self-renewal of spermatogonia and the feedback mechanism-signals present in adult testis (Yoshida et al., 2006), and c) due to the fact that Sertoli cells mature and reach their morphologically comparable characteristics at postnatal day 30 (Sharpe et al., 2003). Moreover, it has been shown that the first round of spermatogenesis exhibits massive apoptosis and is less efficient than that of adults, and it has been questioned whether fertile spermatozoa are produced (Kluin et al., 1982, Mori et al., 1997).

Nevertheless, even though the examination of the level of transcripts in the first spermatogenic wave is not specific for a cellular type, the vast change in the population and number of germ cells in the seminiferous tubule for each postnatal time point examined and the coordinated progression of the first spermatogenic wave allowed us to get an indication of the level of expression and the spermatogenic stage these Notch transcripts are involved.

Consistent with our results examining Notch transcript levels, is a study from Dirami and colleagues (2001) where using western blot analysis they identified the equivalent proteins of the transcripts described above. They detected NOTCH1, NOTCH2, and NOTCH3 receptors and DELTA1, JAGGED1 and JAGGED2 ligands in neonatal mouse testis analysis however, they did not perform a temporal analysis similar to ours but instead they obtained their samples from a single time point (PND 6). Moreover, in line with our data is a northern blot and RT-PCR analysis from Hayashi and colleagues

(2001), which reported the expression of *Notch1* and *Jagged2* mRNAs through out the first 28 postnatal days and also in adult rat testis. Intriguingly, in the same study, expression level of *Notch1* and *Jagged2* transcripts appeared to peak at around 13 to 22 days after birth, similar to the peak detected in our analysis for *Notch1* transcripts, and additionally matching the detection of *Jagged2* mRNAs observed in mouse postnatal testis. Considering the developmental differences between mice and rats in the duration of their spermatogenesis, with the rats producing mature sperm in 48 days compared to 39 days in mice, both peak periods correspond to the transition of spermatogonia to spermatocytes (Malkov et al., 1998).

Having confirmed the transcripts of Notch pathway genes are expressed in adult testis and also a spatial and temporal regulation of some of the components of Notch pathway during the first round spermatogenesis, the next step was to determine the expression of the corresponding Notch proteins.

Using immunohistochemistry in adult testis sections we could detect all Notch proteins, for which their transcripts were present in the mRNA analysis mentioned above. Notch proteins for which their transcripts were absent in the RT-PCR expression analysis were not detected by immunohistochemistry. The cellular distribution of Notch receptors and ligands in seminiferous tubules is summarised in Table 10.

	Sertoli cells	Spermatogonia				Spermatocytes				Spermatids	
		A-Sprg	Int-Sprg	B-Sprg	Pre-L Sprc	Lept, Zyg	Pach	Diplo	S. Sprc	Round Sprt	Elong Sprt
Notch1-ICD	+	—	—	—	—	—	+	+	+	+	+
Notch2-ICD	—	—	—	—	—	—	+	+	+	+	+
Notch3-ICD	—	—	—	—	—	—	+	+	+	+	+
Notch4-ICD	—	—	—	—	—	—	—	—	—	—	—
Delta1	—	—	—	—	—	+	+	+	+	+	—
Delta2	—	—	—	—	—	—	—	—	—	—	—
Delta3	—	—	—	—	—	—	—	—	—	—	—
Jagged1	—	—	—	—	—	+	+	+	+	+	—
Jagged2	—	—	—	+	+	—	—	—	—	—	—

ICD, intracellular domain; A-Sprg, A-Spermatogonia; Int-Sprg, Intermediate Spermatogonia; B-Sprg, B-Spermatogonia; Pre-L Sprc, Pre-Leptotene Spermatocyte; Lept, Leptotene; Zyg, Zygotene; Pach, Pachytene; Diplo, Diplotene; S. Sprc, Secondary Spermatocyte; Round Sprt, Round Spermatid; Elong Sprt, Elongated Spermatid; +, positive staining; —, no staining.

Table 10. Cellular distribution of Notch receptors and ligands in seminiferous tubules

All three Notch receptors proteins (NOTCH1-3) identified as expressed in the RT-PCR analysis could be detected in the adult testis sections and specifically in meiotic germ cells and in post-meiotic spermatids. Signals from the three receptors were not identified

in leptotene and zygotene early spermatocytes. Intriguingly, activation of Notch receptors seemed to take place in pachytene spermatocytes. What was particularly striking was the fact that the localization pattern indicated a sequential activation of the three Notch receptors in primary spermatocytes, with NOTCH1 followed by NOTCH2 and successively by NOTCH3, for summary see Figure 19.

We should note at this point that although the detection method used in these experiments (DAB peroxidase staining, see Materials and Methods section 2.2.2) is not ideal for quantitative analysis and to detect subtle differences in the detection signal, it renders a very strong signal. The sequential activation of these three NOTCH receptors was detected in all testis sections inspected, with about 10 sections examined for each antibody, in testes from two different adult fertile mice. The activation of NOTCH1-3 receptors was observed through out meiosis in all meiotic germ cells and continued in round spermatids. The distribution pattern started to deviate only when the spermatids reached the elongation phase where only NOTCH1 and NOTCH2 persisted for one and two epithelial stages during this phase, respectively (Figure 19).

Investigation of Notch receptors also revealed a stage-dependent activation of NOTCH1 in Sertoli cells. Specifically, it was observed that NOTCH1 receptor was activated in Sertoli cells during stages VI to VIII of spermatogenesis (Figure 19). This stage-dependent cyclic activation coincided with the epithelial stages where B-spermatogonia progress to pre-leptotene spermatocytes and just before meiosis is initiated (Russell et al., 1993). The temporal activation observed could also account for the expression peak of *Notch1* transcripts detected in postnatal days 10 to 15 (Figure 10).

Remarkably, a similar stage-dependent expression pattern was discovered using anti-JAGGED2 antibody. More specifically, immunohistochemistry in adult testis sections provided evidence of JAGGED2 ligand expression in B-spermatogonia and pre-leptotene spermatocytes. This localization pattern not only matched the expression of Jagged2 transcripts in postnatal period but it also complemented the observed stage-dependent expression of NOTCH1 receptor in Sertoli cells (Figure 19).

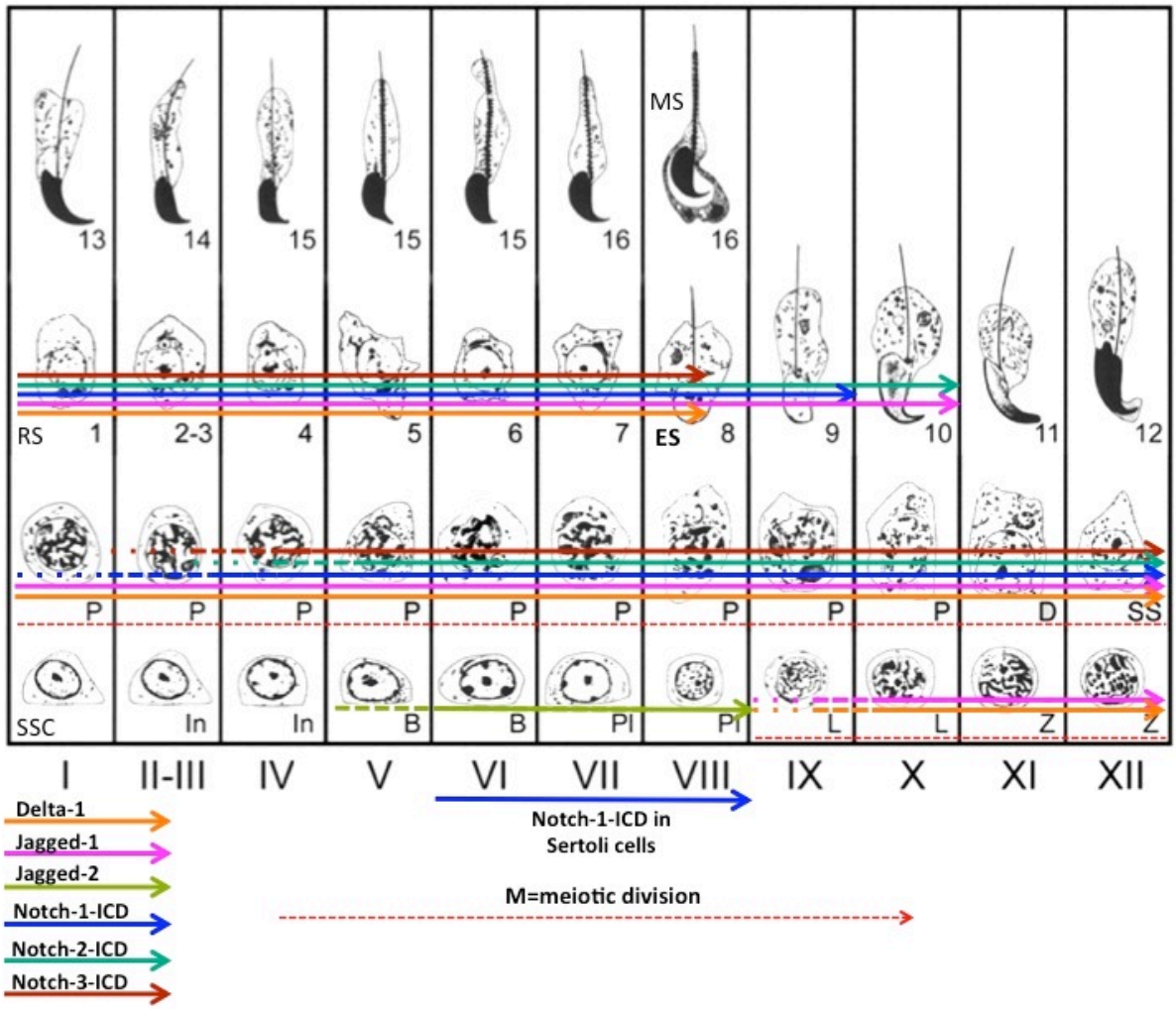


Figure 19. Expression of Notch receptors and ligands during the mouse seminiferous epithelial cycle. The germ cellular associations within each layer of the seminiferous epithelium described above have been used to identify the sequence of stages (I-XII) of the spermatogenic waves in the seminiferous tubules. The stages occur in sequence along the epithelium in a successive order. Stage I is followed by II, followed by III and so on. The red dotted line marks and follows the initiation, progression and completion of meiosis. The Roman numbers represent the stages of the seminiferous epithelial cycle. Arabic numbers represent the steps of spermiogenesis. Spermatogonia stem cells (SSC) differentiate to intermediate spermatogonia (In) to B spermatogonia (B) to pre-leptotene (Pl) spermatocytes to leptotene (L) spermatocytes to zygotene (Z) spermatocytes to pachytene (P) spermatocytes to diplotene (D) spermatocytes to secondary spermatocytes (SS) to round spermatids (RS) to elongating spermatids (ES) and finally to maturing spermatids (MS). Figure modified from (Russell et al., 1993).

Although we can not claim with confidence that JAGGED2 ligand in spermatogonia interacts with NOTCH1 receptor in Sertoli cells, there are however compelling evidences from other studies to justify a suggestion of this interaction. When rat testicular tissues were cultured with antibodies that blocked the activity of NOTCH1 receptor and JAGGED2 ligand, round and elongated spermatids decreased after 5 to 7 days of culture, and disappeared after 9 to 12 days (Hayashi et al., 2001). In addition, when testicular

tissues from human patients with maturation arrest and male infertility problem were examined, the expression of Notch1 and Jagged2 transcripts and proteins were either negative for both of Notch components or positive for just one of them (Hayashi et al., 2004b) implicating the requirement of these two Notch components in spermatogenesis. Moreover, in other cell fate specification and differentiation systems, interaction of Notch1 signals through Jagged2 ligands has been found to regulate limb size development (Francis et al., 2005) and tooth morphogenesis (Mitsiadis et al., 2010).

Consistent with the expression of NOTCH1 receptor in Sertoli cells and JAGGGED 2 ligand in spermatogonia is an investigation by Hasegawa et al. (2012) which also showed the expression of NOTCH1^{-ICD} signal in Sertoli cells of stage VII to VIII tubules and the expression of JAGGED2 ligand in spermatogonia and pre-leptotene spermatocytes in the same epithelial stages (VII to VIII). This result, which again identified the simultaneous expression of NOTCH1 and JAGGED2 in stage VII to VIII seminiferous tubules, adds strength to our initial suggestion of Notch signalling activation in stage specific Sertoli cells by these two Notch components.

It should be mentioned that in the same study (Hasegawa et al., 2012) the investigators failed to detect NOTCH1^{-ICD} signal in any germ cells. This could be attributed either to the different mouse strain used in their study, or difference in the staining procedure or to the different anti-NOTCH1^{-ICD} antibody that detects only cleavage in a specific amino acid (Val1744) and will not recognise NOTCH1 cleaved at other positions, compared to the antibody used in our study that detects any cleaved intracellular active form of NOTCH1.

Analysis of other Notch ligands such as DELTA1 and JAGGED1 showed these to replace the expression of JAGGED2 in germ cells in the subsequent epithelial stages (IX, X) once germ cells had entered meiosis (Leptotene spermatocytes) (Figure 19). The expression continued through out meiosis and could be detected, in the case of DELTA1 in round spermatids until the elongation phase, and in JAGGED1 continued a few stages after that.

The fact that both Notch receptors and ligands are present in spermatocytes and spermatids indicate that these cells are capable of both responding to and stimulating Notch signalling during spermatogenesis, suggesting the possible requirement of these

signalling events for cell fate progression and determination. In this case however, in addition to the traditional trans-activating Notch-ligand complexes, as the Notch receptor and ligand are expressed on the same cell surface, the receptor can form cis-inhibitory complexes with the ligands (see Introduction section 1.3.2). This form of ligand mediated Notch cis-inhibition may serve to limit the zone of Notch activity by mutual inactivation of the receptor in signal-sending cell and also blocking Notch activity in neighbouring cells by restricting ligand to activate receptors in signal-receiving cell. This kind of regulatory mechanism has been shown to have an important role in other developmental programs such as eye and wing disc formation of *Drosophila Melanogaster* (Micchelli et al., 1997, Becam et al., 2010).

In line with our initial hypothesis, the expression analysis of Notch pathway transcripts, the cellular distribution of Notch components and the activation analysis of Notch receptors in seminiferous tubules added supporting evidence of a potential role of Notch signalling proteins in spermatogenesis. Overall these experiments extended previous studies and revealed novel expression data for some Notch receptors and ligands. The cyclical expression of JAGGED2 and NOTCH1 raises the question of whether this is functionally important for spermatogenesis and if it is what processes are regulated by the interaction of these Notch components. One can suggest that the spatially and temporal activation of NOTCH1 receptor might be under the control during the transition from mitosis to meiosis, or, more appealingly, the initiation of meiosis might be under the control of the Notch signal. The mixture of overlapping and spatially and temporally different expression patterns of many Notch components in seminiferous tubules suggests the function of individual Notch family receptors to be different from each other.

Although these data provided the basis for investigating the role of Notch components in spermatogenesis the actual functional involvement of the Notch pathway remains inconclusive. Given that Notch family receptors and ligands were expressed and activated in germ cells, the next stage was to develop an experimental system to investigate their functional role.

Chapter 4. Establishment of ES cell-based model for studying Notch pathway in spermatogenesis

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4.1 Introduction to the chapter

4.1.1 Derivation of germ cells from ES cells (build-up from previous model)

ES cells are known for their capacity to self-renew and their ability to differentiate spontaneously to various cell lineages including the germ line. Previous studies had shown that mouse ES cells could differentiate *in vitro* into primordial germ cells (PGCs) as well as into male and female gametes (Hubner et al., 2003, Kehler et al., 2005, Geijsen et al., 2004). In 2006 a study by Nayernia and colleagues reported the generation of mature male gamete from mouse ES cells that were capable to generate viable transgenic offspring (Nayernia et al.).

Nayernia and colleagues exploited the fact that many markers of male germ cells had been characterised at that time. So, first they used the pre-meiotic promoter of *Stra8* gene to drive the expression of an eGFP reporter construct, and then a second post-meiotic promoter *Prm1* gene to drive the expression of a DsRED reporter (Figure 20). These germ cell-specific-promoter constructs were transfected into mouse ES cells and were used to identify and isolate pre-meiotic-like cells (spermatogonia) and post meiotic-like cells (spermatids) using their novel ES cell differentiation protocol. DsRED positive cells (spermatid-like cells) were then isolated by FACS. These were reported to be able to fertilise mouse oocytes after intracytoplasmic injection, and after implantation of two-cell embryos into oviducts, resulted in live born mice, of which six of seven animals developed into adult mice.

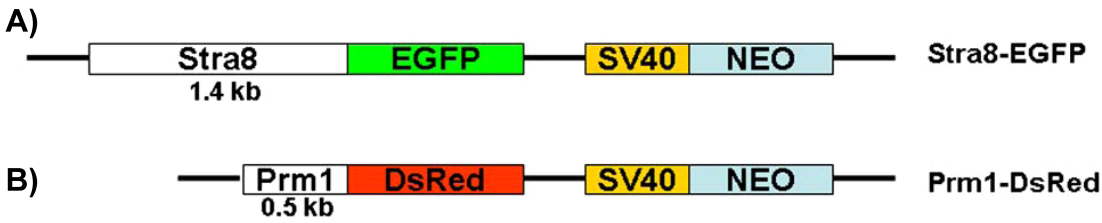


Figure 20. Schematic representation of the *Stra8*-eGFP (A) and *Prm1*-DsRED (B) reporter genes used in Nayernia's study. The germ cell specific promoter regions can activate the expression of the reporter gene expression specifically in pre-meiotic and haploid male germ cells, respectively. Both reporter genes contain neomycin phosphotransferase II gene (NEO), which is driven by the SV40 early promoter and enhancer (SV40). Diagram adapted from (Nayernia et al., 2006).

In an attempt to generate an *in vitro* platform for the investigation of Notch signalling components in spermatogenesis, the strategy used by Nayernia et al was further exploited and evolved in this study.

4.1.2 Aim of the model

The aim was to adopt the already established *in vitro* ES cell-based model that had been shown to produce functional post-meiotic-like haploid germ cells, (and therefore to recapitulate to some extent the *in-vivo* conditions of mouse spermatogenesis), and to use that protocol as a backbone to generate an ES cell-based model system that would allow the identification and isolation of early and late meiotic-like germ cells. This model would then permit me to investigate meiotic spermatogenic stages, and offer a more accessible system for the examination of the role of Notch components during the process of spermatogenesis. In an attempt to generate an *in vitro* platform for the investigation of Notch signalling components in spermatogenesis, the strategy used by Nayernia et al was further exploited and evolved.

4.2 Experimental strategy

4.2.1 Established ES cell based approach

The culture system used by Nayernia and colleagues to demonstrate that ES cells are able to differentiate to mature sperm involved two stages. In the first stage, mouse ES cells were transfected with *Stra8*-eGFP reporter gene construct harbouring the coding region of enhanced green fluorescent protein (eGFP) under the control of the mouse *Stra8* promoter region (Oulad-Abdelghani et al., 1996) (Figure 20 A). *Stra8* is a retinoic acid responsive gene that is expressed in pre-meiotic germ cells (Baltus et al., 2006). Male mutant mice for *Stra8* are infertile as pre-meiotic spermatocytes cannot enter prophase of meiosis I (Anderson et al., 2008). Previous studies showed that *Stra8* promoter could direct the expression of a reporter gene (*Stra8*-eGFP) specifically in pre-meiotic germ cells (Nayernia et al., 2004).

Having established ES cells harbouring the *Stra8*-eGFP construct by selecting them using the *neomycin phosphotransferase II* gene (*Neo*), which was driven by the SV40 early promoter in the reporter gene, cells where then cultured in the presence of RA. RA is known to sustain the survival and self-renewal of mouse germ cells in the absence of somatic cell support, and also to promote the developmental progression of spermatocytes through early stages of meiosis (Koubova et al., 2006). After 10 days of RA induction, eGFP positive cells, (later shown by RT-PCR and immunohistochemistry to express pre-meiotic germ cell markers), were isolated by FACS. Sorted cells were cultured in undifferentiated conditions for 8-10 weeks and then after 12 hrs induction of RA eGFP positive cells were sorted for a second time by flow cytometry.

In the second stage, eGFP positive cells were transfected with a second gene construct that contained the promoter region of the *protamine 1* (*Prm1*) gene fused to red fluorescent protein gene (*Prm1*-DsRED) (Figure 20 B). The *Prm1* promoter region is known to be exclusively active in postmeiotic male germ cells (Zambrowicz et al., 1993). Following transfection with the *Prm1*-DsRED construct, positive colonies where then selected by PCR using primers specific for the DsRED coding region. After a further RA induction for 72 hrs DsRED positive cells that arose from eGFP expressing cells were released in the medium and could be collected from the supernatant (Figure 21). Using this strategy Nayernia et al (2006) were able to generate and isolate post-meiotic spermatid-like cells and injected them into mouse oocytes which in turn gave rise to full-term development embryos and subsequently to viable animals that carried the transgenic allele.

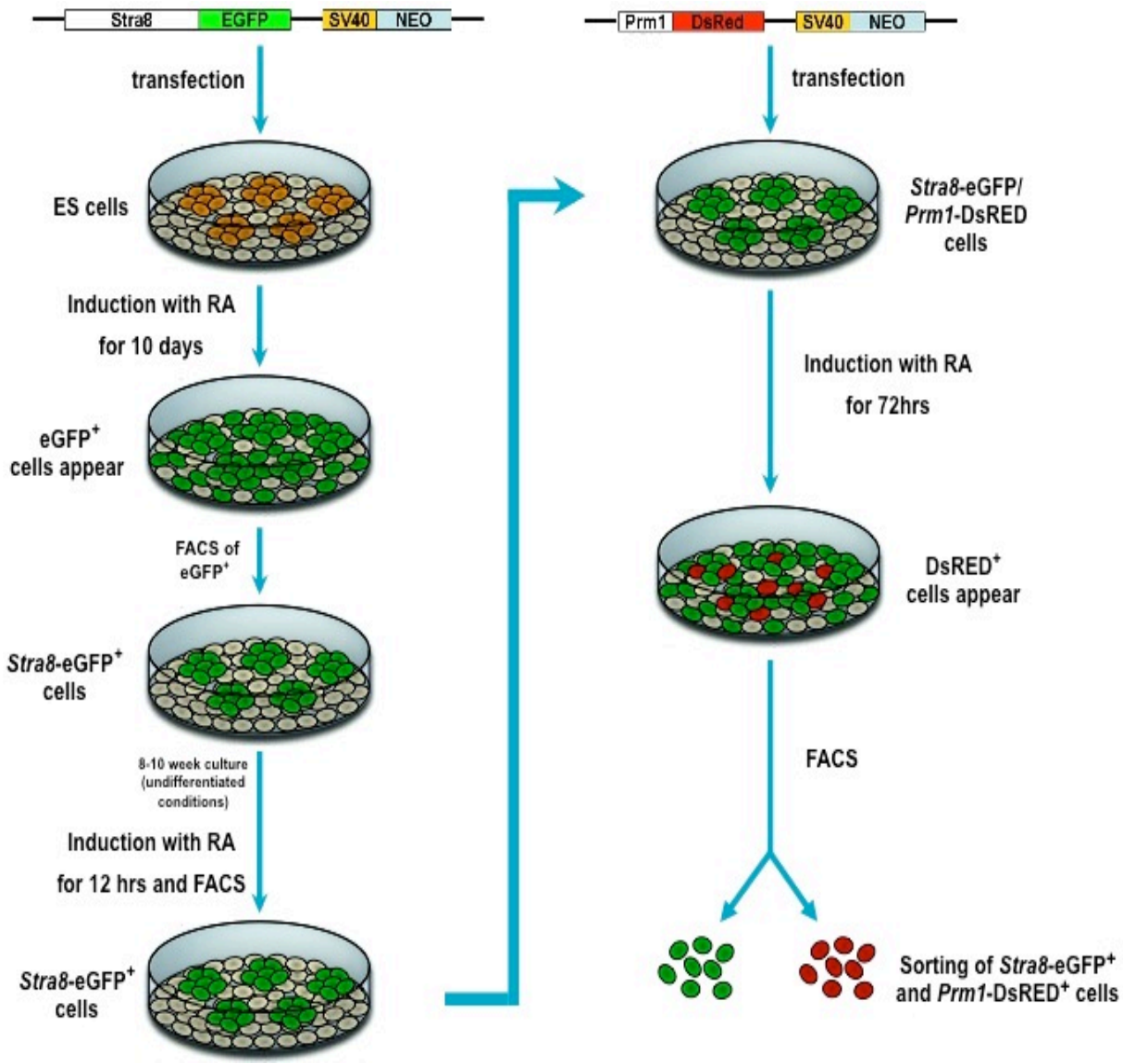


Figure 21. Nayernia's experimental protocol. *Stra8*-eGFP transfected ES cells were induced with RA for 10 days and eGFP positive cells were isolated by FACS. After 8-10 weeks in culture, cells were induced again with RA for 12 hrs and eGFP positive cells isolated by FACS. *Stra8*-eGFP positive cells were then transfected with the second construct *Prm1*-DsRED and further induced with RA for another 72 hrs. After the RA induction *Prm1*-DsRED positive cells appeared which indicated the progression of pre-meiotic cells to post-meiotic stage.

4.2.2 Development of evolved ES cell-based model for the derivation of pre-meiotic, meiotic and post-meiotic germ cells

Although Nayernia's model provided a platform to examine the effect of Notch components during *in vitro* generation of post-meiotic-like cells, intermediate differentiation and maturation steps of spermatogenesis were not accessible. To achieve an ES cell-based model system that could allow the investigation of all major stages of

spermatogenesis (pre-meiosis, meiosis, post-meiosis) the ESC-derived germ cell strategy was re-designed and re-developed. The pre-meiotic and post-meiotic germ cells markers *Stra8* and *Prm1* used previously were exploited again but this time they were combined with known meiotic germ cells markers.

To develop a platform that would provide access to the transition phase of pre-meiosis to meiosis and early and late meiosis the promoter regions of *Synaptonemal Complex Protein 1 (Sycp1)* and *Phosphoglycerate kinase 2* (*Pgk2*) *genes* were selected. Both genes are exclusively active in meiotic male germ cells. SYCP1 protein can be found in the early meiotic stage (leptotene to zygotene spermatocytes) (Sage et al., 1999) and it is a major component of the synaptonemal complexes formed between homologous chromosomes during meiotic prophase (Costa et al., 2005). The *Pgk2* gene on the other hand, has been shown to be expressed at late meiotic stages (Robinson et al., 1989, McCarrey and Thomas, 1987) and the kinase activity of PGK2 is critical to normal motility and fertility of mammalian spermatozoa (Yoshioka et al., 2007).

Thus, combining the pre-meiotic and post-meiotic germ cell markers *Stra8* and *Prm1* with the early and late meiotic germ cell markers *Sycp1* and *Pgk2*, enabled the study of four different spermatogenic-like stages upon *in vitro* differentiation of ES cells to germ-like cells. A summary of the re-designed ES cell-based model is shown in Figure 22.

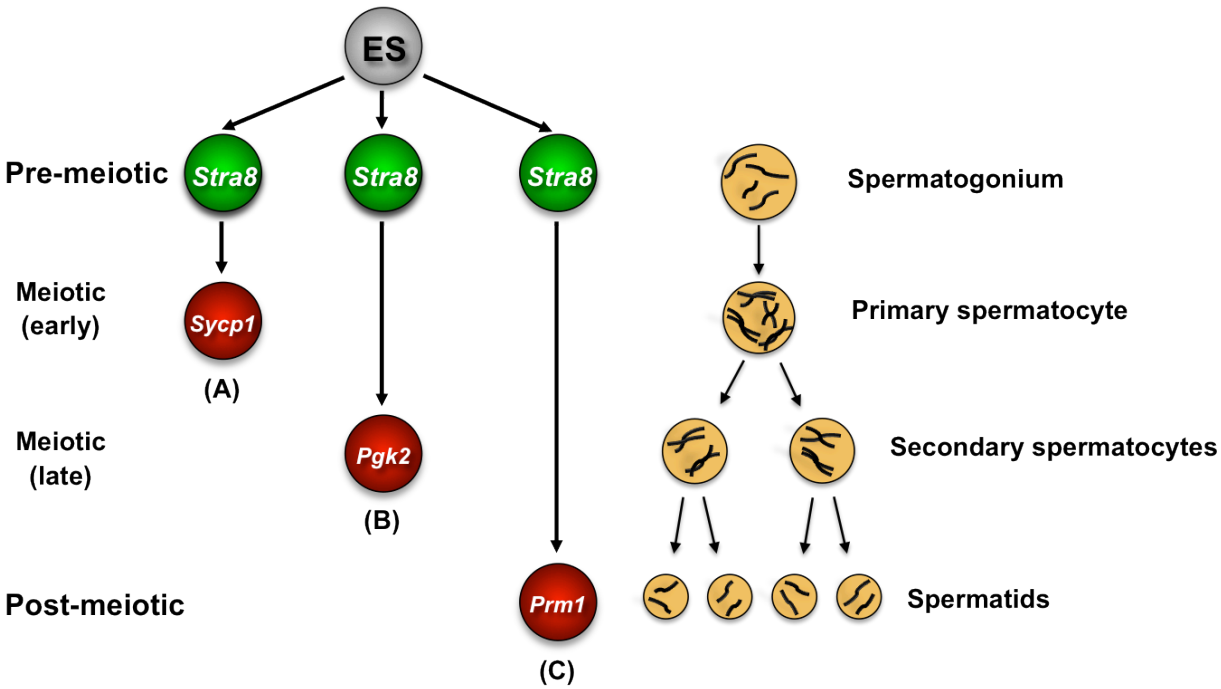


Figure 22. Summary of the ES cell-based model used in our study. Establishment of stable transfected cell lines with expression of different fluorescence proteins (green, red) at different stages of *in vitro* spermatogenesis. (A) *Stra8*-eGFP/*Sycp1*-DsRED (B) *Stra8*-eGFP/*Pgk2*-DsRED and (C) *Stra8*-eGFP/*Prm1*-DsRED. On the right, diagram of spermatogenic progression.

4.3 Generation of reporter constructs and establishment of stable transfected cell lines

The first requirement for the establishment of the ES cell-based *in vitro* model was to generate the meiotic reporter constructs. The *Prm1*-DsRED vector used in the Nayernia study was ready available in the lab therefore it was decided to extract the *Prm1* promoter from the vector with the restrictions sites *XhoI* and *HindIII* and replace it with the other meiotic promoters.

4.3.1 Cloning of Sycp1 and Pgk2 promoters in the DsRED-Express-1 vector

The promoter sequence of *Sycp1* had been characterized and the upstream DNA fragment -722, +102 had been shown to drive the expression of reporter genes directed to pachytene spermatocytes in transgenic mice (Sage et al., 1999). PCR primers flanking the characterized DNA fragment were used to amplify the promoter sequence from mouse genomic DNA (Figure 23 A) with restriction sites *XhoI* and *HindIII* introduced in 5′ and 3′ ends of the amplicon. Both plasmid (DsRED-Express-1) and amplified DNA fragments were double digested with *XhoI* and *HindIII* restriction enzymes and the promoter sequence was ligated to the DsRED vector. The ligation was dialyzed, and 1µl was transformed into *E. coli* DH5α by electroporation (see Materials and Methods sections 2.1.6 - 2.1.9). The transformation mix was then plated out on LB agar plates containing the selective antibiotic kanamycin and incubated at 37ºC overnight. Next day colonies were picked up and PCR screened using the primers used to amplify the promoter fragment from genomic DNA. Insert positive colonies were further grown in LB-kanamycin overnight, and next day the plasmid was purified and digested with the same restriction enzymes (*XhoI* and *HindIII*) used to ligate the promoter to verify the size of the insert (Figure 23 B). Plasmids containing the insert were then sent for DNA sequencing to check for mutations and the appropriate plasmid was selected.

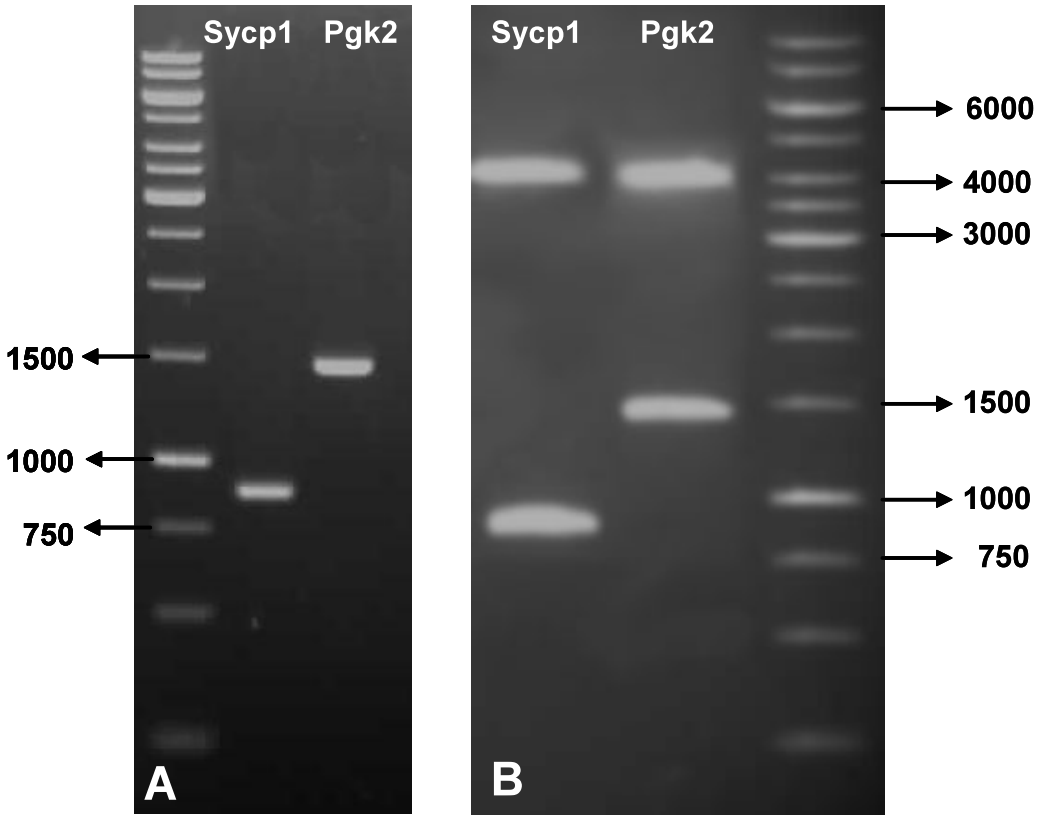


Figure 23. Images from agarose gel electrophoresis (A) Sycp1 and Pgk2 amplified PCR DNA products. (B) Double digestion of *Sycp1*-DsRED and *Pgk2*-DsRED plasmids with *XhoI*/*HindIII* to verify the successful cloning of the promoter sequences to the plasmids. The bands on the top of each column represent the linearized DsRED vectors (4.1kb) and at the bottom the inserted promoter DNA fragments (expected sizes *Sycp1* ~900bp, *Pgk2* ~1400bp).

The transcription regulatory elements of the human *Pgk2* gene had previously been defined (Zhang et al., 1999, Robinson et al., 1989) and it had been shown that a 1.4kb upstream sequence (-1445, -5) could drive the expression of a reporter gene in transgenic mice (Tascou et al., 2001). A bluescript plasmid harbouring the human *Pgk2* promoter sequence was available in the lab. PCR primers flanking the characterized DNA fragment were used to amplify the promoter sequence from the bluescript plasmid (Figure 23 A) and introduce the restriction sites *XhoI* and *HindIII* in 5′ and 3′ ends of the amplicon. Both plasmid (DsRED-Express-1) and amplified DNA fragment were double digested with *XhoI* and *HindIII* restriction enzymes, and the promoter sequence was ligated to the multiple cloning site (MCS) (*XhoI*/*HindIII*) of the DsRED vector. After that, the same procedure as *Sycp1* promoter was followed (dialysis, transformation into *E. coli*, plating, colony screening, plasmid purification and restriction digest testing (Figure 23 B) and DNA sequencing).

The plasmid maps for *Sycp1*-DsRED and *Pgk2*-DsRED vectors are shown in Figure 24.

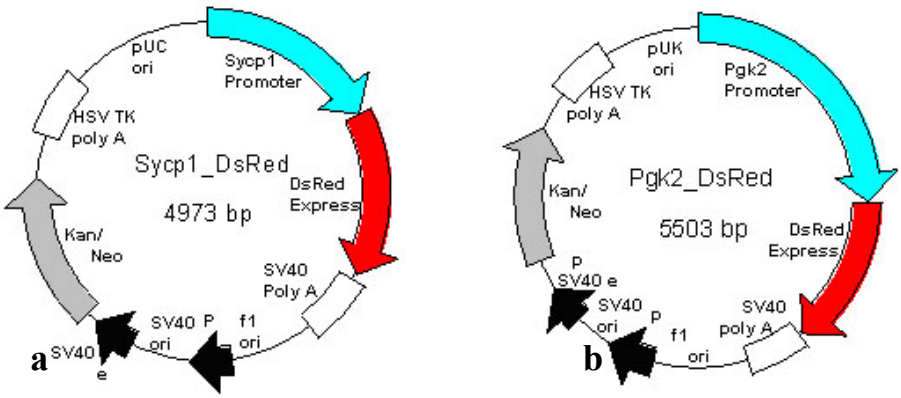


Figure 24. Plasmid maps for (a) *Sycp1*-DsRED and (b) for *Pgk2*-DsRED vectors. (A) The *Sycp1* promoter (866bp) was inserted at the multiple cloning site of the vector to generate a 4.9kb vector. (B) For the *Pgk2*-DsRED construct (5.5kb), 1396bp of *Pgk2* specific promoter sequence was inserted.

4.3.2 Cloning new antibiotic resistant gene into Stra8-eGFP vector

Previously, in Nayernia's study both eGFP and DsRED vectors carried the same antibiotic resistance gene (*neomycin phosphotransferase II)*. This had as a consequence to prohibit the antibiotic selection of DsRED positive ES cell colonies sinces cells had already been transfected with the *Stra8*-eGFP plasmid. To overcome that obstacle Nayernia and colleagues were forced, after transfection, to pick random colonies and screen them by PCR with primers amplifying the *Prm1*-DsRED fragment.

This approach however, was not only very laborious but it was also dropping any selective advantage that antibiotic selection would add. For example, when you transfect a construct in ES cells the linearized vector will randomly integrate in the genome of an ES cell. The more constructs you have integrated in the genome of an ES cell (clone) the more antibiotic resistant that clone (ES cells originated from a single cell) will be and moreover, the more bright the cells will glow. This is because, there will be more reporter constructs inside the cells and therefore, more fluorescent protein will be produced, rendering the clone ideal for the differentiation analysis.

The *Stra8*-eGFP vector was available in our lab so, to maintain this antibiotic selective advantage for our study, the antibiotic resistance gene (*Neo*) of the *Stra8*-eGFP vector was replaced by the *Pac* gene that encodes a puromycin *N*-acetyl-transferase (PAC) (Figure 25).

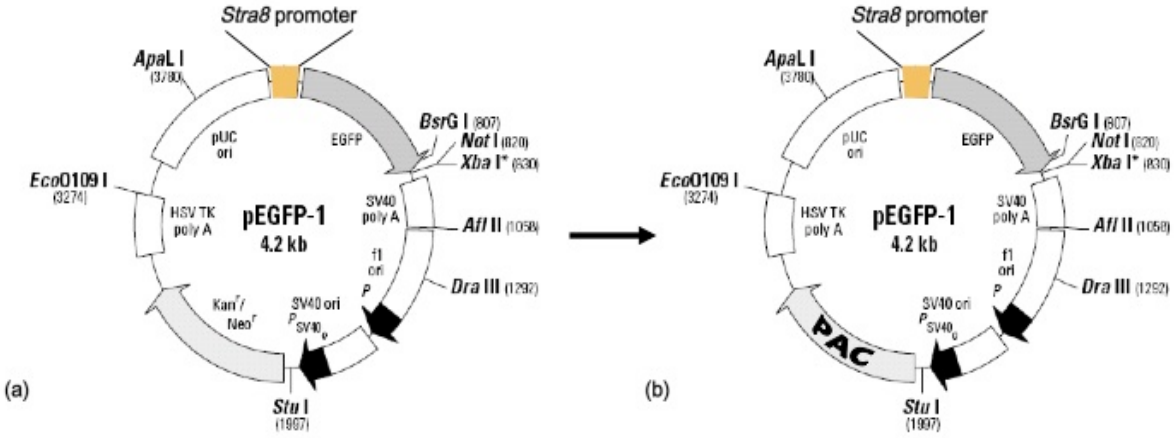


Figure 25. Plasmid maps for (a) *Stra8*-eGFP-*Neo* and (b) *Stra8*-eGFP-*Pac* vectors. The *Neomycin phosphotransferase II* gene (*Neo*) was replaced with the *puromycin N-acetyl-transferase gene* (*Pac*).

In order to achieve the replacement of the *Neo* gene, two identical unique restriction sites (in this case for *KpnI* restriction enzyme) flanking the *Neomycin* cassette had to be introduced. This was done by designing mutagenic oligonucleotide primers that annealed to the same sequence on opposite strands on either site of the *Neomycin* cassette of the *Stra8*-eGFP1-*Neo* plasmid. High fidelity DNA polymerase was used to prevent the generation of new mutations during the plasmid amplification. After DNA (plasmid) amplification, the PCR reaction, containing the parental plasmid (template) and the newly formed un-methylated plasmid (product), was incubated with a restriction enzyme that digested only methylated DNA (template) and left intact the newly formed un-methylated plasmid. The un-methylated plasmid was then purified and digested with the restriction enzyme for which unique restriction sites for *KpnI* had been introduced (Figure 26 a).

The plasmid DNA band on the agarose gel without the *Neo* cassette insert was extracted and used for the ligation of the *Pac* gene to the plasmid. Ligations were dialysed and transformed in methyltransferase deficient heat-shock competent *dam*$^-$/*dcm*$^-$ *E. coli*

suitable for growth of plasmids free of Dam and Dcm methylation. The transformation mix was then plated out on LB agar plates containing the selective antibiotic puromycin and incubated at 37ºC overnight. Next day colonies were picked up and PCR screened using primers for the *Pac* gene. Insert positive colonies were further grown in LB-puromycin overnight, and next day the plasmid was purified and digested with *KpnI* restriction enzyme to verify the size of the plasmid and insert (Figure 26 b). Plasmids containing the insert were then sent for DNA sequencing to check for mutations and the appropriate plasmid was selected (for details see Materials and Methods section 2.1.15).

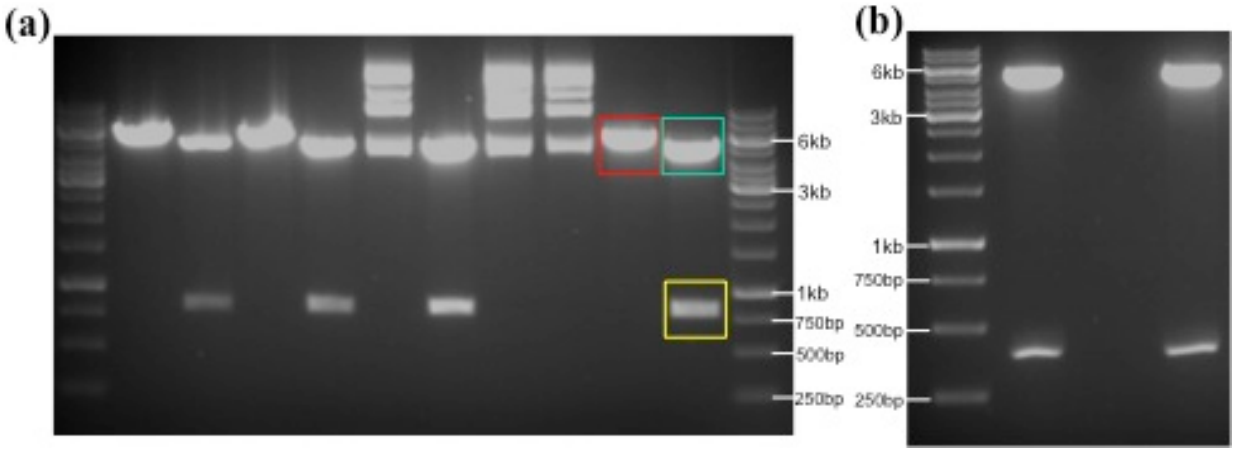


Figure 26. (a) Site-directed mutagenesis screening. After PCR amplification newly formed un-methylated plasmid was digested with *KpnI* restriction enzyme to test that the restriction sites had been introduced to the DNA sequence. Then plasmid (DNA band in green square) without the *Neo* cassette insert (yellow square) was extracted from the agarose gel and used for cloning the *Pac* gene. In the red square linearized plasmid but without insert meaning both restriction sites were not introduced. (b) Screening purified plasmid for successful cloning after digestion with *KpnI* restriction enzyme. The upper band is the digested plasmid without insert and the lower band *Pac* cassette (insert) which signify a successful cloning.

4.3.3 Establishment of Stra8-eGFP-Pac stable ES cell line

ES cells are known for their capacity to self-renew when cultured under undifferentiated conditions (on a layer of MEFs and in the presence of LIF), and also their ability to differentiate into multiple cell lineages including the germ line. Differentiation studies have shown in practise that some ESC lines have a selective tendency for a particular cell lineage upon differentiation and so are preferred from others (Keller, 2005). To avoid, any variation of this kind between ES cell lines, the exact same ES cell line (R1) used in Nayernia's study (2006) was decided to be used in this study (Figure 27).

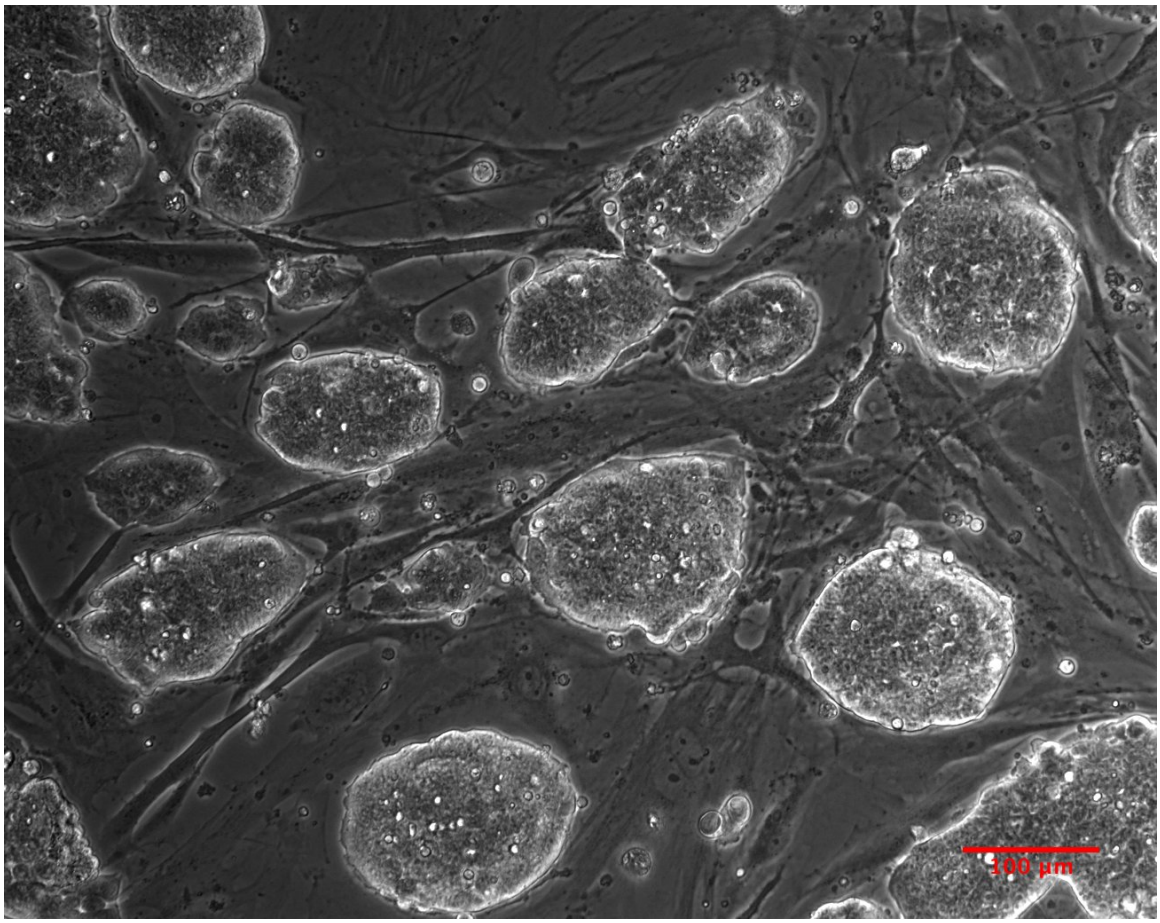


Figure 27. Brightfield image of R1 ES cells cultured on a layer of MEFs under undifferentiating conditions.

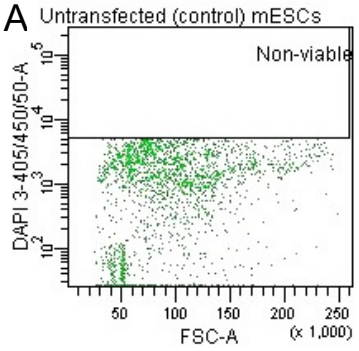
4.3.3.1 Optimization of transfection protocol

To introduce the *Stra8*-eGFP-*Pac* construct into the genome of the ES cells the well-known transfection method that uses electroporation technology was used. In particular, a commercially available device called "Amaxa™ Nucleofector™" which involves a proprietary combination of optimized electrical parameters (programs) that permit the delivery of DNA of interest straight into the nucleus. However, the electrical parameters used for transfection of stem cells vary depending on the cell line. So, in order to determine the parameters suitable for our ES cell line, meaning high efficiency in DNA delivery and also low cell death, four different programs given by the manufacturer were tested (Figure 28). Each program had to be assessed for the efficiency of the transfection (% of GFP positive cells) and the survival rate of cells after the transfection. Five different cell samples were used. Four were transfected with GFP-MAX plasmid (provided in the AMAXA kit) that contains a constitutive promoter for expressing GFP,

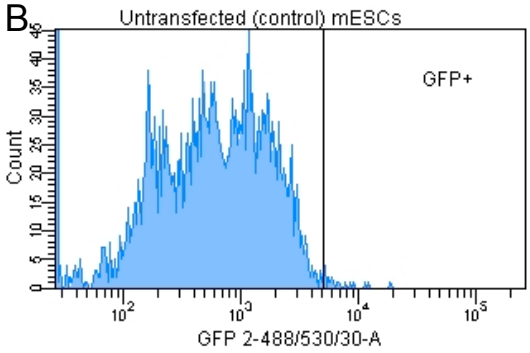
and one cell sample was used as control (non-transfected). Transfections were performed using a different recommended program for each cell sample. 24 hrs later the GFP expression of the cells was examined by FACS analysis. Moreover, to determine how many cells survived after transfection, each cell sample was stained with 4',6-diamidino-2-phenylindole (DAPI) prior to FACS.

I.

A



B



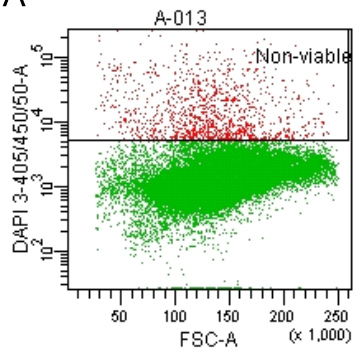
C

Tube Name: Untransfected (control) mESCs

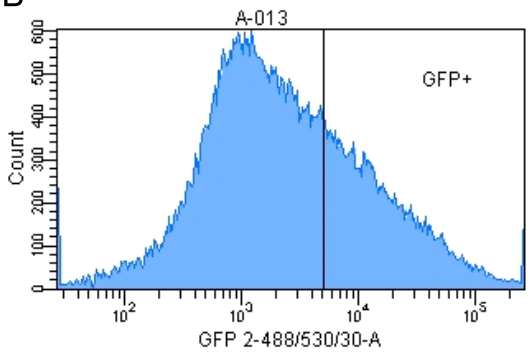
Population	%Parent	%Total	GFP 2-488/530/... Mean
Non-viable	0.0	0.0	####
Viable	100.0	88.2	169
GFP+	0.3	0.0	6,572

II.

A



B



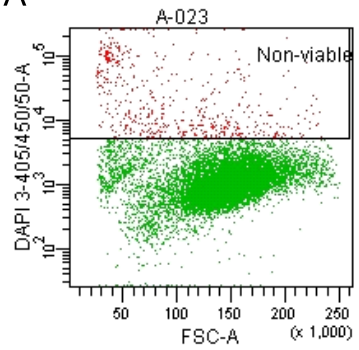
C

Tube Name: A-013

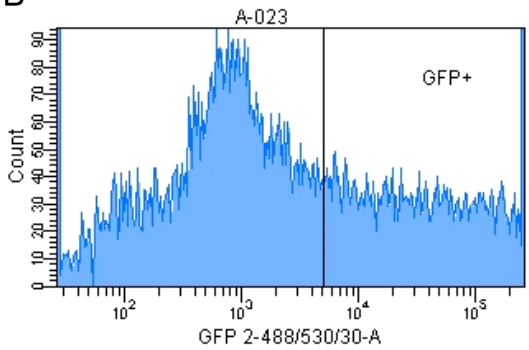
Population	%Parent	%Total	GFP 2-488/530/... Mean
Non-viable	2.2	1.8	12,291
Viable	97.8	79.6	9,831
GFP+	31.4	25.0	27,963

III.

A



B



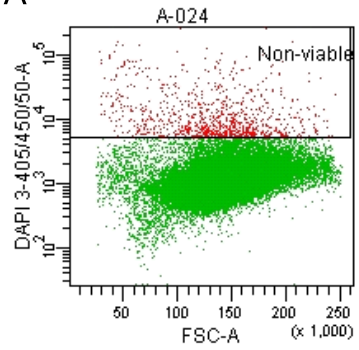
C

Tube Name: A-023

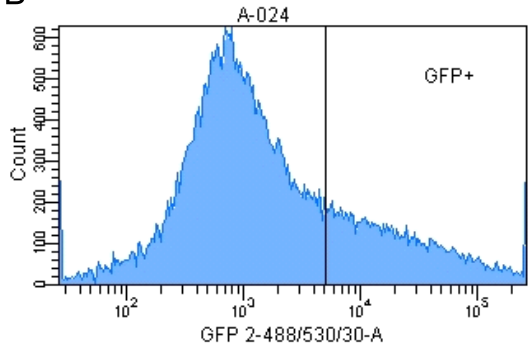
Population	%Parent	%Total	GFP 2-488/530/... Mean
Non-viable	5.0	1.9	44,016
Viable	95.0	35.2	32,053
GFP+	38.6	13.6	81,243

IV.

A



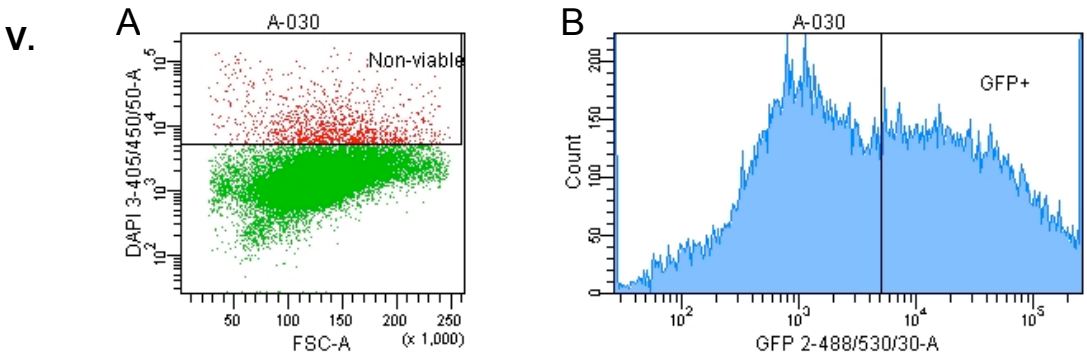
B



C

Tube Name: A-024

Population	%Parent	%Total	GFP 2-488/530/... Mean
Non-viable	2.0	1.6	21,884
Viable	98.0	77.2	11,162
GFP+	24.3	18.8	42,343



C

Tube Name: A-030

Population	%Parent	%Total	GFP 2-488/530/... Mean
Non-viable	5.0	3.3	49,421
Viable	95.0	63.8	28,062
GFP+	48.1	30.7	56,893

Figure 28. Optimisation of electroporation protocol. Testing different "programs" by FACS analysis (I) Non-transfected cells were used as control to set the gate for GFP positive cells. (II) Program A-013 (III) Program A-023 (IV) Program A-024 and (V) Program A-030. (A) Dot plot graph showing the fraction of viable and non-viable cells after incubation with DAPI (a fluorescent stain that binds strongly to A-T rich regions of DNA in dead cells). On the X axis is the Forward light scatter (FSC) relative to the cell size and on the Y axis the DAPI fluorescence intensity in logarithmic scale. The horizontal line separates the viable from the non-viable cells. (B) Histogram showing GFP positive cells. The X axis soes GFP fluorescence intensity and the Y axis represents the cell count. The vertical line indicates the point over which cells are considered GFP positive (C) Summary of the percentages for viable, non-viable and GFP positive over the recorded and total number of cells.

FACS analysis showed that although variation in the survival rate between the programs was very low (95 to 98%) the transfection efficiency varied significantly. Specifically, cells transfected with the A-030 program exhibit 48.6% of GFP positive cells over the viable cell population, therefore this program was selected for subsequent transfection experiments (Figure 28 V).

4.3.3.2 Determination of puromycin concentration

Different cell lines have different sensitivity to puromycin, therefore, the optimal concentration of the selective antibiotic required to maintain and select cells had to be determined for the culture conditions used. So, to establish the optimal concentration a kill curve assay was performed for the R1 ES cell line. Different concentrations of puromycin were added to non-transfected cells and the number of the surviving colonies

was counted after 6 days. The lowest amount of puromycin that killed all the cells within 6 days was used for the antibiotic selection (in this case 1μg/ml (Figure 29)).

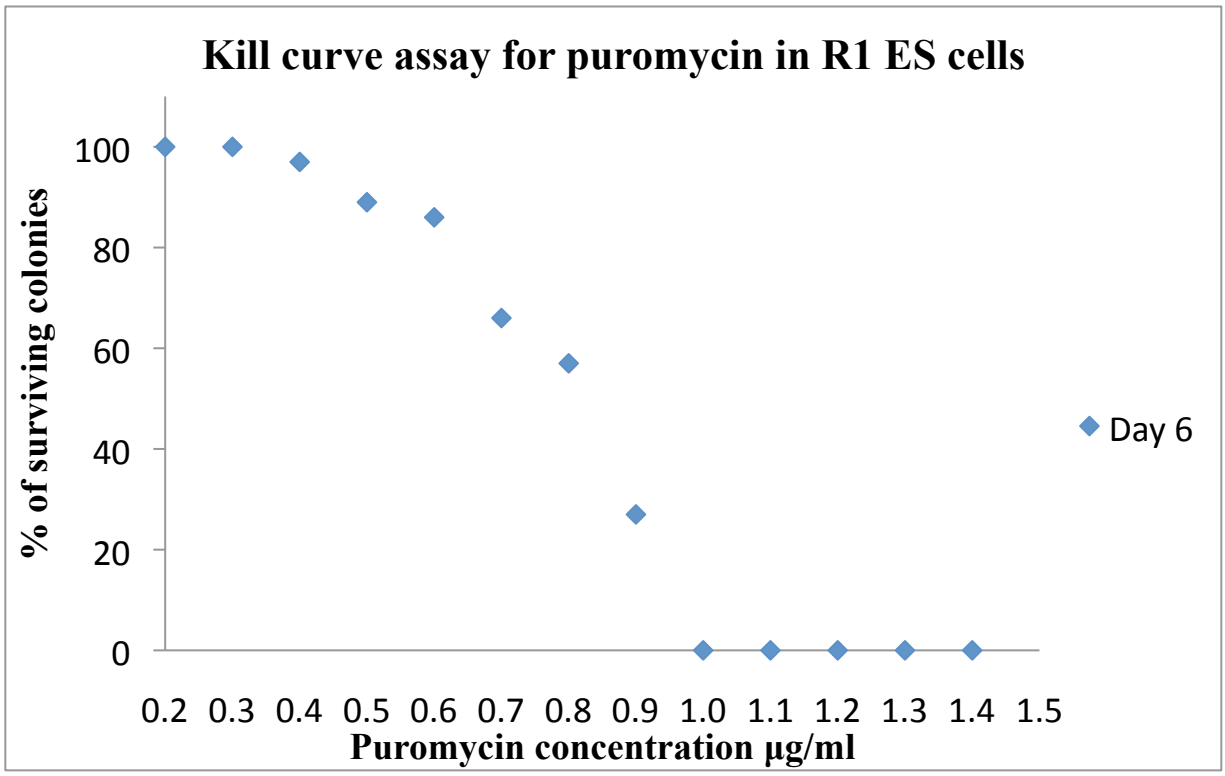


Figure 29. Kill curve assay for determining the optimal puromycin concentration for antibiotic selection.

4.3.3.3 Selection of Stra8-eGFP-Pac positive clones

Having established the transfection parameters and the selective antibiotic concentration the ES cells were transfected with the *Stra8*-eGFP-Pac plasmid and after 24 hrs puromycin antibiotic was introduced to the media (see Materials and Methods section 2.4.3). After 7 days, 12 ES cell colonies/clones (originated from a single cell) were selected and transferred in a separate culture dish for further culture. To confirm existence of the *Stra8*-eGFP-Pac construct, DNA was extracted from each colony and tested by PCR using a forward primer recognizing the *Stra8* promoter sequence and a reverse primer specific for the eGFP DNA sequence. Agarose gel electrophoresis revealed that 7 out of 12 colonies contained the construct of interest (Figure 30).

Stable transfection of cells relies on the integration of the gene construct in to the genome of the cell. As this is a random event and the expression of the construct depends on the inserted position (affected by the heterochromatin or euchromatin enviroment) the only way to determine whether or not these ES cells would express eGFP was by testing their expression after the cells were allowed to differentiate. Therefore, four of these colonies (3, 8, 9 and 12) where selected for further investigation.

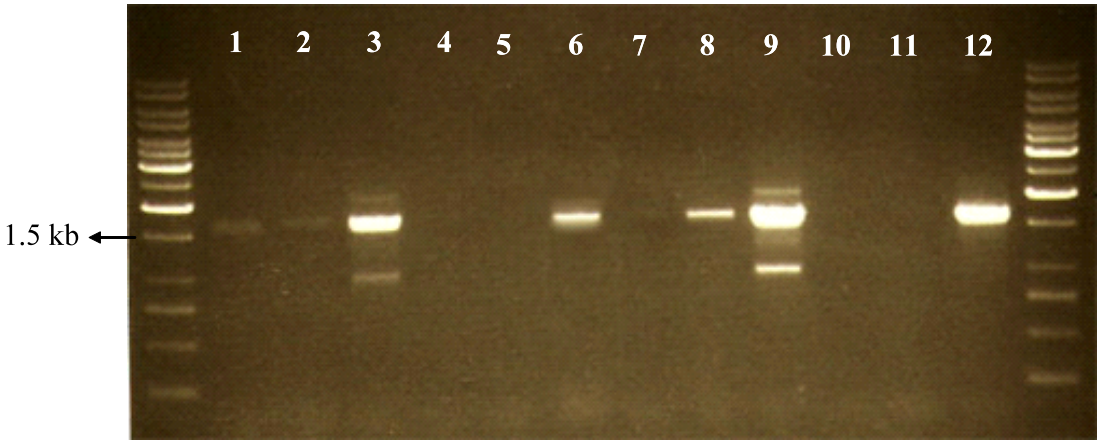


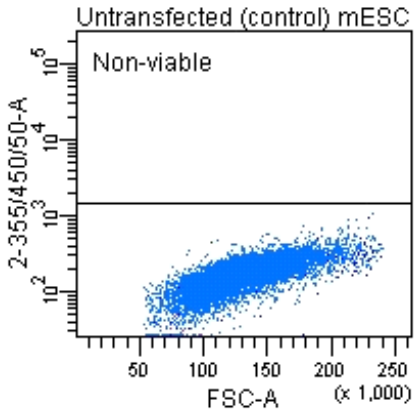
Figure 30. Image from agarose gel electrophoresis. Screening for positively transfected colonies showed that 7 out of 12 colonies contained *Stra8*-eGFP-Pac construct (1, 2, 3, 6, 8, 9, 12). Colonies 3, 8, 9 and 12 were selected for subsequent experiments.

4.3.3.4 Testing Stra8-eGFP-Pac positive clones for eGFP expression

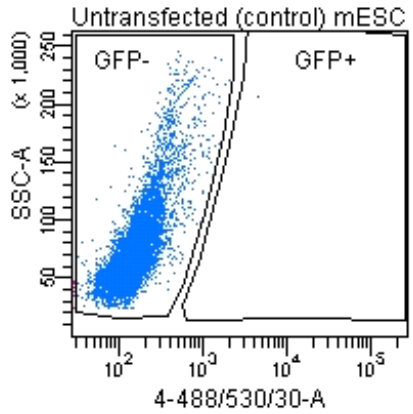
ES cell clones, shown to be resistant to puromycin antibiotic and which tested positive by PCR for the *Stra8*-eGFP-Pac construct, were allowed to differentiate in the absence of LIF and MEF but in the presence of RA. 72 hrs after RA induction, eGFP expression of the clones was analyzed by FACS. A sample of non-transfected cells was used as a control and to set the gate for GFP positive cells (Figure 31 I). In clones 3, 9 and 12 the percentage of GFP positive cells was below 1% of the total viable population (Figure 31 II, IV, V). In contrast, the fraction of GFP positive cells in clone 8 was dramatically higher than the other clones, and accounted for 47.4% of the viable population (Figure 31 III). The viability of cells between different samples did not show a significant fluctuation as all the clones displayed numbers above 99%. In the light of these results it was easy to select the clone for further experimentation and that was clone 8.

I.

A



B



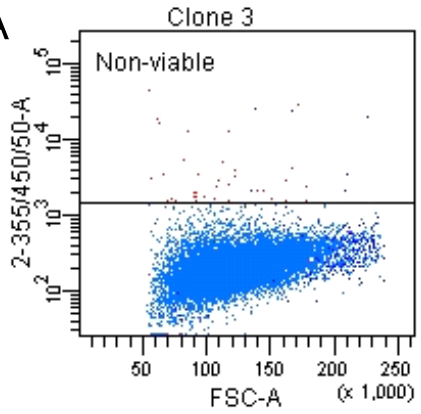
C

Tube Name: Untransfected (control) mESCs

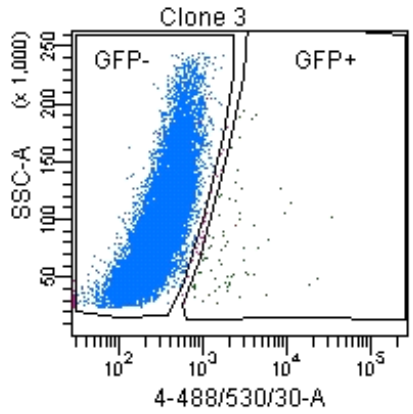
Population	%Parent	%Total	4-488/530/30-A Mean
Non-viable	0.0	0.0	####
Viable	100.0	88.2	169
GFP+	0.0	0.0	4,361

II.

A



B



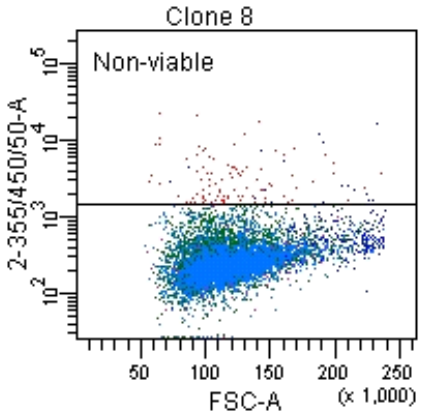
C

Tube Name: Clone 3

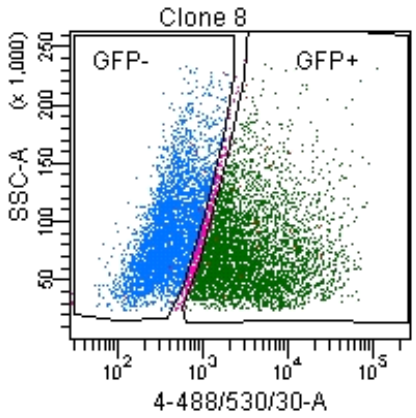
Population	%Parent	%Total	4-488/530/30-A Mean
Non-viable	0.2	0.2	428
Viable	99.8	89.7	319
GFP+	0.4	0.4	3,325

III.

A



B



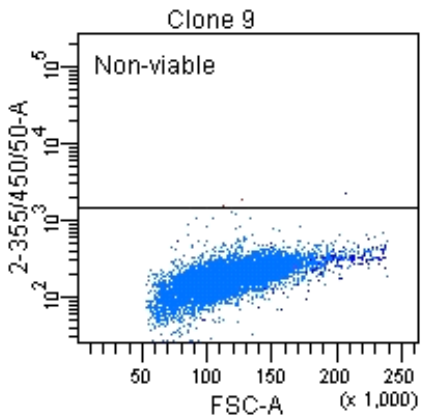
C

Tube Name: Clone 8

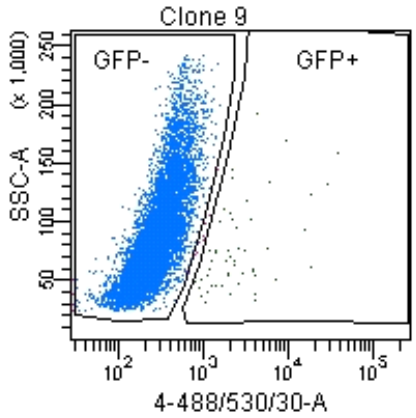
Population	%Parent	%Total	4-488/530/30-A Mean
Non-viable	1.0	0.9	3,725
Viable	99.0	89.7	3,150
GFP+	47.4	42.5	6,089

IV.

A



B



C

Tube Name: Clone 9

Population	%Parent	%Total	4-488/530/30-A Mean
Non-viable	0.0	0.0	399
Viable	100.0	89.6	327
GFP+	0.6	0.5	4,343

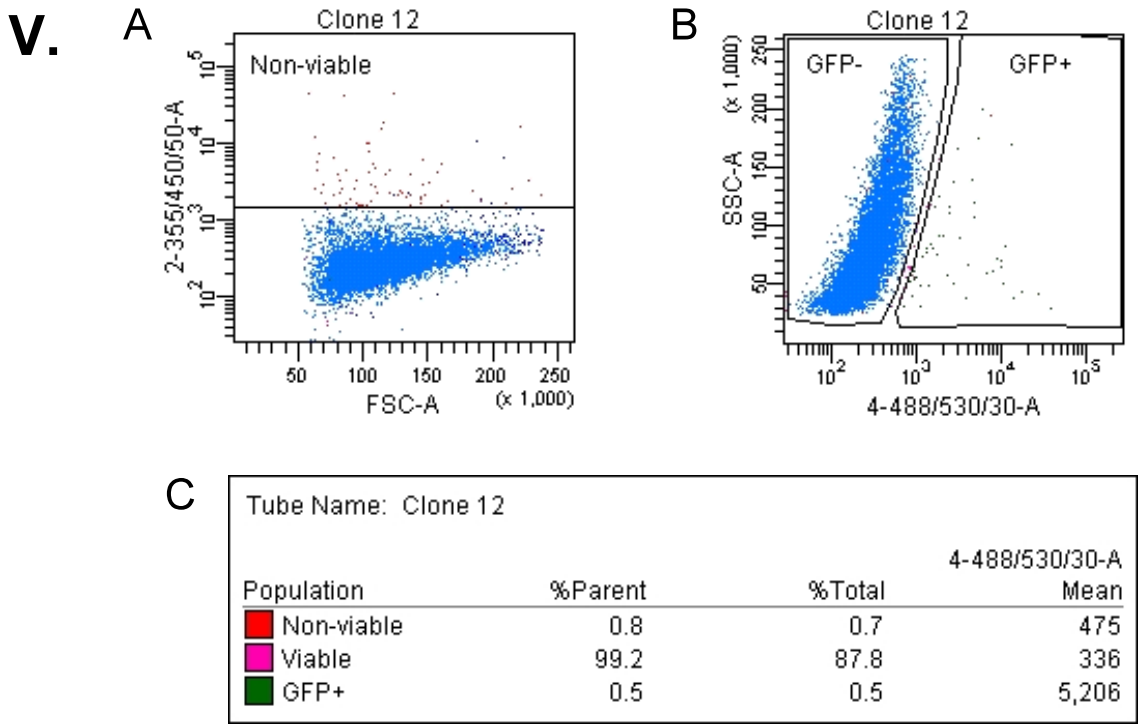


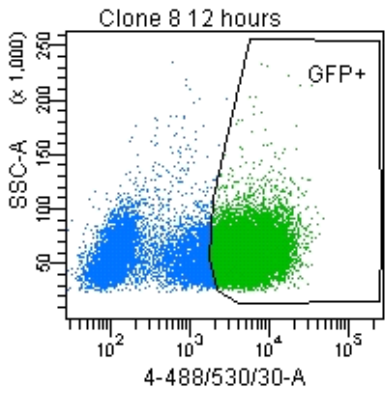
Figure 31. FACS analysis for eGFP expression after differentiation of *Stra8*-eGFP-Pac positive clones (A) Dot plot graph showing the fraction of viable and non-viable cells after incubation with DAPI. On the X axis is the FSC which is relative to the cell size and on the Y axis the DAPI fluorescence intensity in logarithmic scale. The horizontal line separates the viable from the non-viable cells. (B) Dot plot graph displaying negative and positive eGFP cells. The Y axis represents Side light Scatter (SSC) which is relative to cell granularity and the X axis eGFP fluorescence intensity (logarithmic scale). (C) Summary of the percentages for viable, non-viable and eGFP positive cells over the recorded and total number of cells. Mean fluorescent intensity values are shown on the right. (I) An untransfected cell sample was used as control to set the gate for GFP positive cells. (II) clone 3, (III) clone 8, (IV) clone 9, (V) clone 12. The percentage of GFP positive cells (47.4%) in clone 8 over exceeded the other three clones where the fraction of GFP positive cells did not overcome 0.6% and so clone 8 was selected for further experiments.

4.3.3.5 Induction of Stra8-eGFP-Pac clone 8 with Retinoic acid

Having selected the clone that expressed the highest percentage of eGFP positive cells, the next step was to induce the *Stra8*-eGFP-Pac cells with RA for 10 days. eGFP expression peaked after the first 48 hrs of RA induction (Figure 32) and remained at this level until day 10 (Figure 33). On the 10th, day eGFP positive cells were isolated by FACS and then cultured in undifferentiated conditions (MEFs, LIF) and in the presence or puromycin for 8-10 weeks.

I.

A



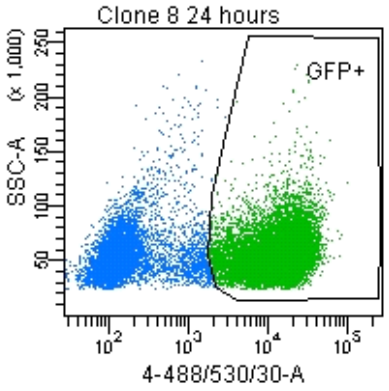
B

Tube Name: Clone 8 12 hours

Population	#Events	%Parent	4-488/530/30-A Mean
GFP+	11,699	59.7	6,249

II.

A



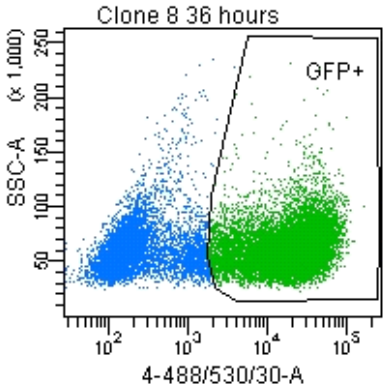
B

Tube Name: Clone 8 24 hours

Population	#Events	%Parent	4-488/530/30-A Mean
GFP+	13,013	67.8	15,253

III.

A

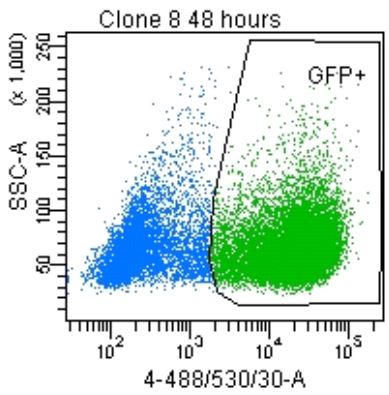


B

Tube Name: Clone 8 36 hours

Population	#Events	%Parent	4-488/530/30-A Mean
GFP+	13,292	68.1	24,694

IV. A



B

Tube Name: Clone 8 48 hours

Population	#Events	%Parent	4-488/530/30-A Mean
GFP+	13,483	69.7	25,293

Figure 32. FACS analysis results after induction of *Stra8*-eGFP-Pac cells (A) Dot plot graph showing the fraction of GFP positive cells (green). (B) Percentage of GFP positive over the recorded number of cells. Mean fluorescent intensity values are shown on the right *(I)* Cells 12 hrs after RA induction *(II)* Cells 24 hrs after RA induction *(III)* Cells 36 hrs after RA induction *(IV)* Cells 48 hrs after RA induction.

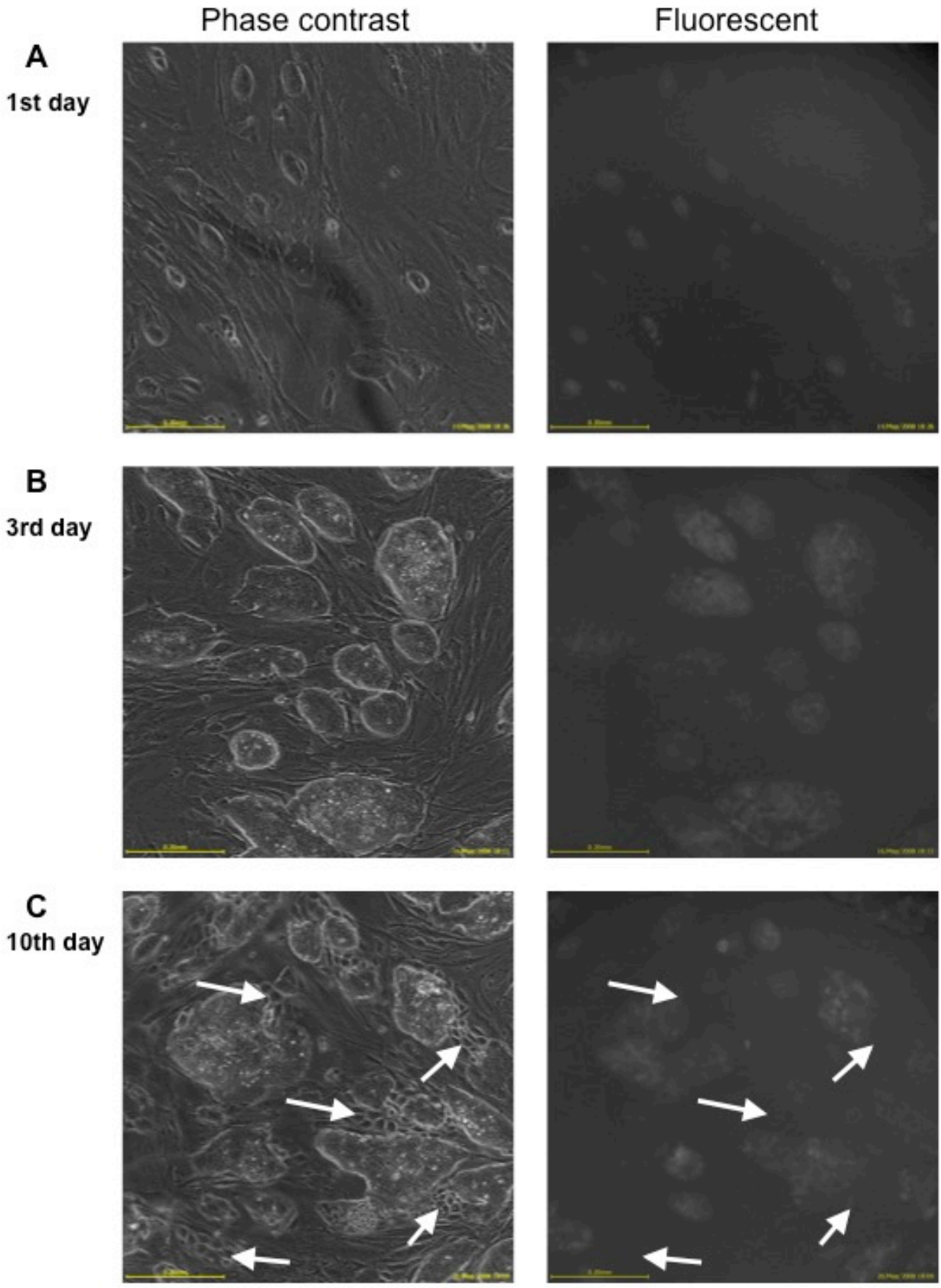


Figure 33. Phase contrast and fluorescent images of *Stra8*-eGFP-Pac cells during induction with RA. (A) after 24 hrs, (B) after 72 hrs and (C) after 10 days. Cells displayed properties of stem cell-like cells and maintained fluorescence activity through out the observation period. Only a small number of differentiated cells (white arrows) appeared at day 10. Bars are 200µm.

After 10 weeks of culture in undifferentiated conditions and in the presence of puromycin, cells were induced once more with RA (Figure 34), and after 12 hrs eGFP positive cells were isolated by FACS (Figure 35). Resistance of cells in puromycin and eGFP expression after 10 weeks of culture indicates the integration of *Stra8*-eGFP-Pac vector into the cells genome and therefore the establishment of a stable *Stra8*-eGFP-Pac cell-line.

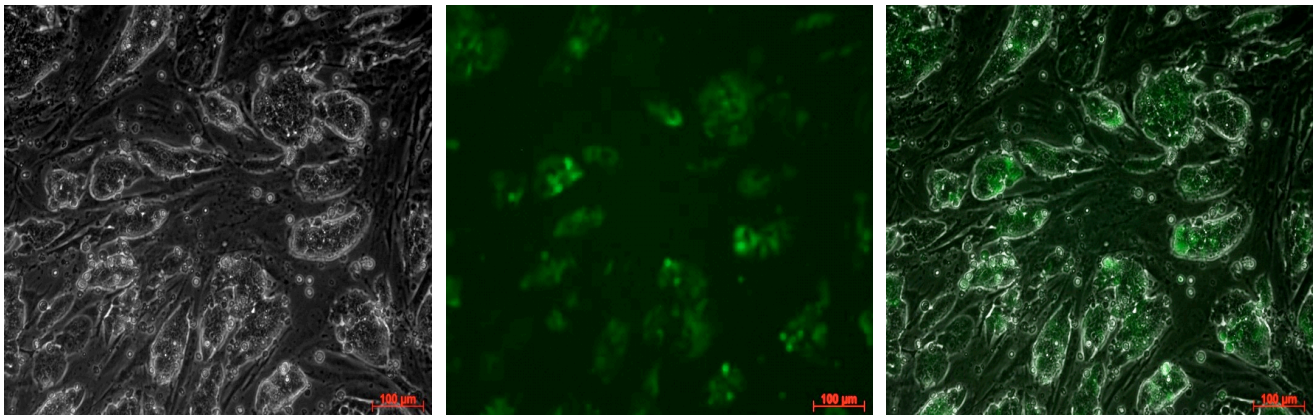
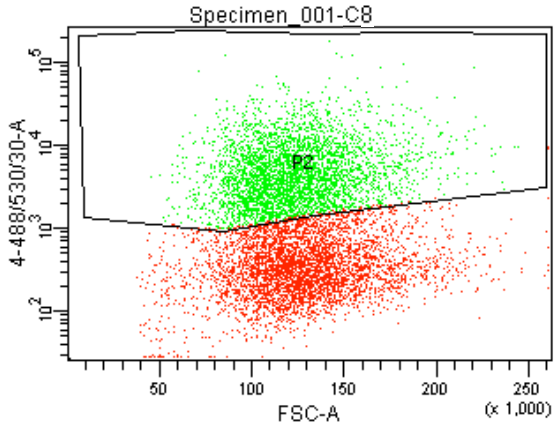
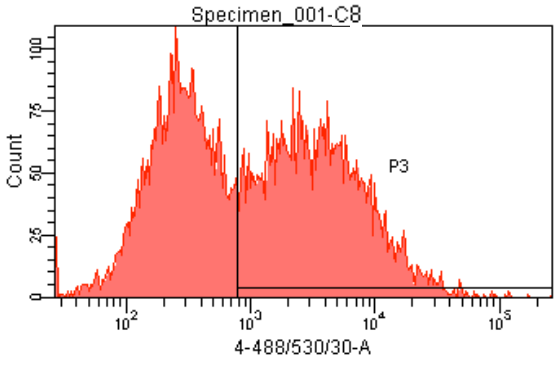


Figure 34. *Stra8*-eGFP-Pac cells after 12 hrs of RA induction express eGFP indicating the activation of the *Stra8* promoter in the cells.

a



b



c

Tube Name:	C8	
Population	#Events	%Parent
All Events	10,000	####
P1	8,401	84.0
P3	4,531	53.9

Figure 35. FACS results after 12 hrs of RA induction of *Stra8*-eGFP-Pac cells (a) Dot plot graph showing the fraction of eGFP positive cells (green), (b) Histograph showing the fraction of eGFP positive cells, P3 gate and (c) Percentage of eGFP positive over the recorded number of cells.

4.3.4 Expression analysis of eGFP positive cells after second RA induction

Stra8 is a pre-meiotic specific gene expressed postnatally, in the spermatogenic lineage (spermatogonia) and their immediate descendants (pre-leptotene spermatocytes), the most advanced cell type before meiotic prophase. Previous studies have shown that meiosis is regulated through retinoic acid induction of *Stra8* (Mark et al., 2008) and also that *Stra8*-deficient male and female mice with the same genetic background are infertile (Anderson et al., 2008).

Knowing that the expression of eGFP was under the control of the *Stra8* promoter and having the indication that the *Stra8* promoter was active (eGFP positive cells), the

expression of pre-meiotic markers was analysed in eGFP positive cells after the second induction of RA by RT-PCR. As the *Stra8*-eGFP-Pac cells were cultured on a layer of MEFs a sample of just MEFs was also included in the analysis as control. RT-PCR analysis confirmed the presence of pre-meiotic genes expressed in eGFP positive cells implying that eGFP positive cells have started acquiring characteristics of spermatogonia-like cells (Figure 36).

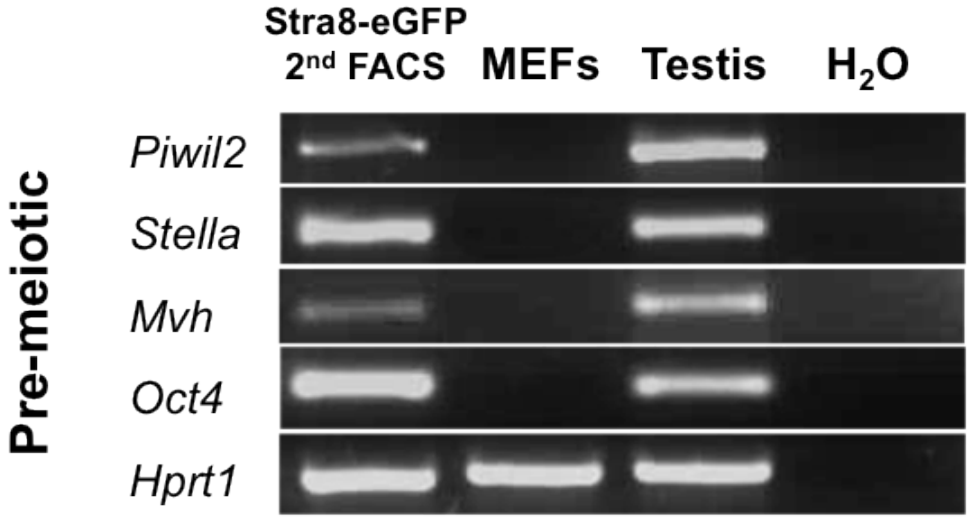
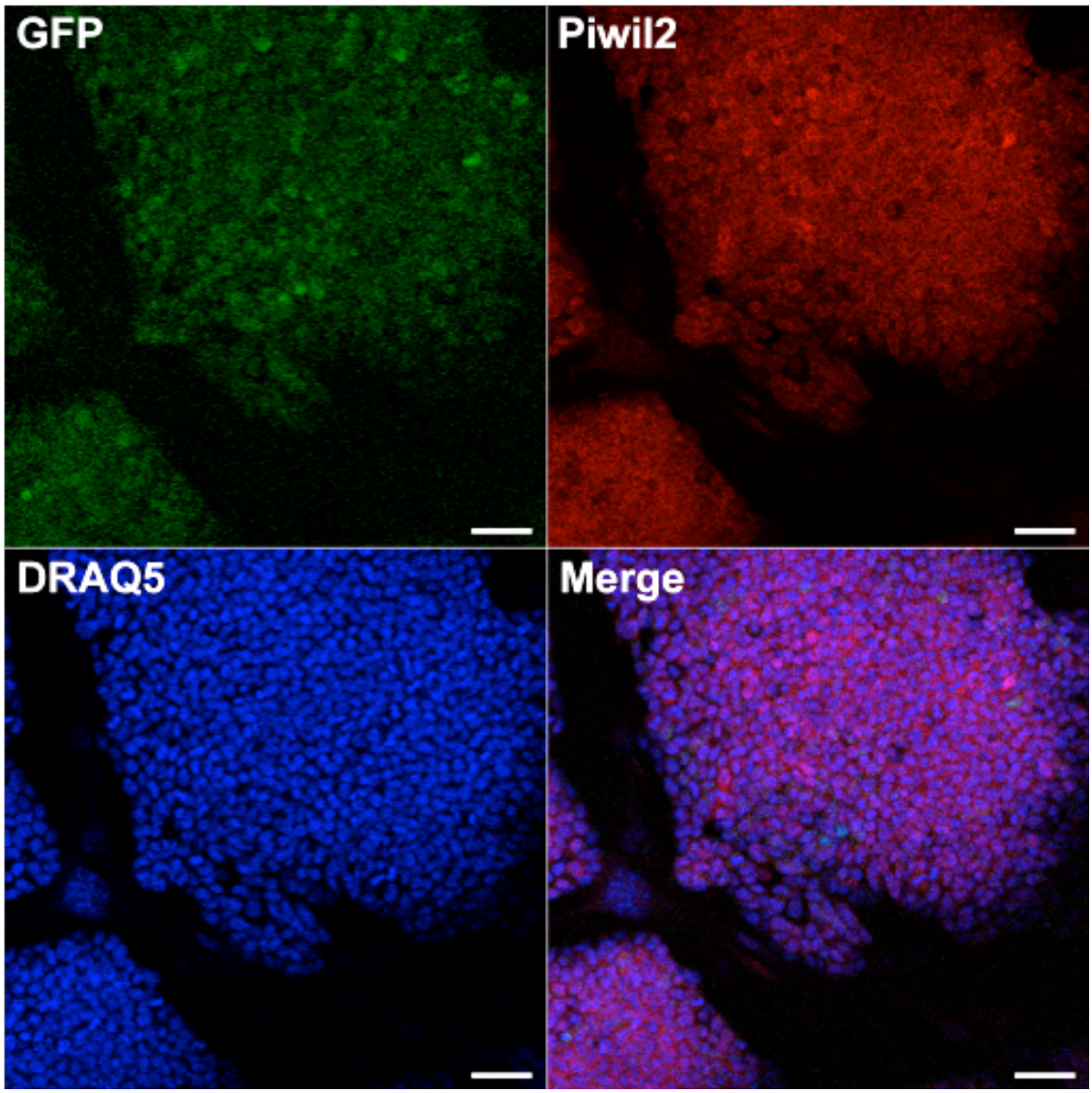


Figure 36. RT-PCR analysis of sorted eGFP positive cells, after 12 hrs induction with RA, for pre-meiotic markers. MEFs and Testis cDNA were used as negative and positive controls respectively. House keeping *Hprt1* gene was also used as positive control. H_20 column no cDNA was present in the PCR reaction.

Stra8-eGFP-Pac cells also tested for the expression of pre-meiotic proteins. In particular, cells were stained with anti-PIWIL2 antibody. *Piwil2* (also known as *Mili*) is known to play an important role in spermatogonia stem cell renewal and germ cell differentiation by modulating the expression of various genes (Lee et al., 2006). In *Piwil2*-null mice PGC development is normal, however, spermatogenesis is arrested in early meiotic prophase (zygotene) (Kuramochi-Miyagawa et al., 2004).

The immunocytochemical analysis showed expression of PIWIL2 in eGFP positive cells after 12 hrs of RA induction (Figure 37) providing more evidence of the spermatogonia-like characteristics that these cells had obtained.

I.



II.

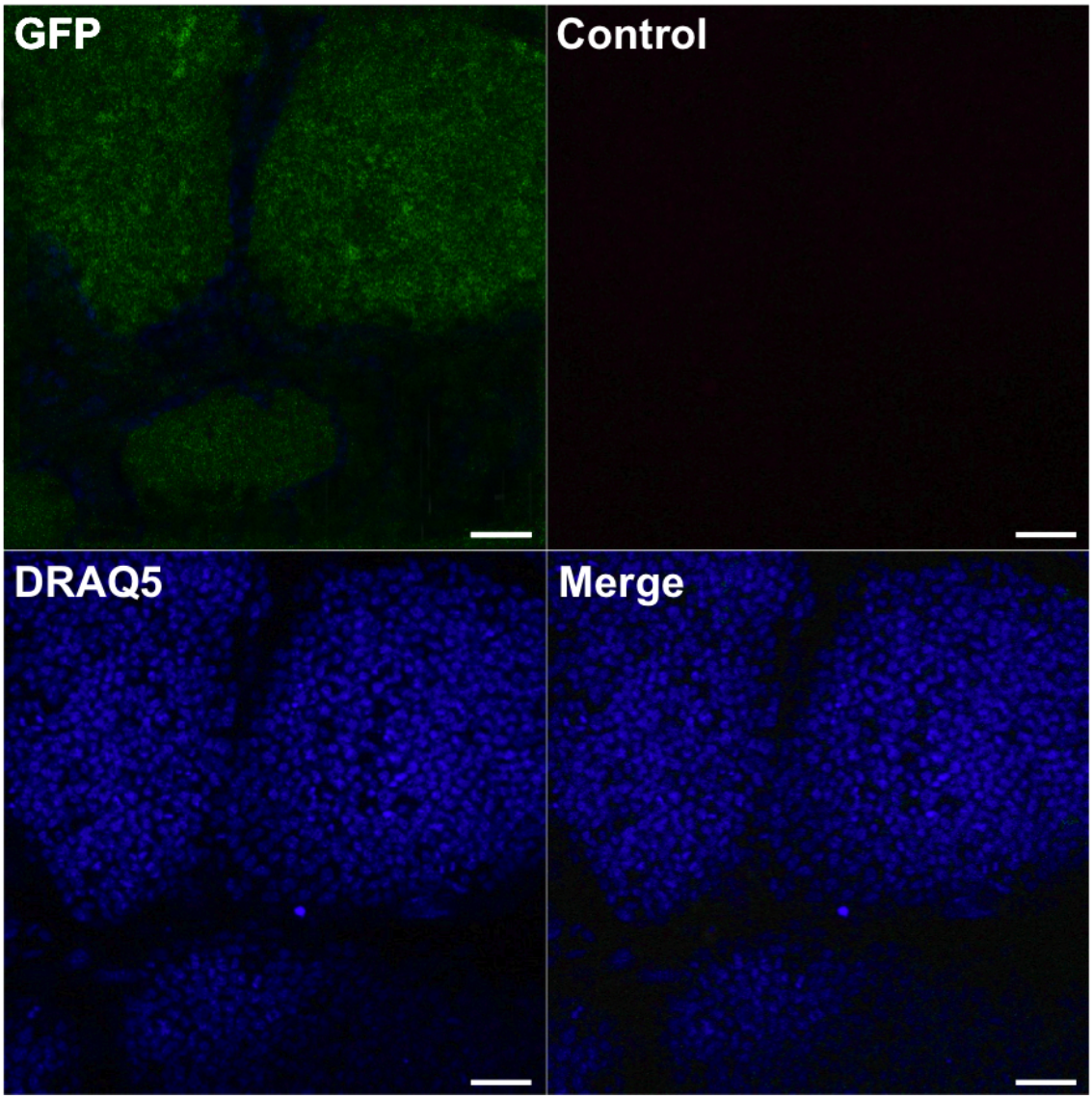


Figure 37. Immunocytochemical analysis of *Stra8*-eGFP-Pac cells after 12 hrs RA induction. (I) eGFP channel shows endogenous eGFP expression. Piwil2 signal was detected throughout the cell colony (red channel). DNA was counter stained with DRAQ5. (II) No signal was observed when primary antibody was omitted in control. Bars are 50µm.

4.3.5 Establishment of double transfected cell lines: Stra8-eGFP/Sycp1-DsRED, Stra8-eGFP/Pgk2-DsRED and Stra8-eGFP/Prm1-DsRED

Having established the *Stra8*-eGFP-Pac cell line and after RA induction and sorting of eGFP +ve cells it was time to transfect the cells with the meiotic (Figure 24) and post-meiotic constructs (Figure 20). Each vector (*Sycp1*-DsRED-Neo, *Pgk2*-DsRED-Neo and

Prm1-DsRED-Neo) was linearized and transfected, as described before, to a separate batch of *Stra8*-eGFP-Pac cells (Figure 38 I).

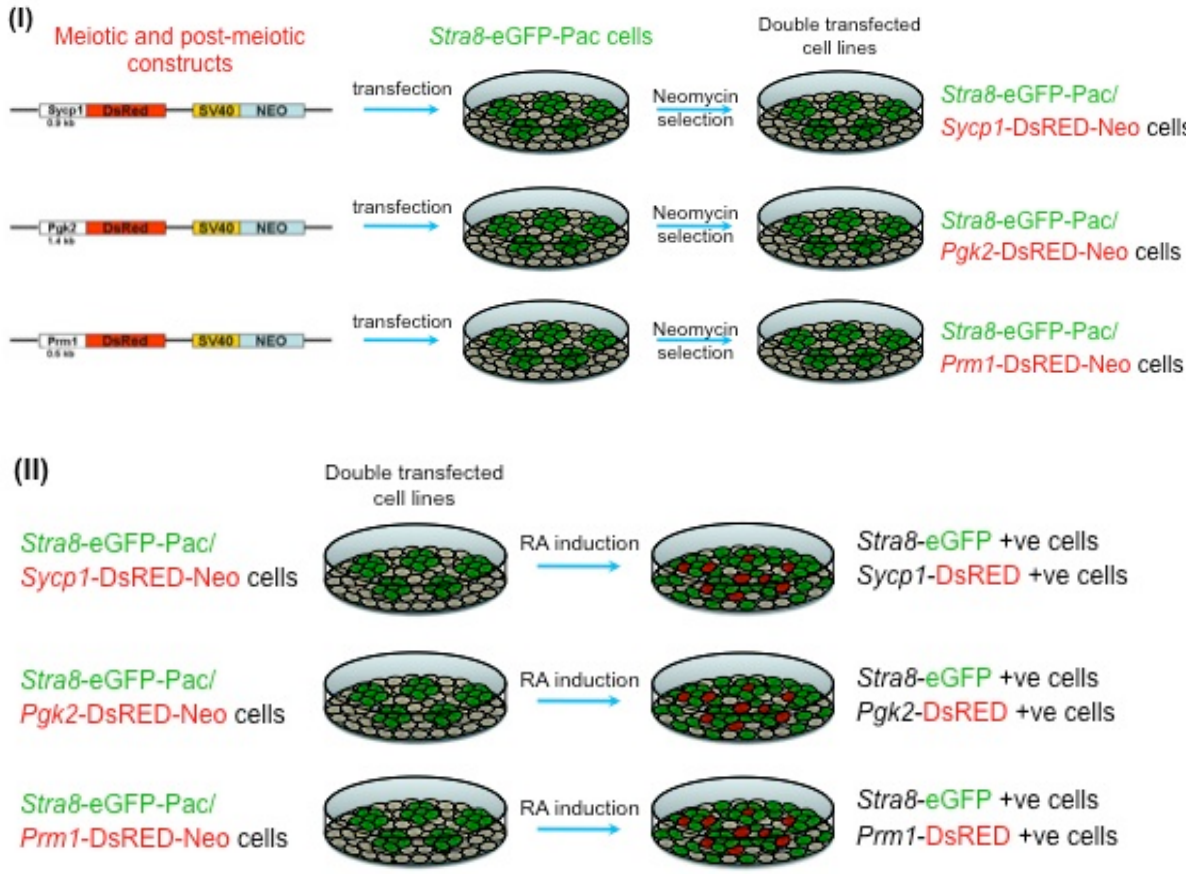


Figure 38. Schematic diagram of experimental steps (I) Transfection of the meiotic and post-meiotic vectors to *Stra8*-eGFP-Pac cells to establish three new stable cell lines, (II) Upon induction with RA DsRED positive cells should appear if the meiotic and post-meiotic specific promoters will become active.

24 hrs following transfection, G418 was added to the media and after 7-8 days of antibiotic selection; colonies with a nice round shaped morphology (Figure 39) were picked up and transferred to individual wells of 96-well cell-culture plate for expansion and PCR screening.

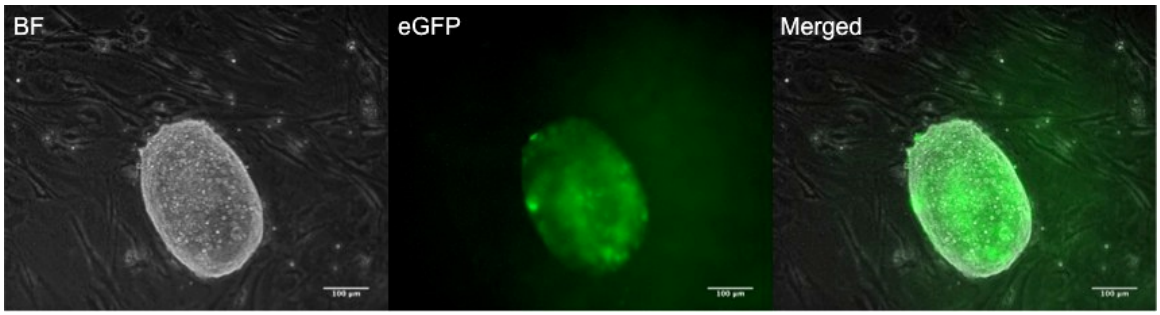


Figure 39. Upon transfection with the second vector *Stra8*-eGFP-Pac cells were seeded on a layer of antiobiotic resistant MEFs and cultured in the precense of G418. After 7-8 days round colonies (like the one shown above) originated from a single cells were picked up. Note the eGFP expression even without the addition of RA indicating the activation of *Stra8* promoter.

4.3.5.1 PCR screening clones for vector construct

To confirm the presence of each vector construct in each clone, DNA was extracted from each colony and tested by PCR using a forward primer recognizing the promoter sequence and a reverse primer specific for the DsRED DNA sequence. Agarose gel electrophoresis results are shown in Figure 40.

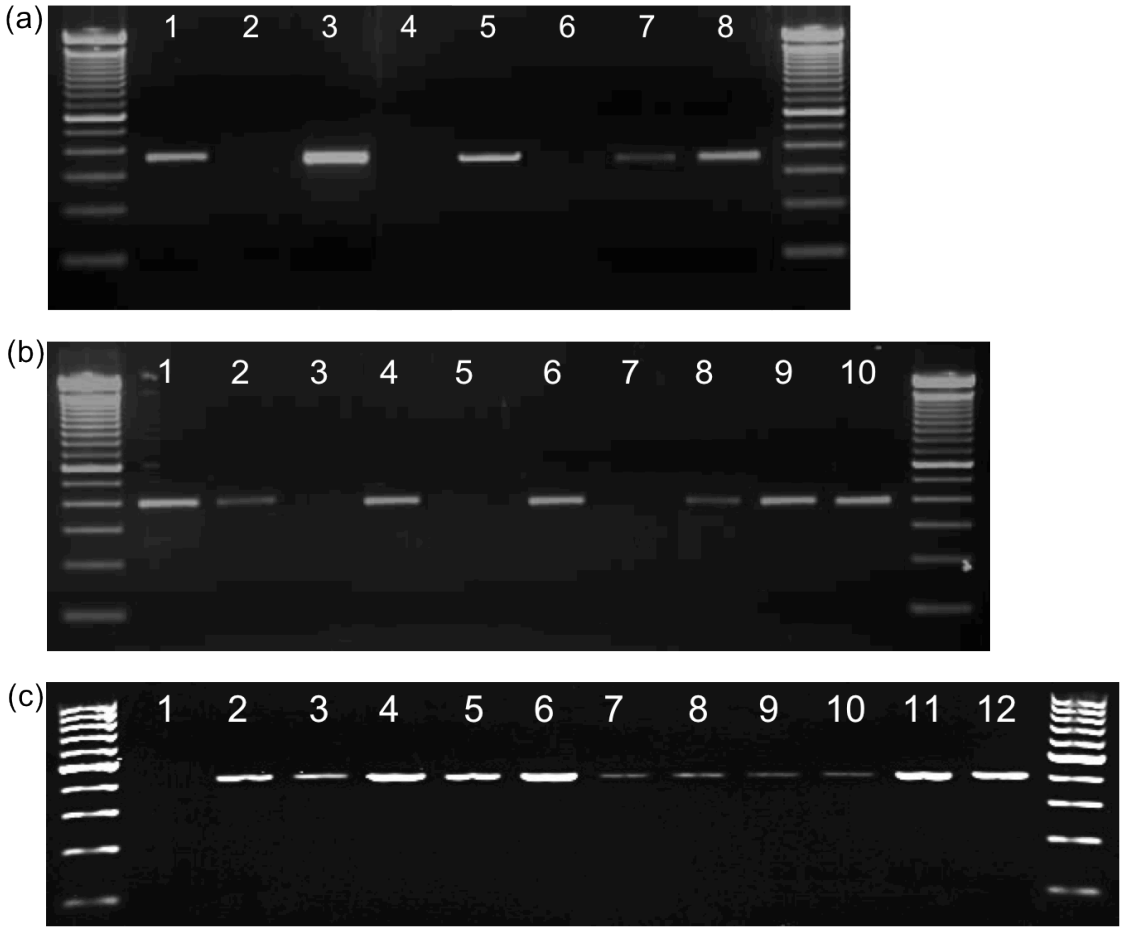


Figure 40. Images from agarose gel electrophoresis. Screening for positively transfected colonies (a) *Sycp1*-DsRED transfected colonies, (b) *Pgk2*-DsRED transfected colonies, (c) *Prm1*-DsRED transfected colonies. Colonies 1, 3 and 5 for *Sycp1*-DsRED, colonies 1, 4 and 6 for *Pgk2*-DsRED and colony 4, 5, 6, 11 and 12 for *Prm1*-DsRED were selected for subsequent experiments.

4.3.5.2 Screening clones for DsRED positive cells after RA induction

Having identified the PCR positive clones we then wanted to select a clone that upon RA induction DsRED positive cells would appear (Figure 38 II). In a personal communication with the senior author (Prof. Karim Nayernia) of the previous study where its protocol was adapted, Prof. Karim Nayernia mentioned that DsRED positive cells appeared in three-dimensional (3D) structures protruding from the two dimensional (2D) cell layer, after about 72 hrs induction/differentiation. These 3D structures were also observed in our culture environment and started to appear after 48 hrs of RA (Figure 41) but they were not very common. Only 5-10 of these 3D structures, with a diameter of around 100μm, appeared in each well of a 48-well plate (0.95 cm^2/well), sugesting their formation was a random spontaneous event.

We tried to enhance the formation of these 3D structures by altering the RA concentration, higher (10^{-4} M) or lower (10^{-6} M), during differentiation and also by altering the initial seeding number of cells in each well, however none of these modifications seemed to alter (increase or decrease) the spontaneous formation of these structures.

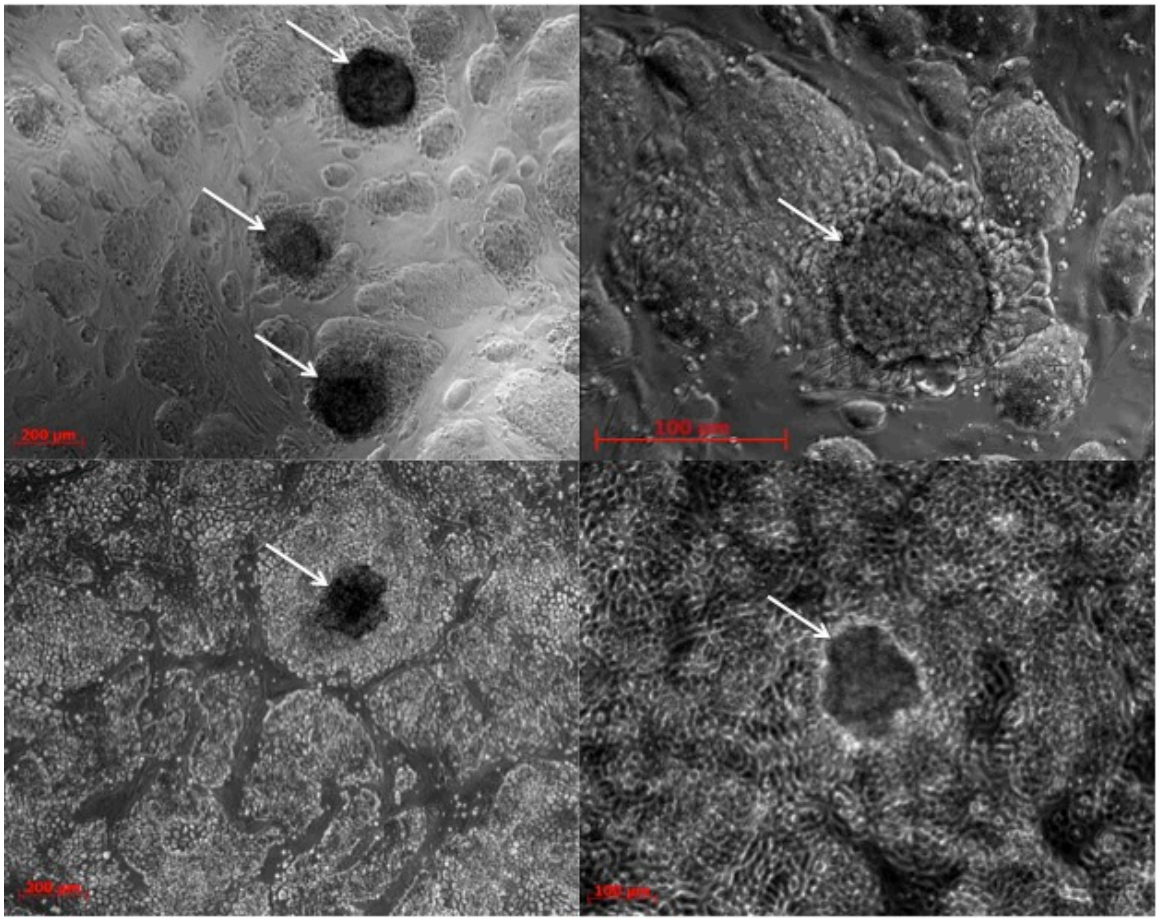
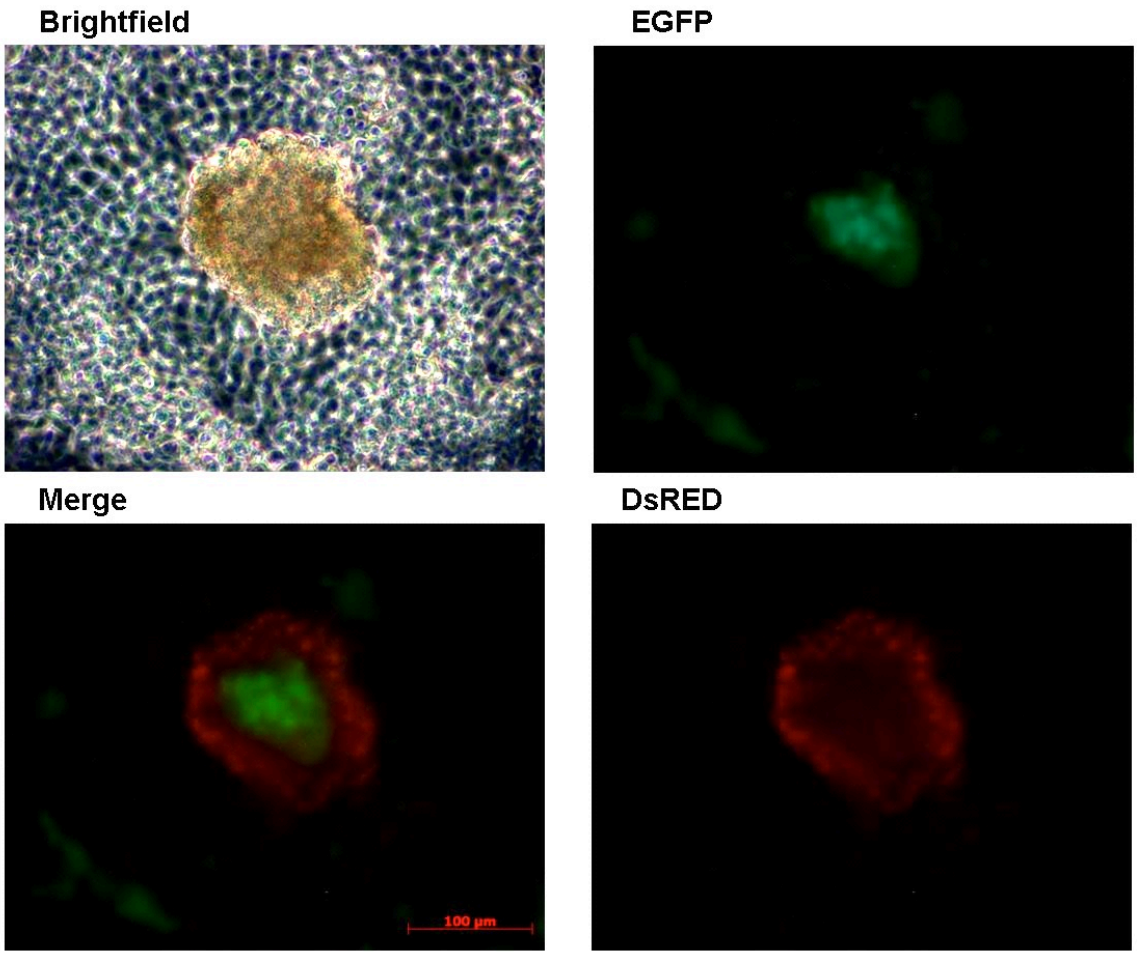


Figure 41. Images showing the three-dimensional (3D) structures (indicated by white arrows) protruding from the two dimensional (2D) cell layer, after the 72 hrs induction/differentiation.

After knowing where to look for DsRED positive cells, the PCR positive clones (Figure 40) were induced with RA for 72 hrs and observed under a fluorescent microscope for the appearance of DsRED positive clones. Both from *Stra8*-eGFP/*Sycp1*-DsRED and *Stra8*-eGFP/*Pgk2*-DsRED clones with bright DsRED positive cells were identified and selected for further expansion and subsequent experiments (Figure 42). However, no DsRED positive cells appeared from all the *Prm1*-DsRED clones tested.

(A)



(B)

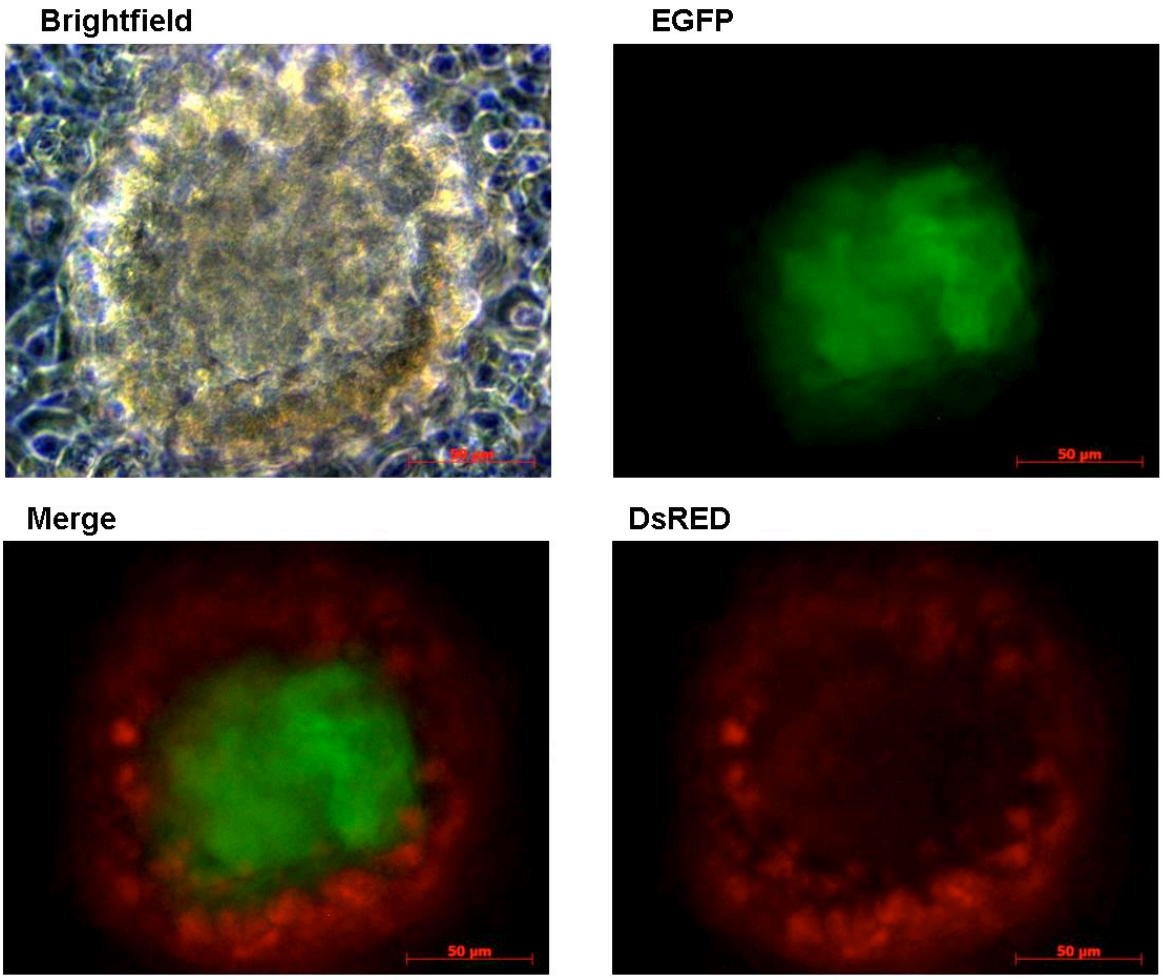


Figure 42. Brightfield and fluorescent images of 3D structures showing the appearance of eGFP and DsRED positive cells after RA induction of Sycp1-DsRED (A) and Pgk2-DsRED (B) transfected clones.

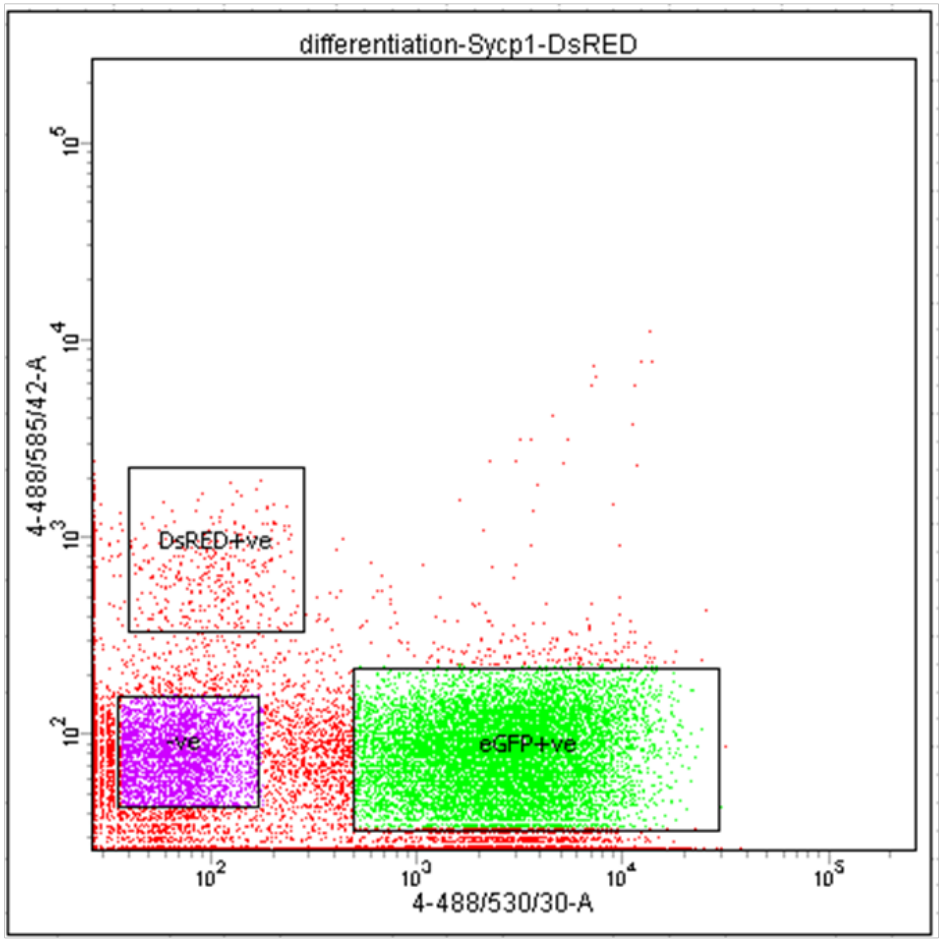
Cells positive for the Stra8-eGFP expression mimicking pre-meiotic cells appeared in the inner side of the 3D round structure and cells positive for the DsRED reporter in the outer parts of the structure. It appeared that as cells progressed in the spermatogenic-like development and differentiation they were "pushed" outwards from the core where the pre-meiotic-like cells (eGFP positive) resided.

4.4 Analysis of *Sycp1*-DsRED and *Pgk2*-DsRED positive cells

4.4.1 RT-PCR analysis of DsRED positive cells for germ cell markers

In order to investigate the molecular profile of ES-derived germ-like cells, *Sycp1*-DsRED and *Pgk2*-DsRED positive cells were sorted by FACS (Figure 43) and RNA was extracted for RT-PCR analysis. RT-PCR analysis showed the expression of meiotic specific genes such as *Sycp1*, *Synaptonemal Complex Protein 3 (Sycp3)*, *Dosage suppressor of mck1 homolog (Dmc1)* and *Pgk2*. The products of *Sycp1* and *Sycp3* genes are important components of the synaptonemal complex assembly that forms between homologous chromosomes during meiotic prophase (de Vries et al., 2005, Miyamoto et al., 2003). *Dmc1* participates in meiotic recombination, and is specifically is required for the resolution of meiotic double-strand breaks (Pezza et al., 2007). The *Pgk2* gene is expressed specifically in the testis during the meiotic stage (Robinson et al., 1989, McCarrey and Thomas, 1987) and the kinase activity of PGK2 is critical to normal motility and fertility of mammalian spermatozoa (Yoshioka et al., 2007). In the case of the *Sycp1*-DsRED and *Pgk2*-DsRED positive cells expression the of *Sycp1* and *Pgk2* genes also verified the activation of the endogenous *Sycp1* and *Pgk2* promoters respectively. Expression analysis from 84 hrs RA-treated cells for the post-meiotic specific gene like *Transition protein 1* (*Tp1)*, an intermediate protein in the replacement of histones by protamines like *Prm1*, showed that these latter transcripts were absent. (Figure 44).

a1)

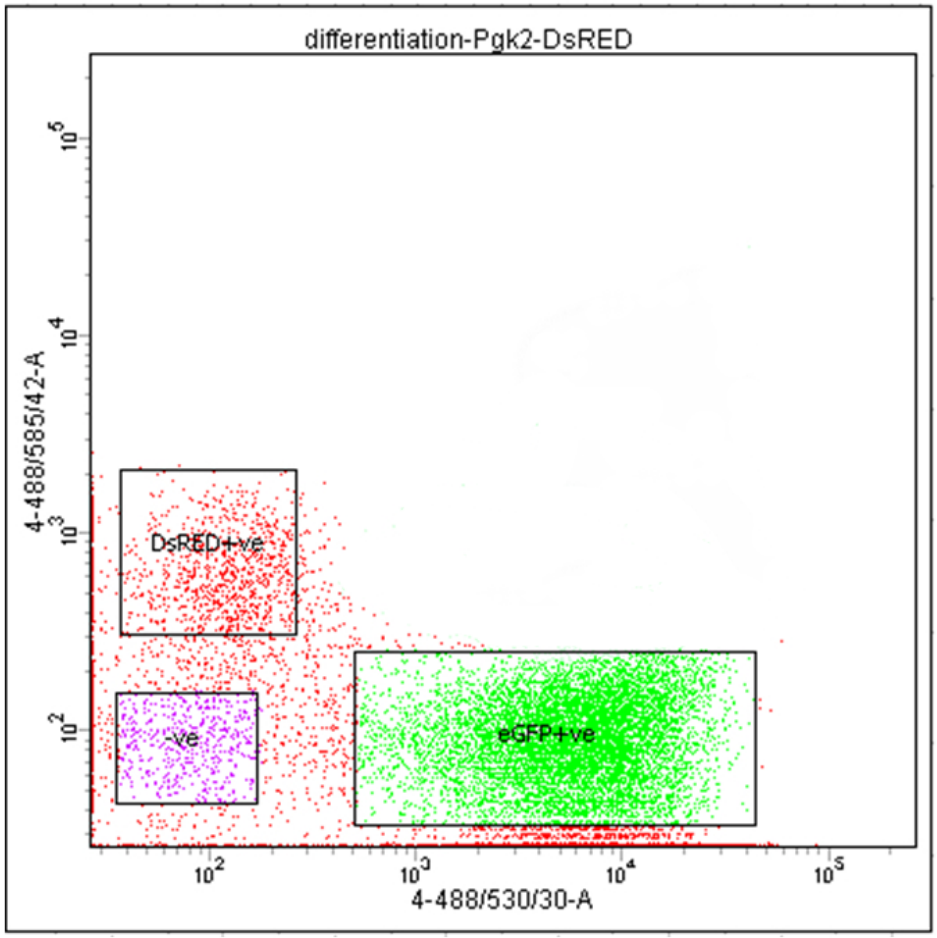


a2)

Tube: Sycp1-DsRED

Population	#Events	%Parent	%Total
All Events	60,000	####	100.0
P1	54,377	90.6	90.6
DsRED+ve	703	1.3	1.2
eGFP+ve	23,693	43.6	39.5
-ve	6,653	12.2	11.1

b1)



b2)

Tube: Pgk2-DsRED

Population	#Events	%Parent	%Total
All Events	50,000	####	100.0
P1	39,964	79.9	79.9
DsRED+ve	564	1.4	1.1
eGFP+ve	21,150	52.9	42.3
-ve	1,898	4.7	3.8

Figure 43. Representative FACS results after 72 hrs of RA induction of *Sycp1*-DsRED (a1, a2) and Pgk2-DsRED (b1, b2). (a1, b1) Dot plot graphs showing the fraction of DsRED and eGFP positive cells, (a2, b2) Percentage of DsRED and eGFP positive cells over the recorded number of cells.

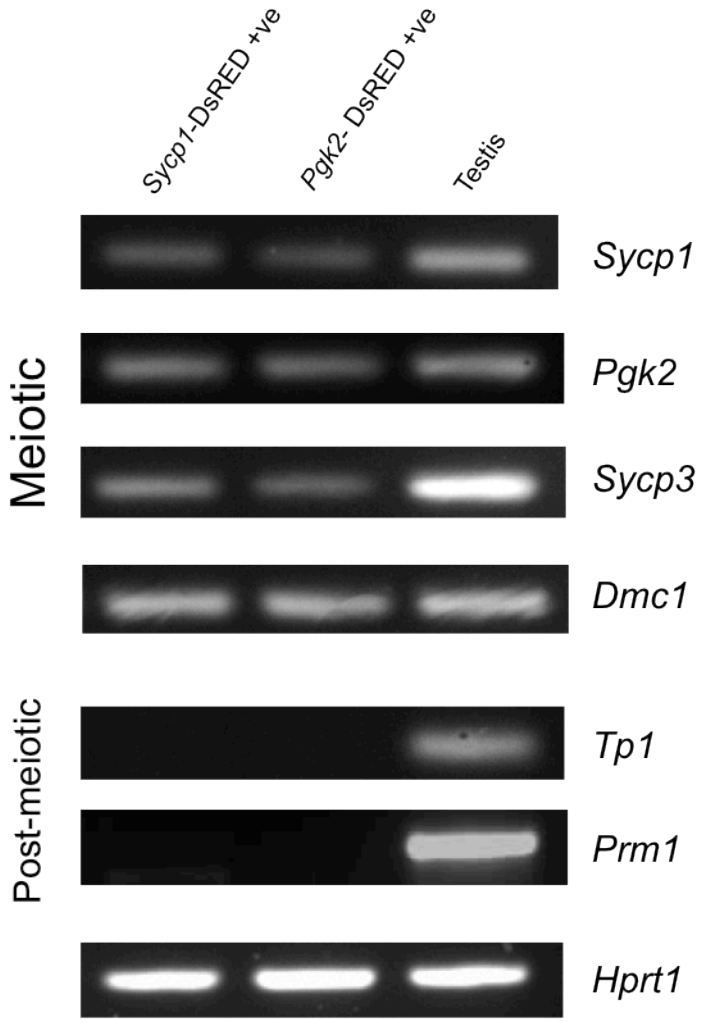


Figure 44. RT-PCR analysis of FACS sorted DsRED positive cells, after 72 hrs induction with RA, for meiotic and post-meiotic markers. Testis cDNA was used as positive control. House keeping *Hprt1* gene was also used as positive control.

4.4.2 Immunocytochemical analysis of DsRED positive cells for germ cell markers

The expression of meiotic specific genes revealed by the RT-PCR analysis indicated that cells had acquired characteristics of meiotic-like cells. To further characterize the DsRED positive cells the expression of meiotic proteins was investigated by immunocytochemical analysis. In particular, *Stra8*-eGFP/*Sycp1*-DsRED and *Stra8*-eGFP/*Pgk2*-DsRED cells were cultured in the presence of RA and after 72 hrs were fixed and stained with antibodies against Heterogeneous Nuclear Ribonucleoprotein G-T (hnRNPG-T), a RNA binding protein involved in pre-mRNA processing which is expressed in spermatocytes through out meiosis (Elliott et al., 2000); and DMC1, a

meiotic recombination protein expressed in early meiosis (meiotic prophase) (Pezza et al., 2007).

Immunocytochemical analysis showed that DsRED positive cells in both *Stra8*-eGFP/*Sycp1*-DsRED (Figure 45) and *Stra8*-eGFP/*Pgk2*-DsRED cells (Figure 46) expressed the meiotic specific protein hnRNPG-T. Moreover, when RA-treated *Stra8*-eGFP/*Sycp1*-DsRED cells where stained with an anti-DMC1 antibody, an early meiotic protein, DsRED positive cells were found positive (Figure 47).

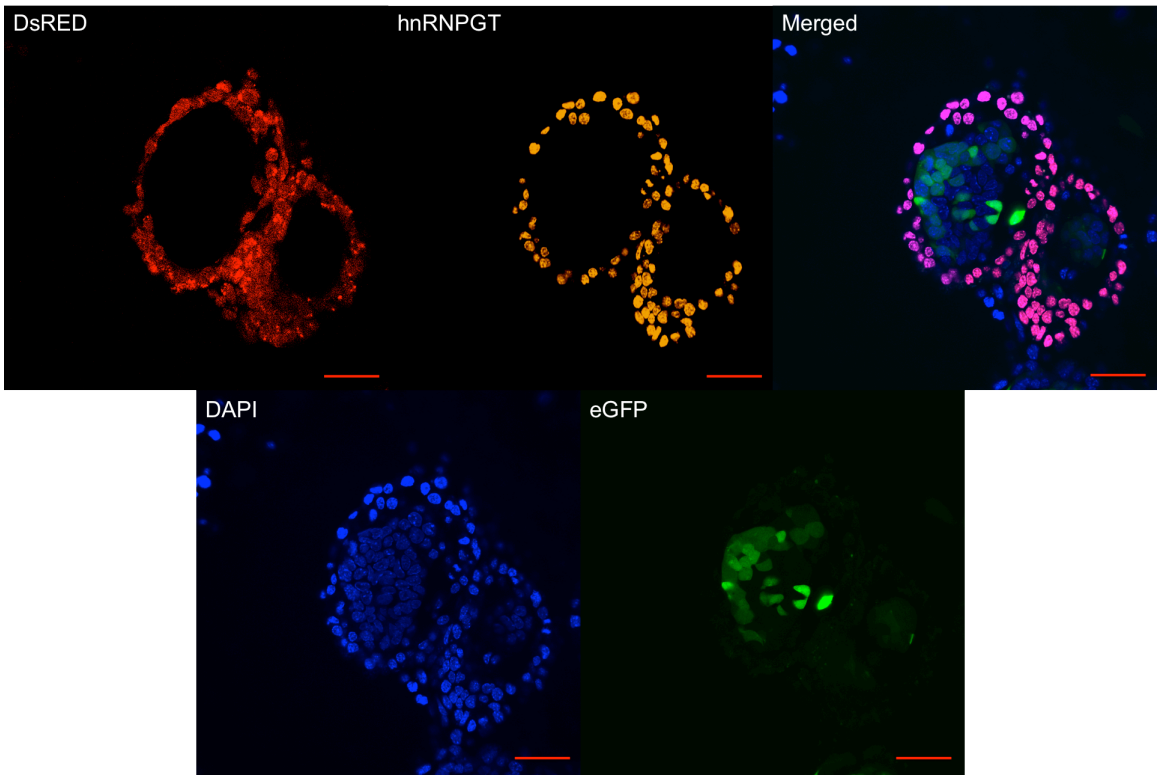


Figure 45. Immunohistochemistry of *Stra8*-eGFP/*Sycp1*-DsRED *cells* using confocal microscopy. Staining using an antibody against hnRNPG-T protein revealed expression in *Sycp1*-DsRED positive cells. Bar is 40µm.

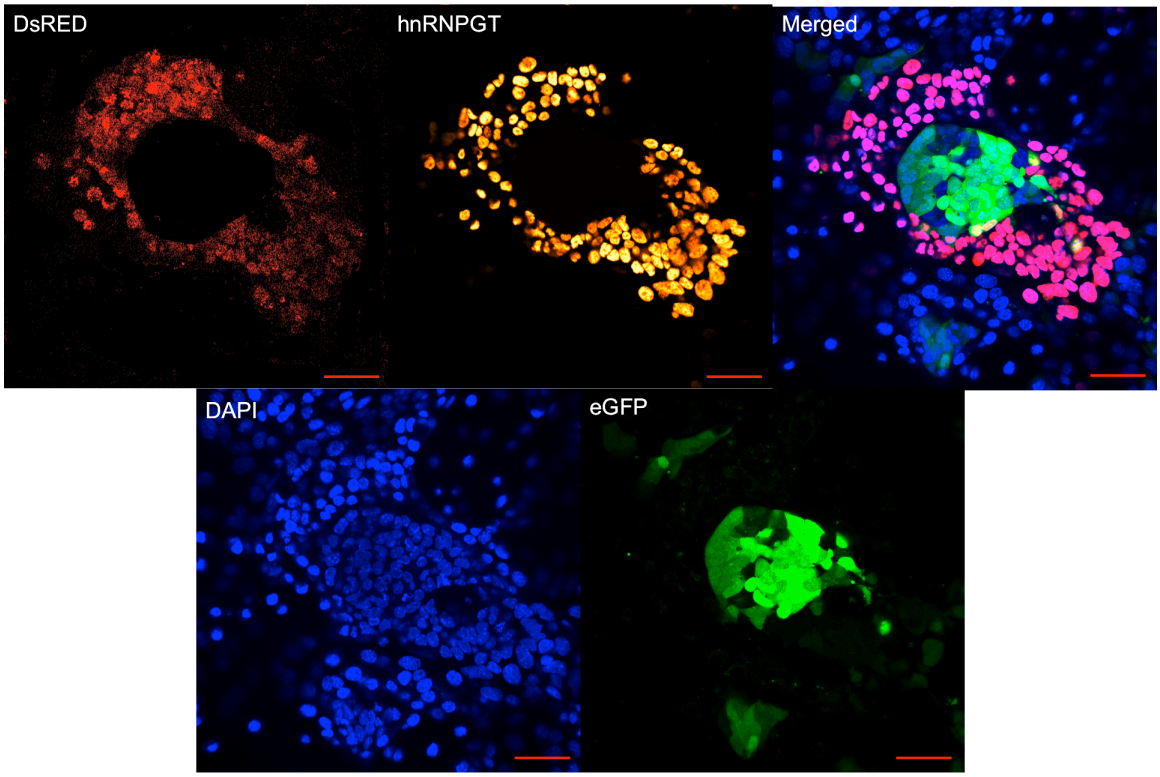


Figure 46. Immunohistochemistry of *Stra8*-eGFP/*Pgk2*-DsRED *cells* using confocal microscopy. Staining using an antibody against hnRNPG-T protein revealed expression in *Pgk2*-DsRED positive cells. Bar is 40µm.

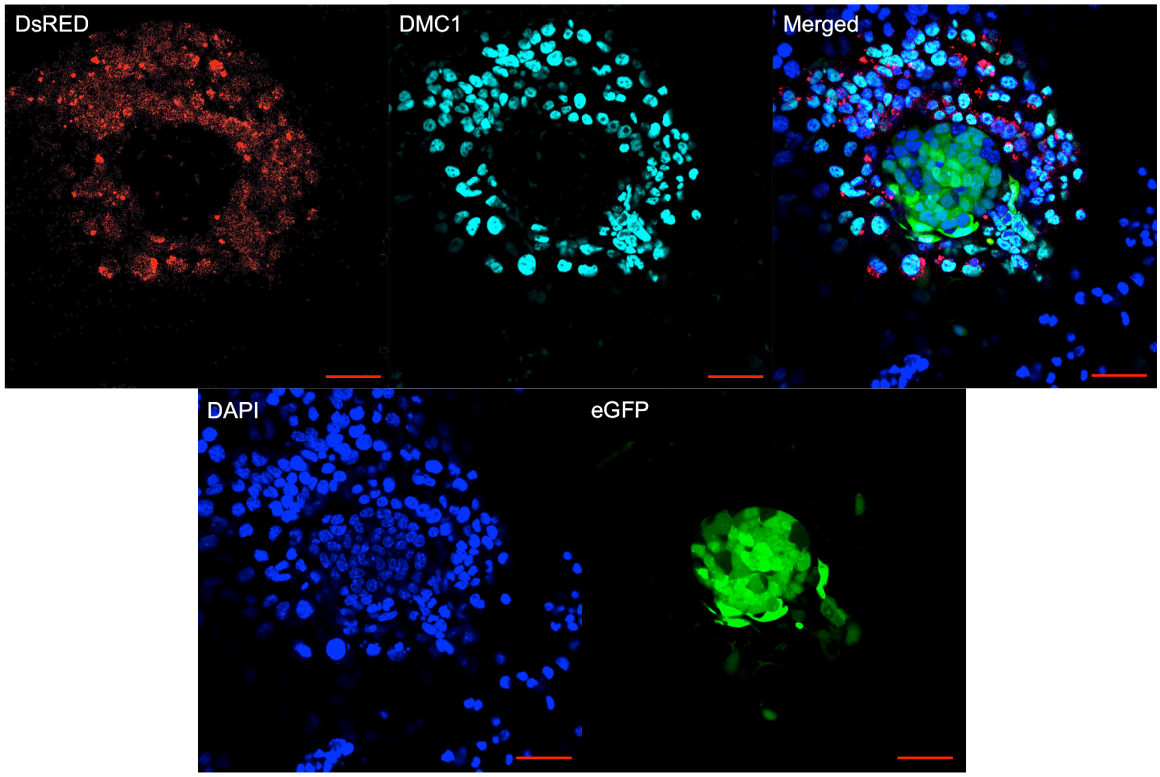


Figure 47. Immunohistochemistry of *Stra8*-eGFP/*Sycp1*-DsRED *cells* using confocal microscopy. Staining using an antibody against DMC1 protein revealed expression in *Sycp1*-DsRED positive cells. Bar is 40µm.

Having determined that 72 hrs after RA treatment DsRED positive cells from both cell lines acquired meiotic-like characteristics we wanted to investigate if eGFP positive cells continued to exhibit pre-meiotic-like properties and also if DsRED positive cells even after meiotic protein expression retained any signs of the pre-meiotic stage. For this purpose, *Stra8*-eGFP/*Sycp1*-DsRED cells were examined for the expression of the pre-meiotic specific protein STRA8. Immunocytochemical analysis showed that eGFP positive cells continued to express pre-meiotic proteins after RA-treatment. Interestingly DsRED positive cells, mimicking the in-*vivo* pattern of germ cell progression, were not found positive for pre-meiotic proteins (Figure 48).

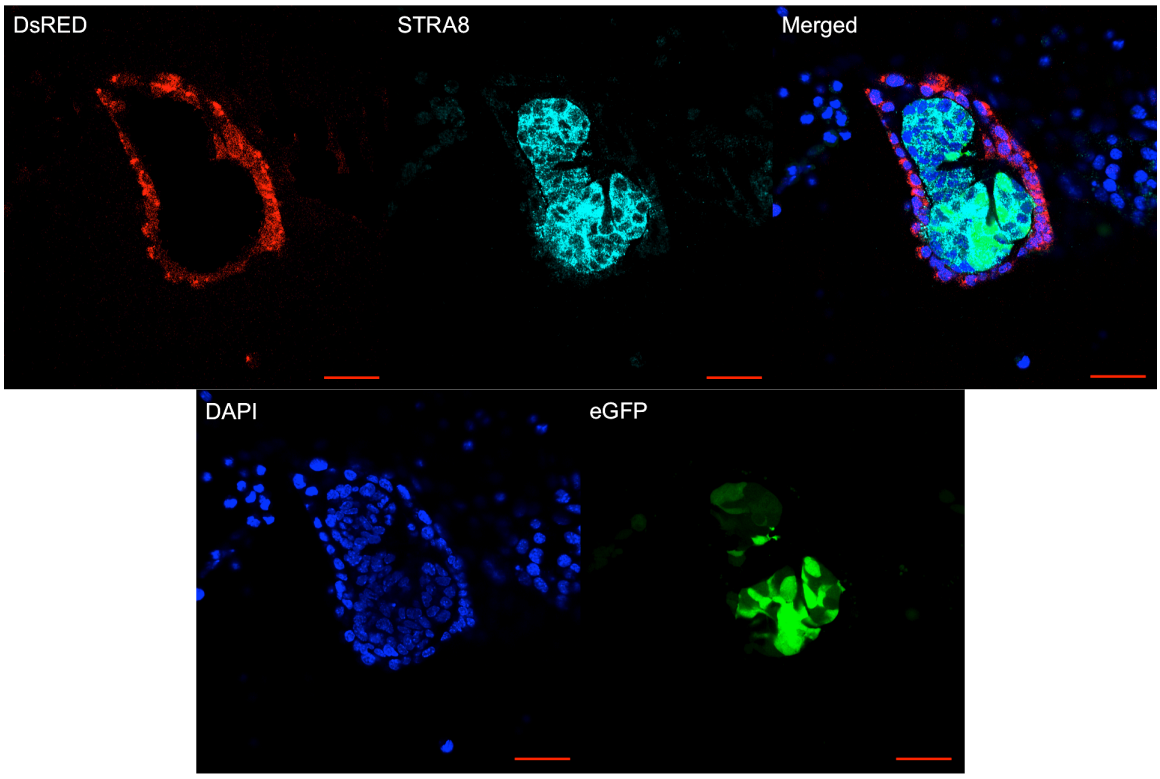


Figure 48. Immunohistochemistry of *Stra8*-eGFP/*Sycp1*-DsRED *cells* using confocal microscopy. Staining using an antibody against STRA8 protein revealed expression in *Stra8*-eGFP positive cells Bar is 40µm.

Stra8-eGFP/*Pgk2*-DsRED cells were also tested by immunocytochemical analysis for the post-meiotic protein TP1, but as predicted by RT-PCR analysis the cells were found negative for this protein.

4.4.3 DNA content analysis of DsRED positive cells

The ultimate goal of meiosis is to produce haploid gametes by reducing the number of chromosomes by half. To investigate whether DsRED positive cells from *Stra8*-eGFP/*Sycp1*-DsRED cell line undergo meiosis *in vitro*, we examined the DNA content of the cells by flow cytometric analysis.

To determine the DNA content and ploidy, mouse sperm and R1 undifferentiated dividing ES cells were used as a reference (Figure 49 A and B-B1). DsRED positive cells were sorted by flow cytometry and their nuclei were extracted and stained with the DNA intercalating agent propidium iodide. Accurate DNA content analysis was then performed using the dedicated software called **ModFit** LT™ (Verity Software House). The DNA content analysis showed about 8.5% of DsRED positive cells to be tetraploid, in other words to have four sets of chromosomes (Figure 49 C-C1).

To further investigate whether any of these tetraploid cells complete meiosis and give rise to haploid cells, *Stra8*-eGFP/*Pgk2*-DsRED cells were examined after 84 hrs of RA treatment. DNA content analysis showed no haploid cell population to be present (Figure 50). This absence of haploid cells was no surprise as the lack of post-meiotic specific genes was also indicated by the RT-PCR analysis (Figure 44).

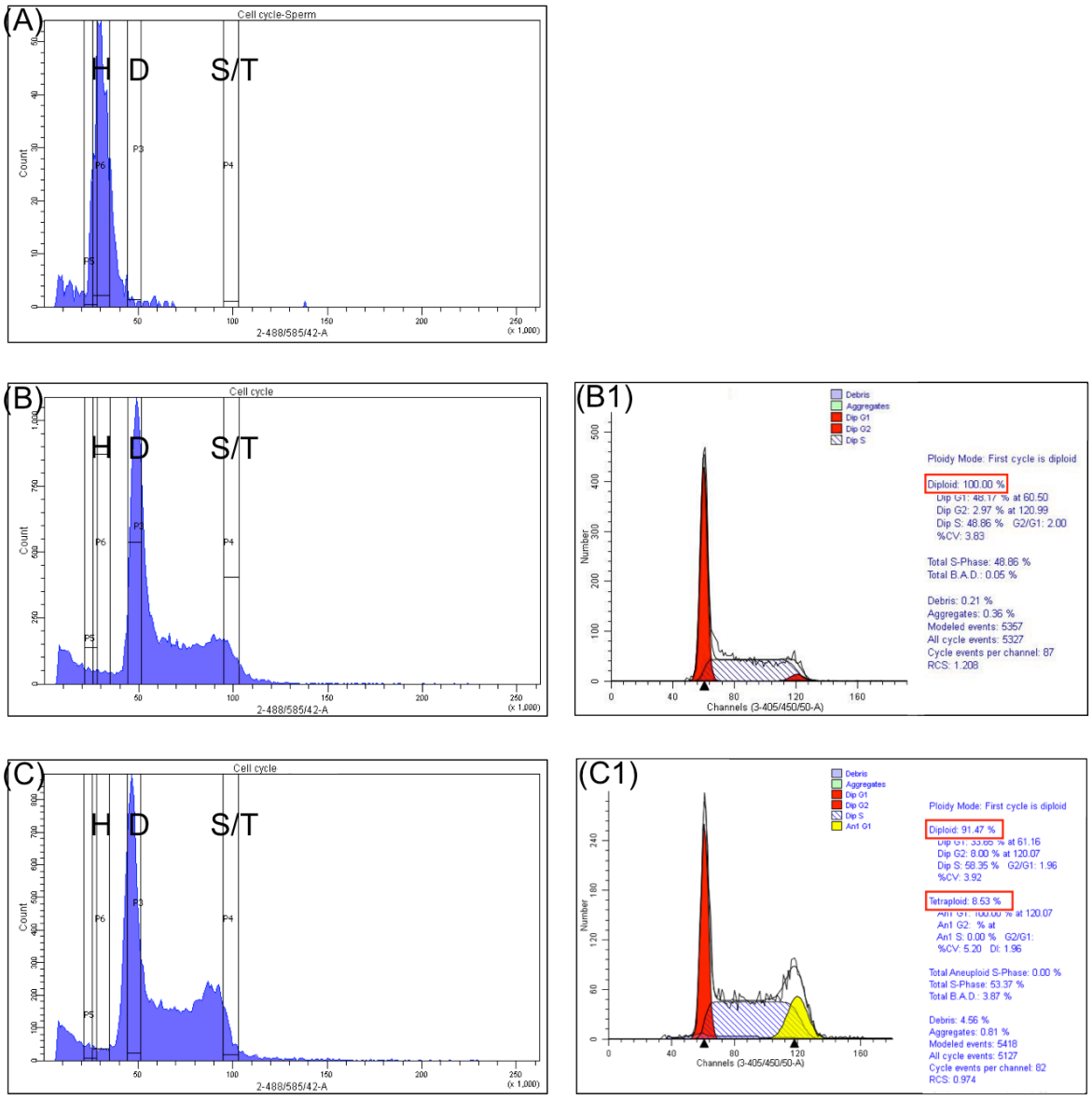


Figure 49. Investigation of ploidy in DsRED positive cells. (A) Mouse sperm was used as haploid reference., (B) R1 undifferentiated dividing ES cells were used as diploid/S-phase reference, (C) DsRED positive cells from *Stra8*-eGFP/*Sycp1*-DsRED cell line. (B1) and (C1) show DNA content analysis using ModFit LT software. H=haploid, D=diploid, S=S-phase, T=tetraploid.

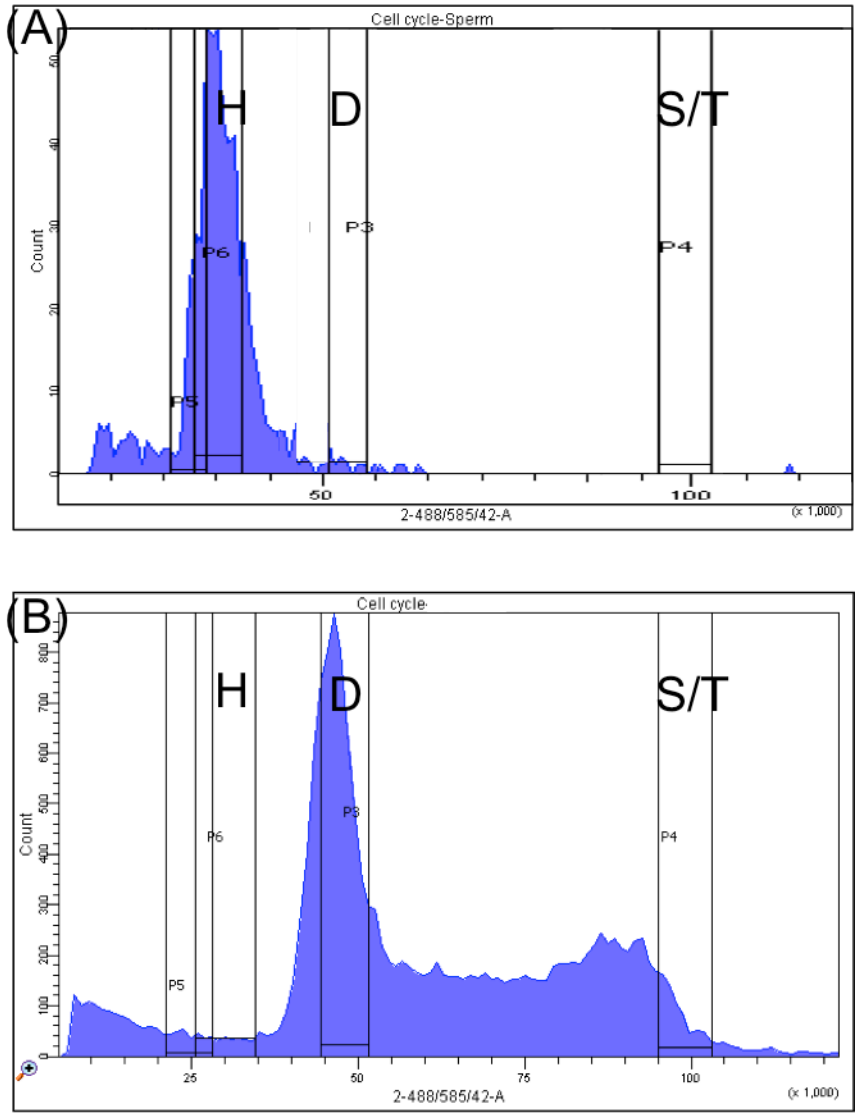


Figure 50. Investigation of ploidy in RA-treated *Stra8*-eGFP/*Pgk2*-DsRED cells. (A) Mouse sperm was used as haploid reference., (B) *Stra8*-eGFP/*Pgk2*-DsRED cells after 80 of RA treatment. H=haploid, D=diploid, S=S-phase, T=tetraploid.

4.5 Summary and discussion

In order to investigate the function and dissect the role of Notch signalling components in spermatogenesis, an already established *in vitro* ES cell-derived germ cell model was adapted. This *in vitro* model, previously shown to be able to generate pre-meiotic-like and post-meiotic-like germ cells and so to mimic to some extent the *in-vivo* conditions of mouse spermatogenesis, was re-designed and re-developed to further allow the identification and isolation of early and late meiotic-like germ cells.

For this purpose, two new meiotic reporter constructs *Sycp1*-DsRED and *Pgk2*-DsRED were generated. These constructs harbored promoters which should be activated in early meiosis, in the case of *Sycp1,* and late meiosis in the case of *Pgk2*. Moreover, a new antibiotic resistant gene (*Pac*) was introduced to the *Stra8*-eGFP reporter construct that allowed antibiotic selection when the meiotic or post-meiotic constructs were transfected, something that was not possible in the approach used by Nayernia and colleagues where PCR screening was used instead.

Having generated the constructs, three stable transfected ES cell lines were established with each one harbouring two reporter constructs (Figure 20, Figure 22). All three cell lines contained the pre-meiotic reporter construct *Stra8*-EGFP-Pac and one of the early meiotic (*Sycp1*-DsRED), late meiotic (*Pgk2*-DsRED) or post-meiotic (*Prm1*-DsRED) reporter constructs.

After selection and establishment of stable cell lines, each cell line was allowed to differentiate in the presence of RA, and eGFP or DsRED positive cells were isolated by FACS. Expression of the fluorescent reporter gene indicated that the cells had reached the spermatogenic-like stage where the corresponding promoter becomes active. This was verified by RT-PCR analysis that showed the expression of pre-meiotic specific transcripts in eGFP positive cells, and also the expression of meiotic specific genes in *Sycp1*-DsRED and *Pgk2*-DsRED positive cells. The pre-meiotic and meiotic-like characteristics of the cells were also confirmed using antibodies against stage specific proteins.

So, the promoter stage-specific activation and the demonstration of pre-meiotic and meiotic specific transcripts and proteins provided enough evidence to support the utilization of the newly developed ES cell-based *in vitro* platform as a model system for the investigation of Notch signalling components in spermatogenic cells.

Although we succeeded our initial aim in developing a novel system that provides direct access to pre-meiotic and meiotic stages, the absence of post-meiotic specific transcripts and proteins suggests that regulatory mechanisms and factors that ensure full progression of germ cells in the *in-vivo* environment might not be present in the *in vitro* culture system.

Meiosis not only requires the coordination of many events and a sophisticated temporal regulation of gene expression (Chu and Herskowitz, 1998, Kassir et al., 2003, Pak and Segall, 2002), but also the existence of meiotic checkpoints that ensure that events have occurred properly (Roeder and Bailis, 2000). The sophistication and tight coordination of this process highlights its importance in fertility. In fact, there are many patients with infertility problems attributed either to defects during meiotic stages (Sun et al., 2007), or in many cases to a complete meiotic arrest which results in azoospermia (absence of sperm) (Judis et al., 2004).

Despite following Nayernia's protocol to the letter and using the exact same ES cell line to avoid cell line variation, we did not observed any DsRED positive cells using the post-meiotic reporter cell line (*Stra8*-eGFP/*Prm1*-DsRED). The nonappearance of post-meiotic cells was also verified by RT-PCR and immunocytochemical analysis where the absence of post-meiotic specific transcripts and proteins was shown, and by DNA content analysis. There are a number of reasons that could be attributed for that.

Although, great care was taken to minimise deviation from the published protocol the usage of fetal bovine serum (FBS) in the culture media adds significant variation to the culture environment. FBS, is a complex mixture, with variability in growth factors and nutrients between different grades, different suppliers, and different lots (Zheng et al., 2006). This was something that was realised from the beginning of the project, and so the supplier that Nayernia used for their FBS was asked to provide us with the same grade and lot number as the one used in Nayernia's study, but unfortunately the company was not able to supply us with the same lot. In addition, the differentiation protocol used in Nayernia's study required 15% of FBS in the cell culture media, where the usual ES cell culture media is supplemented with 10% FBS. This shows that the factors and nutrients in the FBS might have a crucial role in the derivation of germ-like cells, given also the fact that the differentiation media was supplemented only with RA and nothing else.

It's worth mentioning that after 7 years since Nayernia's study was published no other publication has emerged using the same protocol either from the same or a different group. Only one study since then has shown generation of functional gametes from ES cells, but their ES-derived PGCs required transplantation in mouse gonads to give rise to ES-derived haploid cells and could not be done in an *in vitro* environment (Hayashi et al., 2011). This adds up in supporting the hypothesis that generation of haploid cells in

Nayernia's culture conditions was a spontaneous event by undefined factors that wait to be determined.

Of course, we cannot rule out the difference in cell culture practice from lab to lab and from person to person, despite common-standard cell culture aseptic techniques used in our cell culture.

Nevertheless, development of male germ cells from ES cells seemed to be a spontaneous cell-autonomous event, controlled by the microenvironment generated in the observed 3D structures. Even though it could not be considered fully comparable with the *in-vivo* conditions, it can be approached as an *in vitro* model that offers easy access to a spermatogenic-like environment and facilitates immensely the investigation of spermatogenesis *in vitro* before asking the same question in an animal model. For any hypothesis raised this could be the first step before the *in-vivo* experiments, thus helping with better experimental planning and better usage of animals. This would also mean a reduction in animal numbers used, an issue for which many ethical questions have been raised lately.

Overall, our ES cell-based *in vitro* platform provides new possibilities for investigating germ cell development, the mechanism behind the transition from mitosis to meiosis, the hormonal and biological factors involved in spermatogenesis and chemical factors for the development of new drugs against infertility problems.

Chapter 5. Effect of Notch signalling during *in vitro* germ cell development

5.1 Introduction to the chapter

We do not yet fully understand how spermatogonial stem cells are maintained and their activity controlled in the testes, or how the transition of germ cells from mitosis to meiosis is regulated during the seminiferous epithelial cycles.

The seminiferous tubule environment is composed of many cell types, which are capable of communicating with each other and influencing their behaviour. One way in which this communication is mediated is through interaction of proteins on the surface of neighbouring cells. Our expression analysis of Notch components indicated that members of the Notch pathway are likely to play a role in the spermatogenic process.

The role of Notch signalling in adult germ line cell fate has been well characterized in the invertebrate model organism of *C. elegans*. Studies in *C. elegans* showed that LAG-2, a ligand related to Delta, is produced by the somatic gonadal distal tip cell (DTC). The germ line stem cells interact with DTCs via GLP-1, a receptor in the Notch family. It is thought that as the germ line progenitor cells move away from the source of Notch ligand at the distal tip of the gonad, they enter meiosis. In support of this theory, ablation of the DTC or a loss of function mutation components of the Notch pathway result in premature entry into meiosis (Kimble and White, 1981, Austin and Kimble, 1987).

Even though the Notch pathway was shown to be essential in the gametogenesis of *C. elegans*, the role of the Notch signalling system in mammalian spermatogenesis has not been well investigated. In order to understand the role of the Notch signalling pathway in spermatogenesis, it is important that we investigate how this pathway functions during this process. Our ES cell-based platform provided that possibility, because it allowed the examination of the role of Notch pathway components during germ cell development *in vitro*.

The aim was to use the already established *in vitro* ES cell-based model and investigate the Notch pathway in two parts. First, to examine how critical is the role of Notch signalling as a whole during germ cell development by disruption of Notch signalling using chemical treatment and second, to identify specifically the role of separate

components of the Notch pathway during this process by activation or inhibition of specific receptors and ligands using soluble ligands and/or antibodies.

5.2 Notch signalling inhibition during *in vitro* germ cell development

The expression analysis of Notch pathway transcripts, the germ cell distribution of Notch components and the activation analysis of Notch receptors in seminiferous tubules provided enough evidence to support the idea of Notch signalling pathway playing a role in spermatogenesis.

Here, in order to examine the role of the Notch pathway in germ cell development as a whole, we blocked Notch signalling with the chemical DAPT, a γ-secretase inhibitor that blocks Notch cleavage by Presenilin 1 and prevents activation of Notch receptor (see introduction section 1.3.2). DAPT has been widely used in the literature to disrupt Notch signalling (Hellstrom et al., 2007, Yu et al., 2008). It has been often applied in *in vitro* culture systems to study the effects of Notch signalling inhibition as it blocks all forms of Notch receptor/ligand activation (Androutsellis-Theotokis et al., 2006).

Notch signalling requires a receptor-ligand interaction that results in proteolytic cleavage and release of N^{-ICD}, which then migrates into the nucleus and associates with nuclear proteins of the CSL family to form a transcription factor complex. This N^{-ICD}-CSL complex activates expression of primary target genes of the Notch pathway such the *Hairy and Enhancer of split* (*Hes*) family. *Hes* family gene products are transcriptional repressors, known to directly affect cell fate decisions as primary Notch effectors (Iso et al., 2003).

Thus, one way to examine the effect of DAPT as Notch signalling inhibitor was to monitor the expression status of members of the major downstream target gene family of *Hes* like *Hes1* and *Hes3*. To do that we treated *Stra8*-eGFP/*Sycp1*-DsRED cell line with only RA and RA with the commonly used concentrations (0.5μM-2μM) of DAPT described in *in vitro* culture experiments (Androutsellis-Theotokis et al., 2006, Yu et al., 2008) for 72 hrs. RNA was then extracted for RT-PCR expression analysis and the results

showed that 1μM of DAPT in the culture medium was sufficient enough to disrupt the activity of Notch signalling pathway (Figure 51).

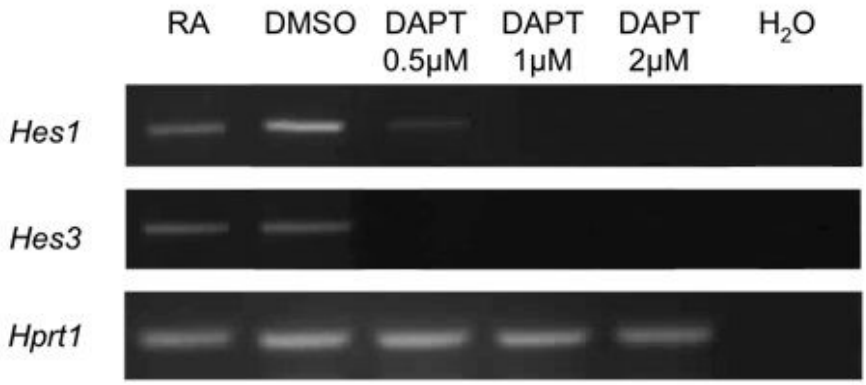


Figure 51. Representative agarose gel from 3 experiments of RT-PCR expression analysis of Notch signalling effecter genes in cultured cells, after 72 hrs induction with RA, DMSO (used as carrier for DAPT) or DAPT. The housekeeping *Hprt1* gene was used as positive control. In the H_20 column no cDNA was present in the PCR reaction.

5.2.1 Notch inhibition effect in pre-meiotic-like cells

Having verified that chemical treatment with DAPT inhibits the activity of the Notch signalling pathway, we then analysed whether disruption of Notch signalling would have any effect in the number of pre-meiotic-like cells after 72 hrs of RA induction. The purpose of this experiment was to examine whether Notch signalling activation is required for the induction and maintenance of the pre-meiotic-like cells in our *in vitro* cell culture system. For this reason we treated both *Stra8*-eGFP/*Sycp1*-DsRED and *Stra8*-eGFP/*Pgk2*-DsRED cell lines with RA and DAPT and 72 hrs later eGFP positive cells were analysed by flow cytometry.

The analysis showed that removal of Notch function during this RA induction period had no effect in the pre-meiotic-like cell induction and maintenance as there was no statistically significant difference between the number of eGFP positive cells within the different conditions tested (Figure 52).

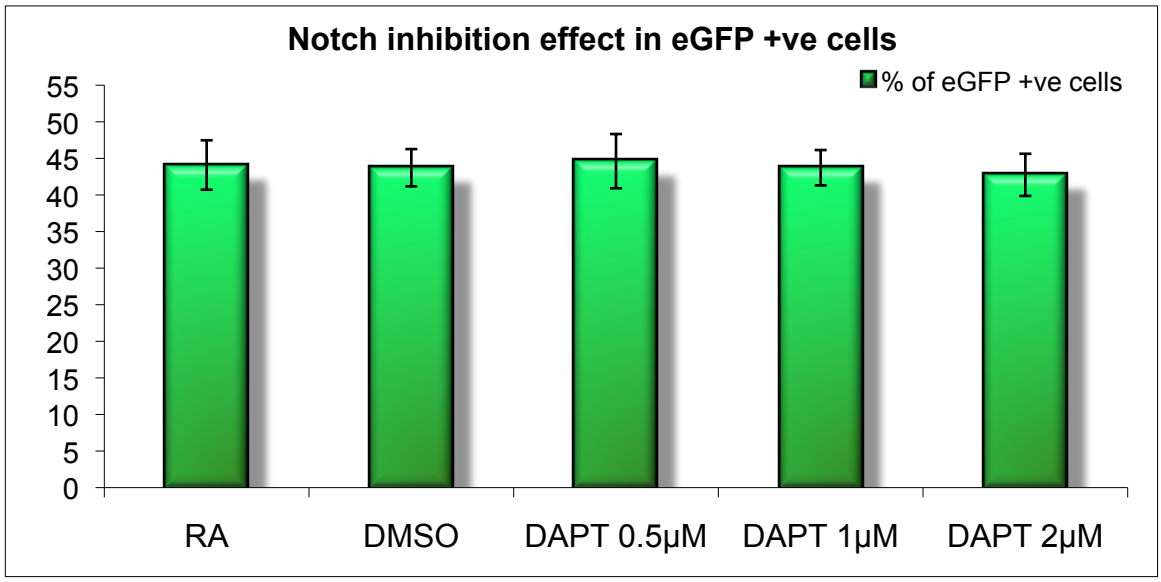


Figure 52. Flow cytometric analysis of Notch inhibition in eGFP positive cells after 72 hrs of RA treatment. Various concentrations of DAPT were used together with RA (10^{-5} M) to determine the effect of Notch inhibition. DMSO was used as carrier for DAPT. RA only treatment was used as control. Error bars indicate standard error of the mean (two biological replicates *Stra8*-eGFP/*Sycp1*-DsRED and *Stra8*-eGFP/*Pgk2*-DsRED cell lines and a technical quadruplicate for each biological sample). No statistically significant difference between the percentages of eGFP+ve cells in each treatment condition was detected.

5.2.2 Notch inhibition effect in early meiotic-like cells

Results from our immunohistochemical analysis of Notch protein components described in section 3.3 revealed expression of Notch receptors and ligands in Sertoli and spermatogonia cells respectively. Specifically, it was observed that NOTCH1 receptor was activated in Sertoli cells during stages VI to VIII of spermatogenesis and curiously, JAGGED2 ligand expression was discovered in B-spermatogonia and pre-Leptotene spermatocytes, germ cell types that are found in the same epithelia stages (V to VIII) where NOTCH1 was activated. The stage-dependent cyclic activation of NOTCH1 receptor and distribution pattern of JAGGED2 suggested the possible requirement of these signalling events for mitosis to meiosis progression and meiotic initiation.

To investigate if Notch signalling function is required in the progression of pre-meiotic germ-like cells to meiotic-like cells we used the *Stra8*-eGFP/*Sycp1*-DsRED cell line. This reporter cell line allowed us to examine the effect of Notch pathway blockage by analysing the number of *Sycp1*-DsRED positive cells in culture conditions where Notch activation was chemically inhibited. For this reason, *Stra8*-eGFP/*Sycp1*-DsRED cells

were treated with γ-secretase inhibitor DAPT during a 72 hrs RA induction and DsRED positive cells were determined by flow cytometry.

Flow cytometry analysis of *Sycp1*-DsRED positive cells showed that meiotic initiation in our *in vitro* culture system doesn't require Notch activation as the percentage of DsRED positive cells in DAPT treated cells didn't show any statistically significant difference with cells treated only with RA (Figure 53).

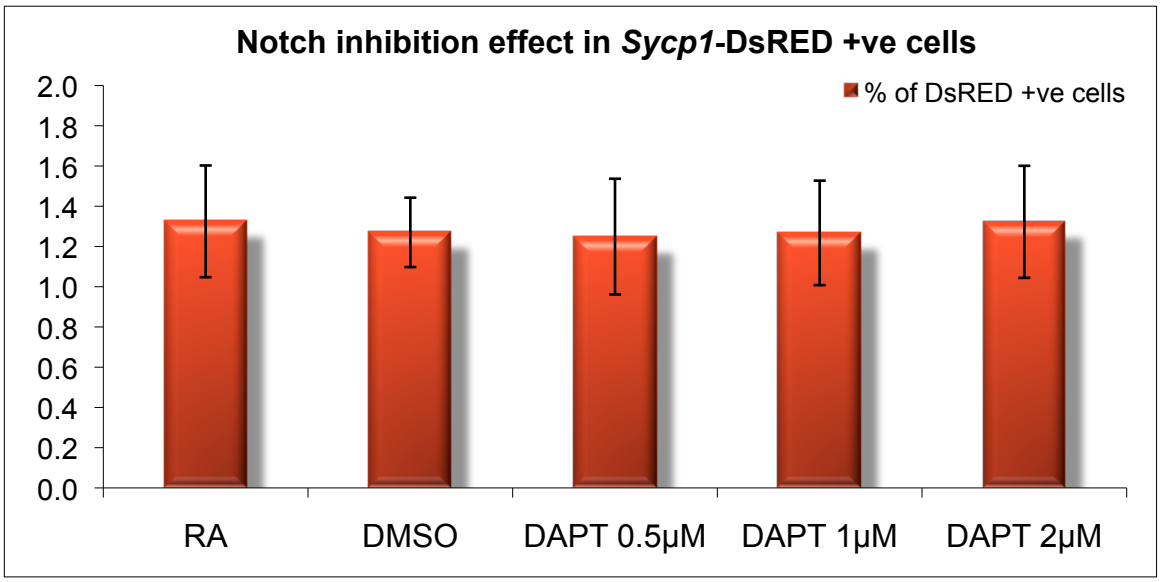


Figure 53. Flow cytometric analysis of Notch inhibition in *Sycp1*-DsRED positive cells after 72 hrs of RA treatment. Various concentrations of DAPT were used together with RA (10^{-5} M) to determine the effect of Notch inhibition. DMSO was used as carrier for DAPT. RA only treatment was used as control. Error bars indicate standard error of the mean (three independent cell culture batches of *Stra8*-eGFP/*Sycp1*-DsRED cell line and a technical triplicate for each batch). No statistically significant difference between the percentages of DsRED+ve cells in each treatment condition was detected.

5.2.3 Notch inhibition effect in late meiotic-like cells

In our current study expression of Notch receptors (NOTCH1, 2 and 3) and ligands (DELTA1 and JAGGED1) in germ cells that had entered meiosis (leptotene spermatocytes) and through out meiotic progression (pachytene, diplotene and secondary spermatocytes) suggested the possible requirement of Notch signalling activation for cell progression and maturation.

In order to determine whether Notch activation is a requisite for meiotic advancement we disrupted activation of Notch receptors in our double transfected cell line bearing the late

meiotic marker, *Pgk2*-DsRED. We treated our *Stra8*-eGFP/*Pgk2*-DsRED cell line with RA and with or without DAPT for 72 hrs before they were harvested for flow cytometric analysis.

Our analysis showed no reduction in the number of *Pgk2*-DsRED positive cells after RA and DAPT treatment in comparison with RA only treated cells. Interestingly, even though we found three Notch receptors (NOTCH1, 2 and 3) to be expressed in meiotic cells in our immunohistochemical analysis (see section 3.3.1), Notch pathway inhibition did not impair the emergence of late meiotic-like cells (*Pgk2*-DsRED positive cells) when γ-secretase inhibitor was used in the culture media.

These surprising results imply that activation of the Notch signalling pathway is not required for meiotic progression of spermatocytes, but on the other hand also do not rule out that Notch pathway might play another less essential role in meiotic progression (Figure 54).

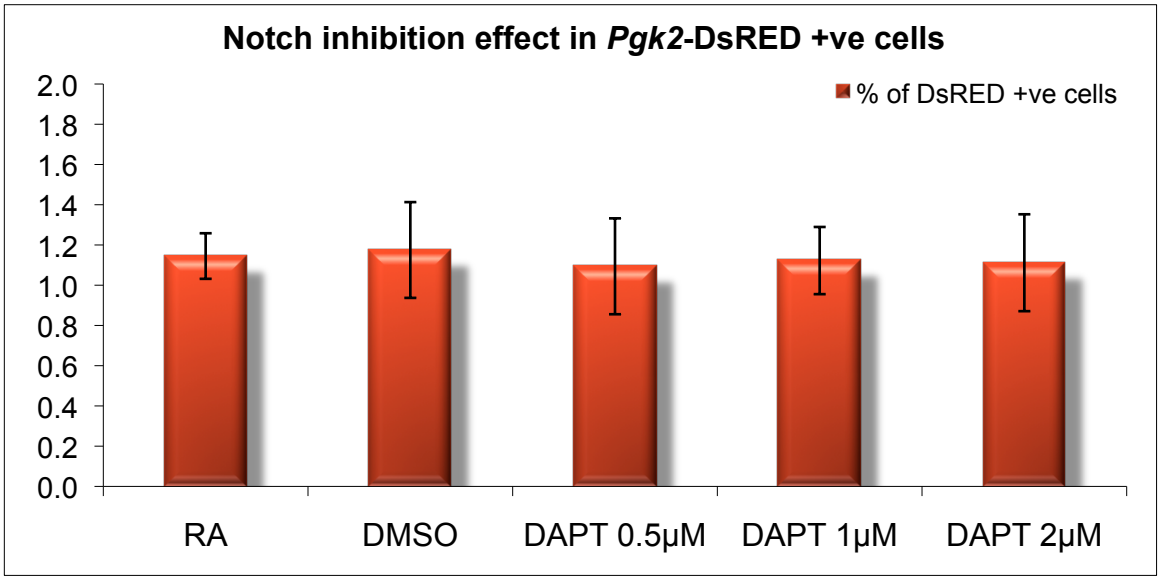


Figure 54. Flow cytometric analysis of Notch inhibition in *Pgk2*-DsRED positive cells after 72 hrs of RA treatment. Various concentrations of DAPT were used together with RA (10^{-5} M) to determine the effect of Notch inhibition. DMSO was used as carrier for DAPT. RA only treatment was used as control. Error bars indicate standard error of the mean (three independent cell culture batches of *Stra8*-eGFP/*Pgk2*-DsRED cell line and a technical triplicate for each batch). No statistically significant difference between the percentages of DsRED+ve cells in each treatment condition was detected.

To further investigate the potential role of the Notch pathway in meiotic progression we examined whether Notch inhibition had any influence in meiotic-specific transcripts

during RA induction in our cell culture system. For this examination, *Pgk2*-DsRED positive cells were isolated by FACS from the previously mentioned various treatment conditions and total RNA was purified. This was then followed by cDNA synthesis and semi-quantitative expression analysis (Multiplex-PCR) was used to analyse the expression of meiotic specific transcripts when Notch signalling is disrupted.

Expression analysis showed that blockade of Notch signalling had no effect in meiotic specific genes (Figure 55).

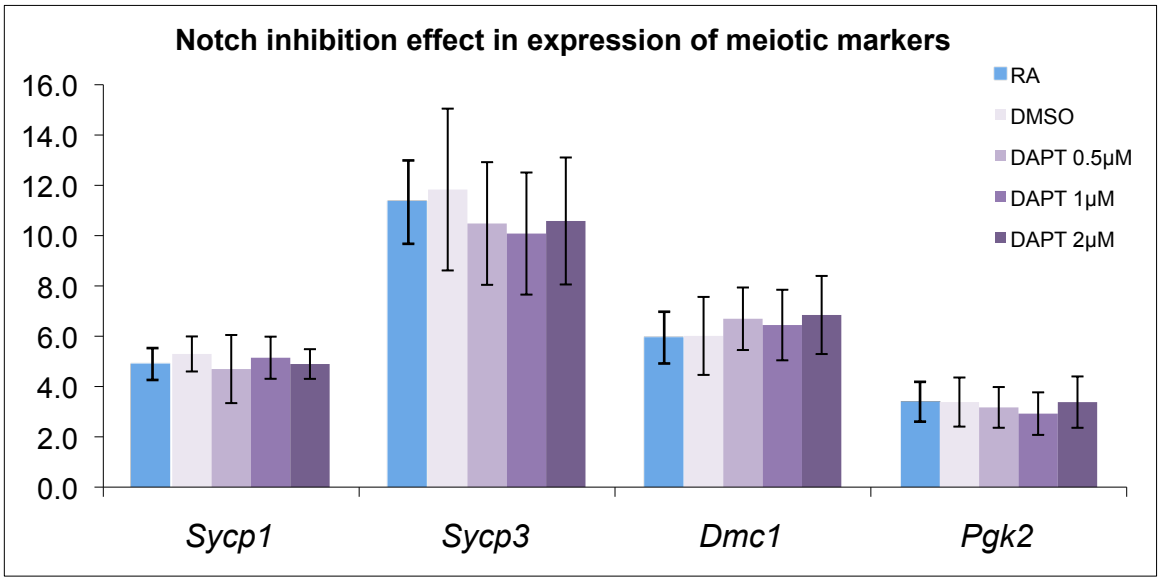


Figure 55. Semi-quantitative expression analysis of meiotic markers in Notch inhibition conditions. The ratio between the meiotic marker and the housekeeping gene *Hprt1* is plotted for each culture condition. RA sample was used as control. Error bars indicate standard error of the mean (RNA was isolated from *Pgk2*-DsRED positive cells from three independent cell culture batches). No statistically significant difference between the expression of each meiotic marker in each treatment condition was detected.

5.3 Notch signalling activation during *in vitro* germ cell development

Having observed that inhibition of Notch activation during *in vitro* germ cell development didn't affect the formation of pre-meiotic, early meiotic or late-meiotic-like cells the next question was to determine the consequences of induced Notch signalling activation in our cell culture system.

To investigate the effect of induced Notch activation we chose to expose our cells to soluble Notch ligands (ligand extracellular domains) that will bind to Notch receptors and activate the Notch signalling pathway cascade. This method has been commonly used to shed light into the roles of Notch pathway and has been reported in many high impact factor journals (Yu et al., 2008, Kuijk et al., 2013)

5.3.1 Notch signalling activation effect in pre-meiotic-like cells

Although chemical treatment with the γ-secretase inhibitor (DAPT) was found to have no influence in *Stra8*-eGFP positive cells, we wanted to examine whether induced Notch signalling activation would have any effect in the number of pre-meiotic-like cells after 72 hrs of RA induction. For this reason both *Stra8*-eGFP/*Sycp1*-DsRED and *Stra8*-eGFP/*Pgk2*-DsRED cell lines were cultured with Notch ligand proteins (DELTA1,3,4, JAGGED1 and JAGGED2) in which their extracellular domain had been fused at the C-terminus to the Fc portion of human IgG. Previous studies have shown that the Notch ligand's extracellular domain must be immobilized to induce Notch activation (Varnum-Finney et al., 2000). Therefore, cells were cultured in previously coated plates with either immobilized ligands or human-IgG as control. After 72 hrs of culturing the cells in various concentrations of Notch ligand-coated plates, and with RA added in the culture media, cells were collected and analysed by flow cytometry and the percentage of eGFP positive cells in each culture condition was recorded (Figure 56).

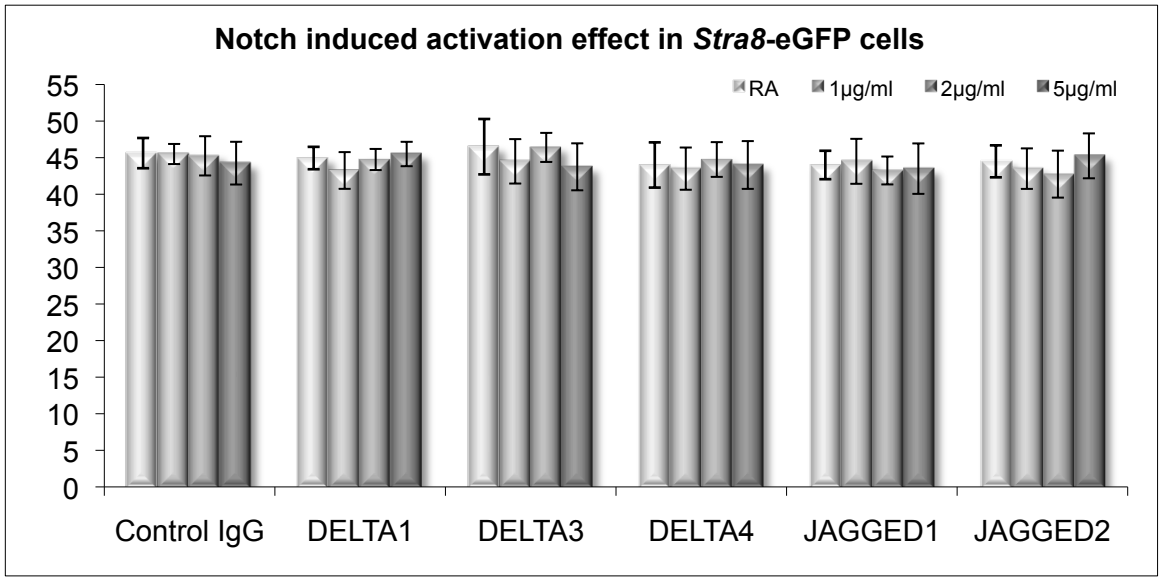


Figure 56. Flow cytometric analysis of Notch ligands activation in *Stra8*-eGFP positive cells during 72 hrs of RA treatment. Immobilized human-IgG coated plates and RA only treated cells were used as controls. Various concentrations of Notch ligand proteins were used together with RA (10^{-5} M) to determine the effect of induced Notch activation. Error bars indicate standard error of the mean (two biological replicates *Stra8*-eGFP/*Sycp1*-DsRED and *Stra8*-eGFP/*Pgk2*-DsRED cell lines and a technical triplicate for each biological sample). No statistically significant difference between the percentages of eGFP+ve cells in each treatment condition was detected.

Again, as in Notch inhibition experiment, induced Notch activation with various Notch ligands did not appear to have any effect on the number of pre-meiotic-like cells after RA induction. In this experiment, as neither DELTA1, 3 and 4, or JAGGED 1 and 2 appeared to enhance the appearance of more *Stra8*-eGFP cells we can suggest that Notch signalling pathway is not involved in the formation of pre-meiotic like cells.

5.3.2 Notch signalling activation effect in early meiotic-like cells

One of the Notch ligands found to be expressed in differentiated spermatogonia and pre-leptotene spermatocytes just before they enter meiosis and become leptotene spermatocytes was JAGGED2. Although, it has been reported in the invertebrate *C. elegans* that Notch signalling is required to maintain the balance between proliferation of progenitor cells (undifferentiated spermatogonia) and differentiating germ cells that are destined to enter meiosis (Kimble and White, 1981, Yochem and Greenwald, 1989), removal of Notch function during RA induction period was found to have no effect in the formation of pre-meiotic-like (Sycp1-DsRED) cells in our cell culture platform (Figure 53).

To investigate further how the observed expression pattern, with Notch components present in pre-meiotic differentiating cells, reflects the role of Notch signalling we treated our *Stra8*-eGFP/*Sycp1*-DsRED cell line with the extracellular domain of JAGGED2 ligand. The purpose of this experiment was to examine whether induced Notch signalling activation would promote pre-meiotic cells to enter meiosis. In this case more *Sycp1*-DsRED positive cells would be observed, if induced Notch signalling would act as regulator/inhibitor, a reduced number of *Sycp1*-DsRED positive cells would appear.

Therefore, *Stra8*-eGFP/*Sycp1*-DsRED cells were cultured in pre-coated plates of various concentrations of JAGGED2 extracellular domain and treated with RA for 72 hrs. This

was followed by trypsinization to detach the cells and achieve a single cell suspension that was then analysed by flow cytometry.

Analysis in *Stra8*-eGFP/*Sycp1*-DsRED cells showed no statistically significant difference in *Sycp1*-DsRED positive cells between cells plated in JAGGED2 coated plates and cells cultured in IgG protein coated plates or uncoated plates (Figure 57).

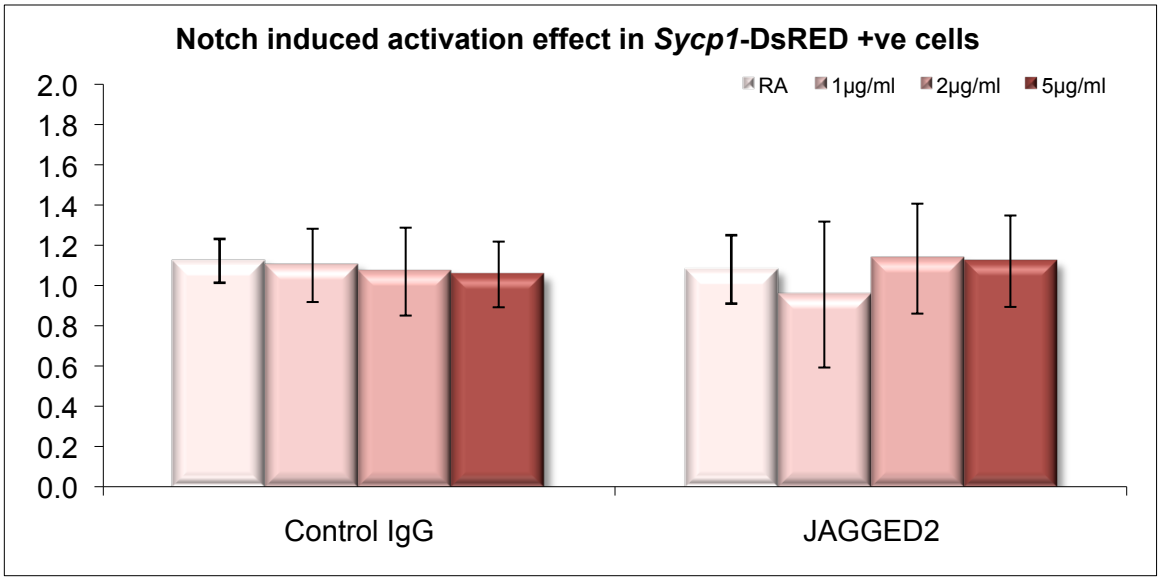


Figure 57. Flow cytometric analysis of induced Notch activation in *Sycp1*-DsRED positive cells during 72 hrs of RA treatment. Immobilized human-IgG coated plates and RA only treated cells were used as controls. Various concentrations of Notch ligand protein were used together with RA (10^{-5} M) to determine the effect of induced Notch-activation. Error bars indicate standard error of the mean (three independent cell culture batches of *Stra8*-eGFP/*Sycp1*-DsRED cell line and a technical triplicate for each batch). No statistically significant difference between the percentages of DsRED+ve cells in each treatment condition was detected.

This result suggests not only that Notch signalling is not required for progression of spermatogonia-like cells from mitosis to meiosis, and also reveals that Notch pathway activation is not involved in the regulation or maintenance of the pre-meiotic-like cells in our *in vitro* cell culture system.

5.3.3 Notch signalling activation effect in late meiotic-like cells

To further evaluate any functional role of Notch signalling during *in vitro* germ cell development, we investigated how induced activation of Notch signalling would affect the progression of germ cells during meiosis. Our expression analysis showed that

testicular germ cells bare two Notch ligands, DELTA1 and JAGGED1 during meiotic progression (see section 3.3.2).

We therefore examined the effect of induced Notch pathway activation during meiotic progression in our cell line that harboured the late meiotic marker *Stra8*-eGFP/*Pgk2*-DsRED. Three independent (different passage number) cell culture experiments were performed and each time a technical triplicate was used for each condition. As before, cells were seeded in culture plates coated with DELTA1 or JAGGED1 ligands, or just coated with IgG protein or uncoated plates as controls. All cells were treated with RA for 72 hrs and after that cells were dissociated, collected and then processed by flow cytometry to examine the number of *Pgk2*-DsRED positive cells.

Notch induced activation during meiotic progression didn't seem to have any effect in *Pgk2*-DsRED positive cells when JAGGED1 was used to activate Notch signalling. Interestingly though, when cells were plated in DELTA1 coated plates the number of Pgk2-DsRED positive cells was found to have a statistically significant increase in comparison with control plates (Figure 58).

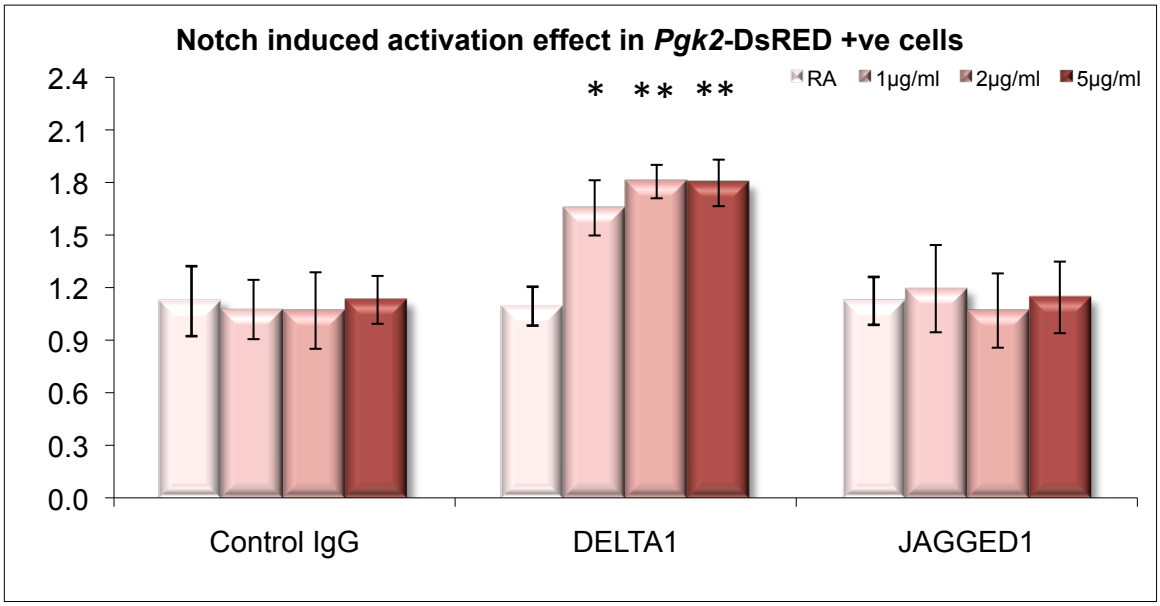


Figure 58. Flow cytometric analysis of Notch ligands activation in *Pgk2*-DsRED positive cells during 72 hrs of RA treatment. Immobilized human-IgG coated plates and RA only treated cells were used as controls. Various concentrations of Notch ligands proteins were used together with RA (10^{-5} M) to determine the effect of induced Notch-activation. Percentages of DsRED positive cells are plotted. Error bars indicate standard error of the mean (three independent cell culture batches of *Stra8*-eGFP/*Pgk2*-DsRED cell line and a technical triplicate for each batch). Star marks represent P-values statistically different from control (RA), * P≤0.05, **P≤0.01.

We then wanted to test that the extracellular domains of DELTA1 ligands mediate their effect through the Notch signalling cascade, and not via another biological effect. For this reason we cultured the *Stra8*-eGFP/*Pgk2*-DsRED cells in DELTA1 ligands coated plates and with or without the addition of DAPT. If DELTA1 ligands exert their effect in promoting progression of meiotic-like cells by Notch signalling receptors, the addition of DAPT should abolish this effect. And indeed, investigation showed that the addition of γ-secretase, a Notch inhibitor, in the culture media, resulted in similar percentage of *Pgk2*-DsRED positive cells as in control conditions (Figure 59).

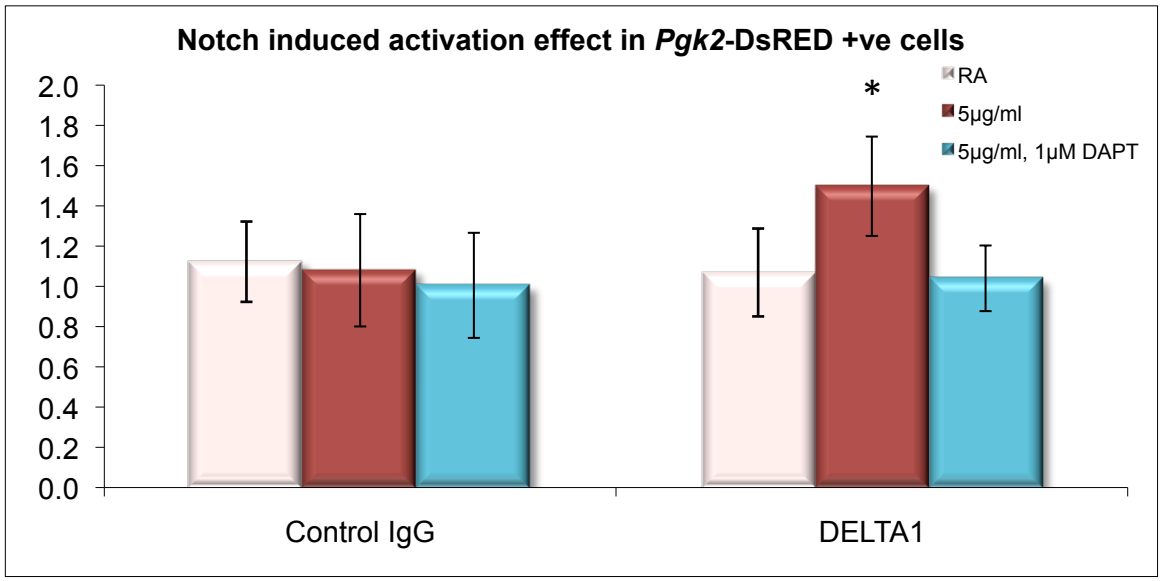


Figure 59. Flow cytometric analysis of Notch ligands activation by DELTA1 ligands in *Pgk2*-DsRED positive cells during 72 hrs of RA treatment. Immobilized human-IgG coated plates and RA only treated cells were used as controls. DAPT was used together with DELTA1 ligands and in the presence of RA (10^{-5} M) to determine the effect of induced Notch-activation. Percentages of DsRED positive cells are plotted. Error bars indicate standard error of the mean (three independent cell culture batches of *Stra8*-eGFP/*Pgk2*-DsRED cell line and a technical triplicate for each batch). Star marks represent P-values statistically different from control (RA), * P≤0.05.

We also went a step further and tested the expression of Notch target genes *Hes1* and *Hes3* to show that DELTA1 ligands activate Notch signalling pathway receptors and in turn the intracellular domains of Notch receptors activate the expression of the known downstream Notch target genes. To examine that, total RNA was isolated from *Pgk2*-DsRED positive cells and was followed by cDNA synthesis and semi-quantitative expression analysis (Multiplex-PCR) as described before (see materials and methods section 2.1.12).

Expectedly, the results showed that DELTA1 ligands increased the expression of Notch effector genes in meiotic-like cells and also that the expression was abolished when DAPT was added (Figure 60).

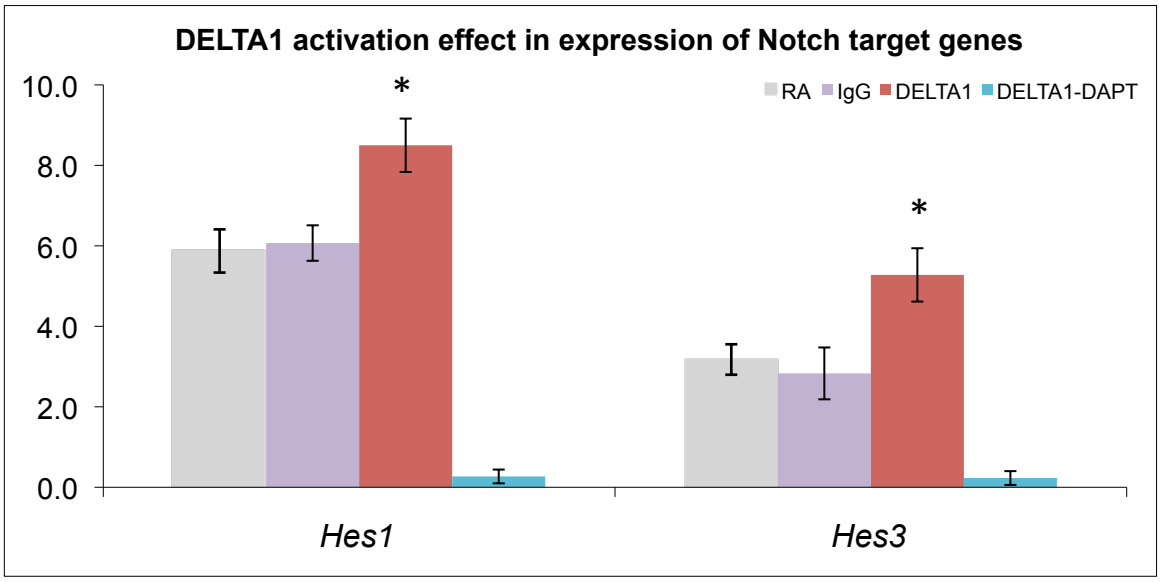


Figure 60. Semi-quantitative expression analysis of Notch downstream target gene transcripts in *Pgk2*-DsRED positive cells under DELTA1 induced Notch activation conditions. cDNA from cells treated with just RA or IgG was used as a positive control. The ratio between the gene of interest and *Hprt1* housekeeping gene is plotted. Error bars indicate standard error of the mean (three independent cell culture batches of *Stra8*-eGFP/*Pgk2*-DsRED cell line and a technical triplicate for each batch). Star marks represent P-values statistically different from control (RA), * P≤0.05.

Additionally, we examined what effect the DELTA1 and JAGGED1 induced Notch pathway activation had on meiotic-specific genes during RA induction in our cell culture system. Hence, *Pgk2*-DsRED positive cells were sorted by FACS from four different treatment conditions and total RNA was purified. cDNA was then synthesised and expression was analysed by semi-quantitative expression analysis (Multiplex-PCR).

Interestingly, in our expression analysis we did not observe any changes in the expression of meiotic transcripts, meaning that DELTA1 or JAGGED1 activation have no direct influence in meiotic genes (Figure 61).

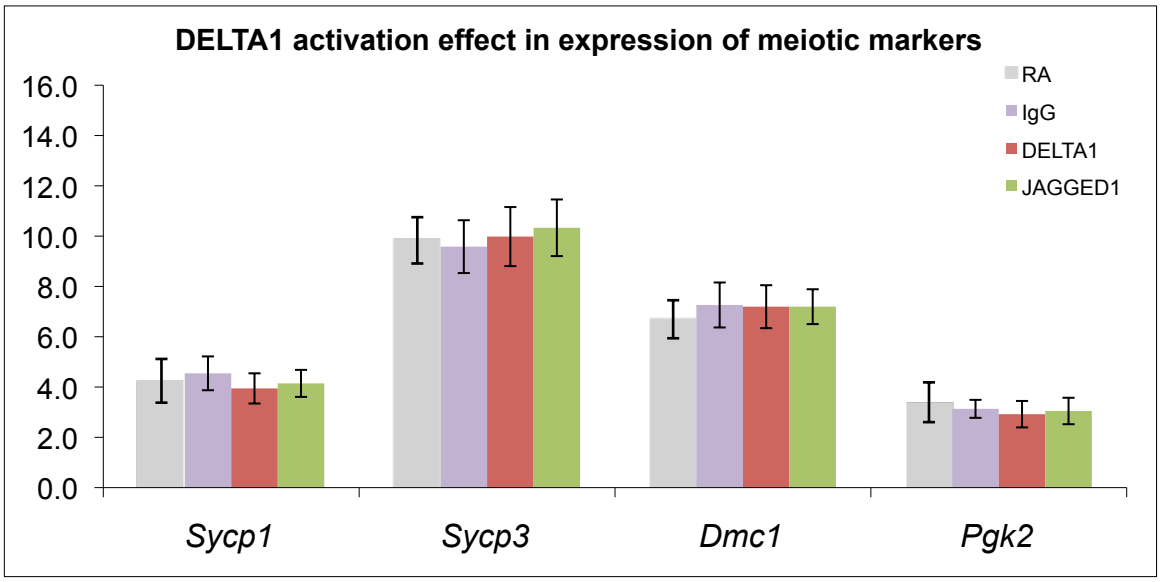


Figure 61. Semi-quantitative expression analysis of meiotic markers in DELTA1 and JAGGED1 activation conditions. The ratio between the meiotic marker and the housekeeping gene *Hprt1* is plotted for each culture condition. RA sample was used as control. Error bars indicate standard error of the mean (RNA was isolated from *Pgk2*-DsRED positive cells from three independent cell culture batches). No statistically significant difference between the expression of each meiotic marker in each treatment condition was detected.

These results suggest that JAGGED1 and DELTA1 ligands play a different role during meiosis but also more importantly demonstrate that activation of Notch pathway via DELTA1 ligands promotes meiotic progression. However, this promoting effect doesn't seem to also increase the expression of specific meiotic genes more than RA treatment condition.

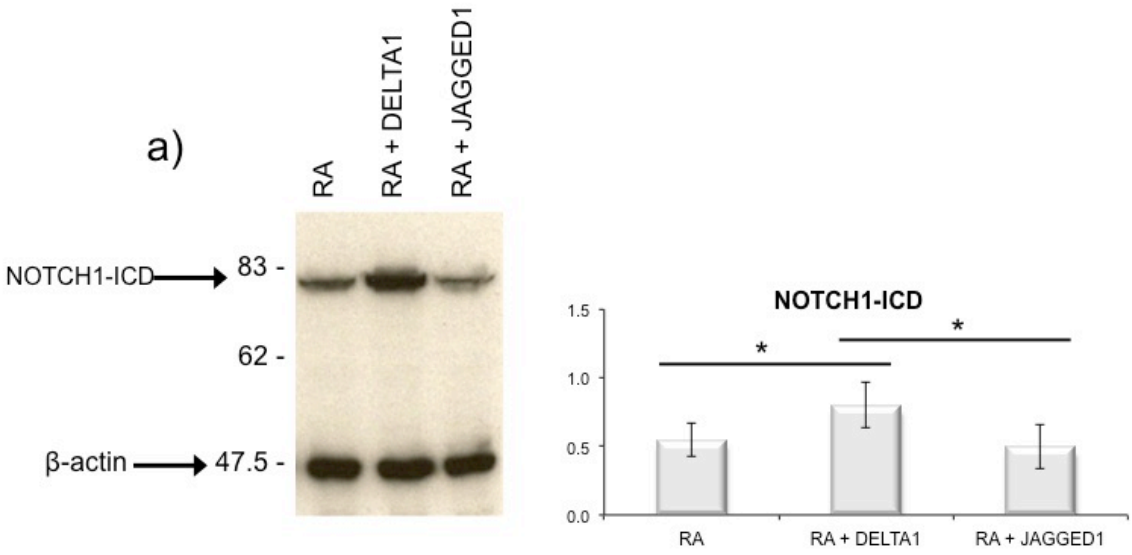
5.3.4 Investigation of Notch receptors during meiotic progression

The finding that Notch signalling activation via DELTA1 ligands resulted in more meiotic-like cells after 72 hrs of RA induction, and the confirmation that this effect utilizes the known Notch signalling cascade, brought the next question: Which Notch receptors are utilized by DELTA1 and JAGGED1 ligands?

We addressed this question by examining the NOTCH^{-ICD} of the receptors found to be expressed in our immunohistochemical analysis NOTCH1, 2 and 3. In induced-Notch activation conditions, with the cells cultured in pre-coated plates with Notch ligands, the cleaved "activated" NOTCH^{-ICD} protein would be expected to increase if a Notch ligand would bind and activate that particular type of Notch receptor. Therefore, to assay the

quantity of each NOTCH^{-ICD} receptor protein in *Pgk2*-DsRED positive cells, cultured with different Notch ligands, we used a western blot (immunoblot) technique.

The analysis of NOTCH^{-ICD} protein using antibodies against activated Notch intracellular domains showed an increase in NOTCH1^{-ICD} when cells were culture in DELTA1 coated plates indicating a preferential Notch-ligand binding of these two components (Figure 62 a). Similarly, NOTCH2^{-ICD} was found to have a statistically significant increase when cells were plated with JAGGED1 ligand (Figure 62 b). Finally, NOTCH3^{-ICD} did not show any change in both culture conditions, although the presence of protein indicates activation of the receptor in all culture conditions (Figure 62 c).



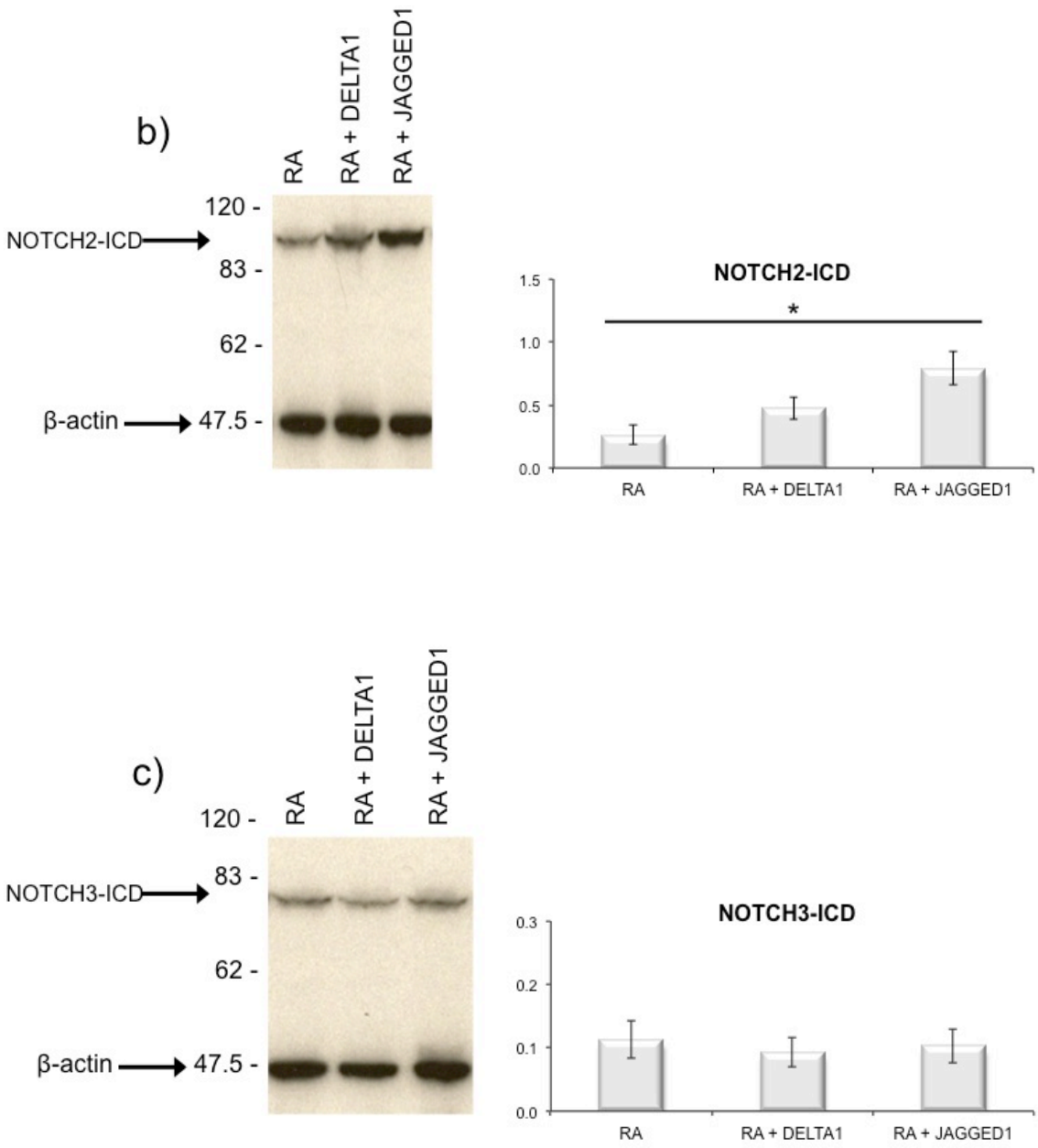


Figure 62. Representative westerns of ligand-induced activation assay for various Notch receptors in the presence or absence of Notch ligands during RA induction. Cells were harvested 72 hrs after induction and analysed by western blotting. Blots were probed with a NOTCH^{-ICD} primary antibody and an HRP-conjugated secondary antibody. The migration of molecular weight markers is indicated on the left of the Westerns. The graphs show the relative amount of the NOTCH protein in each of the culture conditions compared to the β-actin loading control. Data is presented as the mean ± SE and is representative of three experiments. Star marks represent P-values statistically different from control (RA), * P≤0.05.

In the light of these observations we can suggest that NOTCH1-DELTA1 interaction might be responsible for the increase in *Pgk2*-DsRED positive cells observed when cells were cultured in DELTA1 ligand pre-coated plates.

5.3.5 Investigation of Notch activation in post-meiotic-like cells

Considering that induced Notch activation was found to increase late-meiotic-like cells in our cell culture system, we investigated whether induced activation by any of the NOTCH ligands would result in post-meiotic-like cells, something that we did not observed with just RA treatment.

We examined this by plating the *Stra8*-eGFP/*Prm1*-DsRED cell line in pre-coated plates with one of the following Notch ligands DELTA1, DELTA3, DELTA4, JAGGED1 and JAGGED2.

Despite our efforts we did not observe any *Prm1*-DsRED positive cells that would indicate post meiotic-like cell generation in our *in vitro* platform.

5.4 Summary and discussion

Having determined the Notch pathway components expressed during the process of spermatogenesis, we then exploited the versatility of ES cells and established an *in vitro* ES cell-based system that facilitates the investigation of germ cell development and provides direct access to germ-like cells. Thus, in this chapter we took advantage of the capabilities of the *in vitro* platform and used it to address the role of Notch signalling in spermatogenesis to help us understand the effect that receptors or ligands of this pathway have on this process.

Initially, we used a chemical treatment to disrupt Notch signalling activity in our *in vitro* model system. The aim was to investigate the importance of the Notch pathway during germ cell development, and for this reason we analysed the effect that Notch signalling disruption had at various time points of our *in vitro* germ cell development platform.

But first we verified that the γ-secretase inhibitor used in our study (DAPT) efficiently blocked Notch signalling activation when applied in the *in vitro* culture system, by

examining the expression of Notch target genes. Then, we chose to inhibit the Notch signalling pathway and examine the effect at specific developmental points, using our stage-specific reporter cell lines established in this project. This allowed us to separate early and late functions of Notch components.

We started by testing the effect of a blockade of Notch signalling on the formation of pre-meiotic like cells (*Stra8*-eGFP positive cells) from ES cells. Disruption of Notch signalling was not found to alter the number of pre-meiotic like cells emerging after 72 hrs of RA, indicating that the Notch pathway activation is not required in this process. This result did not come as a surprise, given that we did not find Notch components present in spermatogonia progenitors in our expression analysis. In fact, in other ES differentiation systems such as in neural stem cell differentiation from ES cells, Notch inhibition was not only shown not to prevent neuronal differentiation but was found to result in acceleration and enhancement of neuronal differentiation (Borghese et al., 2010, Crawford and Roelink, 2007). Although in our case we did not find Notch inhibition to enhance pre-meiotic like cell formation, acceleration of the process by Notch inhibition is something that remains to be tested.

A result that also supports the idea of Notch not playing a role in pre-meiotic cell induction emerged when we investigated the activation of Notch signalling by various Notch ligands. Similarly to the result obtained when Notch inhibition was tested, we did not observe activation of Notch to have an impact in pre-meiotic-like cell formation.

Next, following our finding that activation of Notch1 receptor in Sertoli cells, in a stage-dependent manner, coincided with the expression of Jagged2 in differentiating spermatogonia we examined whether disruption of Notch signalling would influence the formation of early meiotic-like cells. To test this, we analysed the percentage of *Sycp1*-DsRED cells in RA and RA plus DAPT treated cultured cells. The analysis indicated that removal of Notch signalling function does not promote or impair meiotic induction of germ-like cells.

A similar result was observed when immobilised JAGGED2 ligands were used to induce Notch signalling activation in our *in vitro* cell culture system. Jagged2 activation was not found to increase or decrease the number of early meiotic cells, another indication that the NOTCH1-JAGGED2 interaction is not required for mitosis to meiosis transition.

Given the fact that Sertoli cells span from the basal membrane to the lumen of the seminiferous tubule, censoring and tightly controlling the maturation process by responding to hormones and other signals (see introduction section 1.2.2), it's most likely that cyclic Notch1 activation in Sertoli cells by Jagged2 ligand in differentiating spermatogonia serves a monitoring purpose and provides another feedback step to the process. Knowing also that Sertoli cells support and regulate the maintenance of the spermatogonia stem cell progenitor pool (Payne et al., 2010), the NOTCH1-JAGGED2 feedback step might be linked with this response.

This suggestion is supported firstly by our examination on Notch inhibition and activation having no effect in the formation of meiotic-like cells, and secondly by another recent study where NOTCH1 constitutive activation in Sertoli cells drastically affected the physiology and population of gonocytes (the early form of spermatogonia stem cells). They found that mutant mice constitutively expressing NOTCH1^{-ICD} exhibited premature exit of gonocytes from mitotic arrest and premature differentiation before birth. In their molecular analysis they demonstrated that expression of Sertoli-specific genes involved in spermatogonia stem cell maintenance was disrupted. However, in the same study they reported that the morphology of mutant Sertoli cells was normal (Garcia et al., 2013).

In an ex-vivo study where rat testicular tissue sections were culture with anti-NOTCH1 or anti-JAGGED2 antibody, the round spermatid number decreased after 5-7 days, and in contrast spermatocytes number increased after 11 days (Hayashi et al., 2001). Although these results are in a different model organism, they lack credibility. Instead of using IgG antibody to treat control samples the researchers only used testicular tissues cultured without antibodies. In this way one cannot conclude that the effect observed was not due to a non-specific response to the antibodies and not because of their specificity in blocking Notch components.

We then examined the role of Notch receptors and ligands during meiotic progression after expression analysis revealed 3 Notch receptors and 2 Notch ligands during the meiotic phase. The first assay, which involved investigation of Notch inhibition, showed that disruption of Notch signalling had no impact in the generation of late meiotic-like cells (*Pgk2*-DsRED cells). Furthermore, the molecular analysis of the expression of meiotic specific genes under Notch blockade condition also showed no difference in comparison to untreated conditions. However, when Notch induced activation was

examined for the two Notch ligands revealed to be expressed in meiotic progression (DELTA1 and JAGGED1) the results were quite different.

Remarkably, induced activation of Notch pathway by Delta1 ligands was found to increase the number of late meiotic-like cells by almost 40%. Additionally, an increase in the expression of Notch effector genes provided more evidence that DELTA1-induced Notch signalling activation took place, supporting in this way the likelihood that this was a Notch pathway mediated effect. Notch induced activation by JAGGED1 on the other hand, was not found to exhibit any effect in the generation of late-meiotic cells.

These exciting results first imply that Notch pathway components are involved in meiotic progression and second that not all Notch components play similar role in meiotic progression of spermatocytes, something that has been observed in many developmental systems (Benedito et al., 2009) and reviewed here (Andersson et al., 2011). But on the other hand induced activation by Delta1 ligands also reveals that Notch pathway is interacting with the machinery that controls meiotic progression. Although, no alteration in the expression of meiotic specific transcripts during DELTA1 induced activation, suggests that Notch pathway doesn't directly influence meiotic mechanism.

Interestingly, in another ES-derived *in vitro* system researchers observed that transient Notch signalling pathway activation enhanced generation of hematopoietic cells from committed ES cells (Yu et al., 2008). Although this finding suggests that Notch pathway has a promoting role in generation of differentiating cells from progenitor cells it also implies that the effect observed in our culture system, with DELTA1, might have to do with the fact that our progenitor cells in our culture system are not real spermatogonia stem cells but close resemblant of them.

Based on our Western blot analysis DELTA1 ligand seemed to favour the interaction with NOTCH1 receptor from the other two NOTCH2 and NOTCH3 in late meiotic-like cells. In contrast JAGGED1 was shown to interact mostly with NOTCH2. NOTCH1, NOTCH2 and NOTCH3 receptors found to be expressed in an overlapping pattern in spermatocytes of the seminiferous tubules. Homozygous *Notch3* knockout mice appear viable and fertile with normal spermatogenesis (Krebs et al., 2003). The same is also true for *Notch4* (Krebs et al., 2000). Mice that carry a knockout mutation of Notch2 die mid-gestation, precluding analysis of a gonad phenotype. However, mice with a hypomorphic

mutation in Notch2 are viable and no defects in fertility or spermatogenesis have been reported (McCright et al., 2001). Therefore, only Notch1 and most likely Notch2 receptor appear primarily to be involved in germ cell development.

Although, further studies are required to attribute the role of each receptor, our current data nonetheless suggest a Notch1-Delta1 interaction might promote meiotic progression and thus, these two components would be ideal candidates for *in-vivo* studies.

We should point out that although inhibition of Notch using γ-secretase inhibitor DAPT is widely used, it's function is not limited in Notch signalling and it may also inhibit signalling by other γ-secretase dependant signalling pathways such as the ephrins (Tomita et al., 2006).

We shall also not forget at any point that in our study we are dealing with an *in vitro* environment that might not mimic exactly the *in-vivo* situation, and the results should be treated with caution and as guidance for *in-vivo* experiments. We should also keep in mind the complexity of Notch pathway, its regulation in cis and trans and the co-localization of ligands and receptors in the same cell (Fehon et al., 1990). Similarly, we should consider the ligand-receptor interaction and how this controls endocytosis of the receptor of the ligand (Hansson et al., 2010) and the Notch signalling crosstalk with other signalling pathways such as receptor tyrosine kinase (RTK)–RAS, WNT, Hedgehog, transforming growth factor-β (TGFβ) and Janus kinase (JAK)–signal transducer (Hurlbut et al., 2009, Flaherty et al., 2009).

Recently anti-Notch receptor specific antibodies have been generated (Falk et al., 2012) so it would be very informative to use those antibodies in our culture system to dissect the role of Notch receptor in germ cell development.

Overall these experiments suggest that a Notch1-Jagged2 interaction might be responsible for providing a feedback signal to Sertoli cells to regulate and control the progenitor pool, and revealed that Delta1 ligand can promote meiotic progression and most likely by interacting with Notch1 receptor. Both findings can provide a working framework to investigate the Notch signalling pathway in genetic models of infertility in the mouse. If Notch interactions discovered in our ES cell based *in vitro* system prove to have the same pivotal influence *in-vivo* then specific substances that would trigger or mimic these interactions could help to cure patients with male infertility.

Chapter 6. Concluding remarks and future work

From the first description of cells involved in the creation of male gametes (sperm) by Enrico Sertoli an Italian physiologist in 1865, it was apparent that spermatogenesis is not a simple process. Since then, there has been a rapid expansion in our understanding and spermatogenesis is now is considered one of the most complex cellular differentiation processes, which is endowed with the task of generating the highly specialized cells called spermatozoa that transmit the genetic information to the next generation. This complicated process that involves mitotic cell division, meiosis and the process of spermiogenesis is maintained by male germline stem cells (spermatogonia stem cells) that self-renew and give rise to large number of differentiating germ cells. The regulation of the differentiation and maturation of the germ cells largely depends on both endocrine, such as follicle stimulating hormone and luteinizing hormone, and paracrine signals such as retinoic acid. In the last decade however a few studies have emerged that suggest also the involvement of juxtacrine signals in the process of spermatogenesis. The signalling mechanism of cell-cell communication that requires direct contact of adjacent cells was shown to be the Notch signalling pathway (Hahn et al., 2005, Hayashi et al., 2004a, von Schonfeldt et al., 2004).

The Notch pathway, first identified in *Drosophila melanogaster,* has been studied extensively in many organisms and has been found to contribute to the formation, growth and development of embryonic tissue and to control numerous cell-fate specification events and differentiation of progenitor cells in various cell types. Studies in *Drosophila* and *C.elegans* have reported receptors of the Notch family to regulate germ line stem cell differentiation and entry of the germ cells to meiosis respectively (Hardy et al., 1979, Francis et al., 1995). However, the precise role of Notch signalling components in spermatogenesis is not well understood.

The complexity of the Notch pathway and the numerous Notch family members make the genetic analysis on all the Notch receptors and ligands very laborious and hugely expensive. Therefore, in this study, we aimed to shed light to the function of Notch pathway components during the spermatogenic process using an *in vitro* approach. We exploited and evolved a previously reported protocol which demonstrated the potential of ES cells to differentiate to germline cells (Nayernia et al., 2006). Thus, to advance our

understanding of Notch function first we identified the Notch receptors and ligands expressed during spermatogenesis, second we established an ES cell-based germline differentiation *in vitro* platform which provided access to different spermatogenic stages, and third we investigated the function of specific Notch components via manipulation of the Notch signalling pathway in our *in vitro* system. The experimental approach taken for each step and the findings have been discussed at the end of each chapter. In this section we summarise the major discoveries and we discuss future directions.

Given the discrepancy on the expression of Notch pathway receptors and ligands in the seminiferous tubules of previous studies and also the fact that not all components had been investigated we performed our own expression analysis in postnatal and adult testis. First we identified the Notch pathway mRNA transcripts expressed during the first round of spermatogenesis. We showed that the gene expression of receptors the *Notch1*, *Notch2*, and *Notch3* and the ligands *Delta1*, *Jagged1*, and *Jagged2,* are spatially and temporally regulated during the first round spermatogenesis and are also present in adult spermatogenesis. We next performed an immunohistochemical investigation for the Notch components discovered in our mRNA analysis.

Examination of sections of adult fertile mouse testes revealed the time points at which activation of Notch receptors takes place during the epithelial cycle of spermatogenesis. We found a stage dependent cyclic activation of Notch1 receptor in Sertoli cells that coincided with the stage-dependent expression of Jagged2 ligand in the adjacent differentiating spermatogonia germ cells. We then investigated the role of these two Notch components using our *in vitro* system and addressed the question whether their cyclical expression and activation were functionally important during germ cell differentiation. Our analysis showed that chemical ablation of Notch pathway or induced activation via Jagged2 ligand does not affect the differentiation of germ cells. In fact a recent study presented similar results, that inactivation of Notch1 signaling in Sertoli cells using the cre-loxP system is dispensable for mouse spermatogenesis (Hasegawa et al., 2012). Moreover, whist writing this thesis, a study published by Garcia and colleagues (2013) showed that constitutive activation of Notch1 signaling in Sertoli cells causes exit of gonocytes from mitotic arrest and premature differentiation. Knowing that Sertoli cells regulate cell fate decisions of undifferentiated spermatogonial cells by secretion of glial cell line-derived neurotrophic factor (GDNF) (Meng et al., 2000,

Hofmann et al., 2005), it can be suggested that activation of Notch1 in Sertoli cells is may be part of a feedback mechanism that senses the presence and progression of pre-meiotic germ cells to regulate the re-initiation of a new spermatogenic wave (Figure 63).

Our immunohistochemical examination of testis also revealed the presence of Notch1, Notch2 and Notch 3 receptors together with Delta1 and Jagged1 ligands in meiotic cells. Examining these Notch components in our cell culture platform did not reveal any change in the differentiation of germ-like cells when Notch pathway activity was blocked. Intriguingly though, induced Notch pathway activation by immobilised ligands led us to discover that Delta1 ligands enhance the formation of late meiotic germ-like cells, an effect that was shown to be mediated mainly via Notch1 receptor. We speculate that the Notch1-Delta1 interaction has a plausible role in the meiotic commitment process, by preferential ligand usage of meiotic cells in a relatively heterogeneous environment of cells in the seminiferous tubules. This idea of intrinsic signals in meiotic cells is also supported by the observation that when rat germ cells are transplanted to a mouse testis, spermatogenesis proceeds with the cycle characteristics of a rat (Franca et al., 1998). A schematic diagram that illustrates the essence of the proposed functions of notch components elucidated from this study is shown in Figure 63.

Moreover, the presence of three Notch receptors in the same cell type does not rule out the possibility of Notch receptors having redundant properties and overlapping functions, something that has been shown in *C. elegans* (Lambie and Kimble, 1991) and might also explain why knockout mice for Notch receptors are viable and fertile (Krebs et al., 2000, Krebs et al., 2003, McCright et al., 2001).

Undoubtedly, the Delta1 mediated effect in meiotic cells should be investigated further. One way we could use in our *in vitro* model to identify the role of each Notch receptor would be to inhibit the function of each receptor in an exclusive manner using the recently developed anti-NOTCH specific antibodies (Falk et al., 2012, Tran et al., 2013, Sharma et al., 2013). This way we could examine germ cell differentiation in different combinations of Notch ligand-coated plates and incubation in various anti-NOTCH specific antibodies to dissect the role of each Notch component and identify possible receptor-ligand interactions. Another way could be to use the Cre-LoxP approach and existing strains of transgenic mice (*Pgk2-Cre* (Bhullar et al., 2001)) to generate conditional knockin mice in which Lox sequences would appropriately allow the

activation of the *Delta1* gene in meiotic germ cells. A similar methodology could also be used to investigate the Notch1-Jagged2 interaction in Sertoli and differentiating spermatogonia using a *Stra8-Cre* (Sadate-Ngatchou et al., 2008) transgenic mouse strain to drive the expression of *Cre* in spermatogonia and an *Amh-cre* (Lecureuil et al., 2002) mouse strain to drive the expression in Sertoli cells. However, using our *in vitro* system might prove to be more cost effective than starting with animal studies.

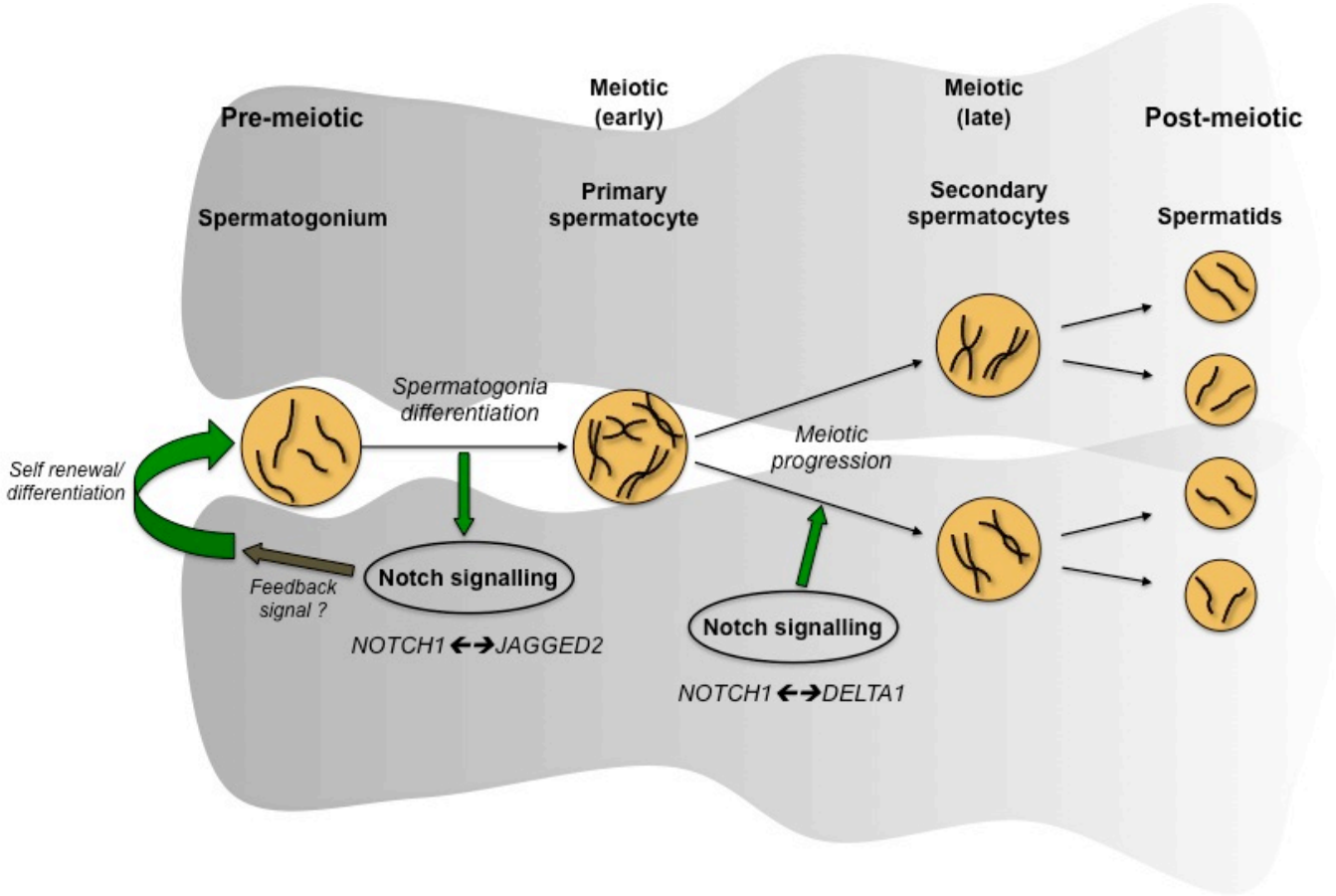


Figure 63. Schematic diagram of a proposed model showing the suggested functions of Notch receptors and ligands during spermatogenesis discovered in this project. The Notch1-Jagged2 interaction is thought to be involved in a feedback mechanism for the regulation of differentiated spermatogonia and the initiation of a new spermatogenic wave. Notch1-Delta1 is suggested to promote meiotic progression of germ cells via a meiosis commitment signal.

Although, the ES cell-based *in vitro* platform established in this project cannot be considered fully comparable with the *in-vivo* conditions, it can be approached as an *in vitro* model that offers easy access to a spermatogenic-like environment. In addition, the fact that adult spermatogonia stem cells have been shown to be closely related to pluripotent stem cells (Guan et al., 2006) supports the usage this model for *in vitro*

studies in spermatogenic-like cells. However, one of the questions not fully addressed in this project is to what extent the germ-like cells generated in the cell culture system mimic real spermatogenic cells.

Although, expression profiling for stage-specific transcripts and proteins have been accepted as a method of characterizing ES derived cells in many studies, it does not always show the real nature of the cells. One example is the induced pluripotent stem (iPS) cells from differentiated fibroblasts. Genome-wide data suggested that the iPSC miRNA expression signature differs from embryonic stem cells (Chin et al., 2009). Therefore, large-scale gene expression surveys such as RNA-sequencing could provide a detailed analysis of the character of the germ-like cells. Recent advances have also shown that single cell RNA-sequencing analysis is possible (Tang et al., 2010). This method will greatly help us to explore also the heterogeneity of cells sorted by FACS for the same reporter from the *in vitro* system. This analysis could also help us to discover what prevented the generation of post-meiotic cells in the ES cell-based system. Answering this question could perhaps help us to cure a similar problem in infertile patients.

Nevertheless, investigation of Notch signalling in an *in vitro* ES cell-based system which develops male germ-like cells provides an important step towards our understanding of the role of the Notch proteins in the process of spermatogenesis. Further investigation of the function of Notch components suggested in this project could lead to significant discoveries that could contribute to therapies of patients with arrested spermatogenesis or other forms of male infertility. In addition, further analysis of the generation of germ-like cells from ES cells and the characteristics of those cells could further our understanding of germ cell specification and maturation.

Appendix A. Primers used in this thesis

Primer name	Sequence (5′-3′)	Purchased from
Hprt1-F	CCTGCTGGATTACATTAAAGCACTG	Eurogentec
Hprt1-R	GTCAAGGGCATATCCAACAACAAAC	Eurogentec
Piwil2-F	TTGGCCTCAAGCTCCTAGAC	Eurogentec
Piwil2-R	CATGCCACGGAACATGGAC	Eurogentec
Stella-F	GACCCAATGAAGGACCCTGAA	Eurogentec
Stella-R	GCTTGACACCGGGGTTTAG	Eurogentec
Mvh-F	CACCGGCAATTTTGACTTTT	Eurogentec
Mvh-R	GTTTGAGCACAAGCCATCAA	Eurogentec
Oct4-F	CACCATCTGTCGCTTCGAGG	Eurogentec
Oct4-R	AGGGTCTCCGATTTGCATATCT	Eurogentec
Sycp1-F	TGAAAAGAAGGATCATTTAACATCAG	Eurogentec
Sycp1-R	TGTTGAGTTCTTCCATTTGAGC	Eurogentec
Sycp3-F	TGCCAAGAGGAAAAGAATAGAAA	Eurogentec
Sycp3-R	CACTGCTGCAACACATTCAT	Eurogentec
Dmc1-F	ACCGCTTCAACGTAGACCAT	Eurogentec
Dmc1-R	CCACTCGAAAAAGTGCCATT	Eurogentec
Pgk2-F	GGTCGGCCTGATGGTATCC	Eurogentec
Pgk2-R	GCAGGGTCAGCACTAATCTTTT	Eurogentec
Tp1-F	AAGAACCGAGCTCCTCACAA	Eurogentec
Tp1-R	GGGGAGAAACAGCCAACATA	Eurogentec
Prm1-F	ATGGCCAGATACCGATGCT	Eurogentec
Prm1-R	CAGCATCTTCGCCTCCTC	Eurogentec
Hes1-F	TCAACACGACACCGGACAAAC	Eurogentec
Hes1-R	ATGCCGGGAGCTATCTTTCTT	Eurogentec
Hes3-F	GCACGCATCAACGTGTCAC	Eurogentec
Hes3-R	TGAGTTCTGGAGGCTTCTCAT	Eurogentec
Notch1-F	AGGGTGGTCAGGAAAATCAT	Eurogentec
Notch1-R	CGATAGGAGCCGATCTCATT	Eurogentec
Notch2-F	AGTGTGCCACAGGTTTCACT	Eurogentec
Notch2-R	TTGGCAGTTGCACTGGTAAC	Eurogentec
Notch3-F	CCGTGTGGCCTCTTTCTACT	Eurogentec
Notch3-R	CAATCGAGCACTCATCCACA	Eurogentec
Notch4-F	AGTGTCTCCCAGGCTTTGA	Eurogentec
Notch4-R	GTGTTCCTGACCTTGGCATT	Eurogentec
Delta1-F	GGTTTGTGTGTGACGAGCAC	Eurogentec
Delta1-R	CTCCCCTGGTTTGTCACAGT	Eurogentec
Delta3-F	CCGGTCTATACGGAGCACC	Eurogentec
Delta3-R	CAGGTTTCAATGACGAGGGAG	Eurogentec
Delta4-F	ACCTGCGGCCAGAGACTTC	Eurogentec
Delta4-R	CATCTGGCTGGCACTCATAA	Eurogentec
Jagged1-F	TGTCGGGATTTGGTTAATGG	Eurogentec
Jagged1-R	CTCGCAGTAATCGATGTCCA	Eurogentec
Jagged2-F	ATGCAAAGAAGCCGTGTGTA	Eurogentec
Jagged2-R	TGGCTGCCACAGTAGTTCAG	Eurogentec

Primer name	Target	Sequence (5′-3′)	Mutation
D-MutUpstATG-F	upstream	AGACAGGATGAGGATCGTTCCGCATGATTGAACAAGATGGA	A➔C, C➔G
D-MutUpstATG-R	upstream	TCCATCTTGTTCAATCATCCGGAACGATCCTCATCCTGTCT	A➔C, C➔G
D-MutDwnstTGA-F	downstream	CTTCTTGACGAGTTCTTCTGATCCGGACTCTGGGG	A➔C, C➔G
D-MutDwnstTGA-R	downstream	CCCCAGAGTCCGGATCAGAAGAACTCGTCAAGAAG	A➔C, C➔G

Primer name	Target	Sequence (5′-3′)	Site	Purchased from
Stra8-Puro cl1F	Puro	GCCTCTCTTCTTATTCTGCGACGAATTCGGTAC	*Eco*RI	Eurogentec
Stra8-Puro cl1R	Puro	GTACCGAATTCGTCGCAGAATAAGAAGAGAGGC	*Eco*RI	Eurogentec
PUROcloningF	Puro	AAAAAAAATCCGGATGACCGAGTACAAGCCCAC	*Kpn21*	Eurogentec
PUROcloningR	Puro	AAAAAAAATCCGGATCAGGCACCGGGCTTGC	*Kpn21*	Eurogentec
Sycp1promoF	Sycp1	GACCTCGAGTGCTAACCCTGATGCTCAAA	*XhoI*	Eurogentec
Sycp1promoR	Sycp1	TAGAAGCTTACACCCTCCACACCCTCAC	*HindIII*	Eurogentec
PUROScreenF	Puro	GCCCCATGGCTGACTAATTT	-	Eurogentec
PUROScreenR	Puro	AGTTCTTGCAGCTCGGTGAC	-	Eurogentec
Pgk2promoF	Pgk2	CCCTCAACAGCAAGTTGGTT	-	Eurogentec
Pgk2promoF	Pgk2	GGGTGCTTCACGTACACCTT	-	Eurogentec
Sycp1ScreenF	Sycp1	GTTCCGTTCCATGTCGTCTT	-	Eurogentec
Sycp1ScreenR	Sycp1	GCTTCTTGTAGTCGGGGATG	-	Eurogentec
Pgk2ScreenF	Pgk2	CCCTCAACAGCAAGTTGGTT	-	Eurogentec
Pgk2ScreenR	Pgk2	GGGTGCTTCACGTACACCTT	-	Eurogentec
Prm1ScreenF	Prm1	AAGCAGGTGTGTGGCACTTA	-	Eurogentec
Prm1ScreenR	Prm1	AAGCGCATGAACTCCTTGAT	-	Eurogentec
Stra8-F2	Stra8-eGFP	AGTTGAGCTCTGGAAACCCACAACGAAAGG	-	Eurogentec
EGFP-R	Stra8-eGFP	GGTGGTGCAGATGAACTTCAG	-	Eurogentec
EGFPcasset-seq-F	eGFP	GGGGTACCAGGTCAGTTTTT	-	Eurogentec
EGFPcasset-seq-R	eGFP	TTGCATACTTCTGCCTGCTG	-	Eurogentec
DsREDcassetteF	DsRED	GGCCTTTTGCTCACATGTTC	-	Eurogentec
DsREDcassetteR	DsRED	CTCCATGCGCACCTTGAA	-	Eurogentec
PUROcasset-seq-F	Puro	CAGCAGGCAGAAGTATGCAA	-	Eurogentec
PUROcasset-seq-R	Puro	GCAGAGCGAGGTATGTAGGC	-	Eurogentec
Stra8promoF	Stra8	GGCCTTTTGCTCACATGTTC	-	Eurogentec
Stra8promoR	Stra8	TCCAGCTCGACCAGGATG	-	Eurogentec

Appendix B. Antibodies used in this thesis

Antibody name	Source	Mono/ Polyclonal	Species	Dilution for IHC/IF	Dilution for WB
α-NOTCH1-ICD	Rockland	Polyclonal	Rabbit	1:100	1:500
α-NOTCH2-ICD	Abcam	Polyclonal	Rabbit	1:100	1:500
α-NOTCH3-ICD	Dr Keith Brennan (Manchester University)	Polyclonal	Rabbit	1:250	1:400
α-NOTCH4-ICD	Dr Keith Brennan (Manchester University)	Polyclonal	Goat	1:100	-
α-DELTA1	Dr Keith Brennan (Manchester University)	Polyclonal	Goat	1:500	-
α-DELTA3	Dr Keith Brennan (Manchester University)	Polyclonal	Rabbit	1:200	-
α-DELTA4	Dr Keith Brennan (Manchester University)	Polyclonal	Goat	1:100	-
α-JAGGED1	Abcam	Polyclonal	Rabbit	1:100	-
α-JAGGED2	Abcam	Polyclonal	Rabbit	1:50	-
α-PIWIL2	Abcam	Polyclonal	Rabbit	1:300	-
α-DMC1	Santa Cruz	Polyclonal	Goat	1:200	-
α-STRA8	Santa Cruz	Polyclonal	Rabbit	1:400	-
α-hnRNP G-T	(Ehrmann et al., 2008)	Polyclonal	Sheep	1:500	-
α-HES1	Abcam	Polyclonal	Rabbit	-	1:500
α-HES3	Santa Cruz	Polyclonal	Goat	-	1:500
α-Tp1	Santa Cruz	Polyclonal	Rabbit	1:200	1:500
α-β Actin	Sigma	Polyclonal	Rabbit	-	1:1000
α-mouse IgG(HRP)	Amersham	Polyclonal	Sheep	1:500	1:1000
α-goat IgG(HRP)	DAKO	Polyclonal	Rabbit	1:1000	1:1000
α-rabbit IgG(HRP)	Jackson Lab	Polyclonal	Goat	1:1000	1:1000
α-sheep IgG(HRP)	DAKO	Polyclonal	Rabbit	1:1000	1:1000
α-rabbit IgG(488)	Molecular Probes	Polyclonal	Donkey	1:400	-
α-sheep IgG(594)	Molecular Probes	Polyclonal	Donkey	1:400	-
α-mouse IgG(594)	Molecular Probes	Polyclonal	Donkey	1:400	-
α-rabbit IgG(Biotin)	DAKO	Polyclonal	Goat	1:300	-

Appendix C. Publication: Book chapter (1st author)

REPRODUCTIVE MEDICINE & ASSISTED
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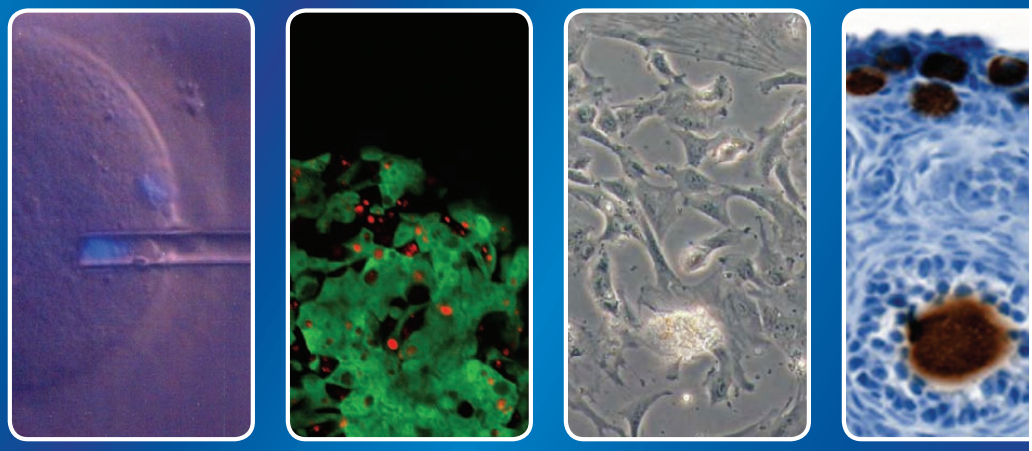
REPRODUCTIVE TECHNIQUES SERIES

Stem Cells in Human Reproduction

Basic Science and Therapeutic Potential

Second Edition



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10 Stem Cell–Based Therapeutic Approaches for Treatment of Male Infertility

Vasileios Floros, Elda Latif, Xingbo Xu, Shuo Huang, Parisa Mardanpour, Wolfgang Engel, and Karim Nayernia

INTRODUCTION

Stem cells have the capacity for self-renewal and the ability to differentiate to various cell lineages. Thus, they represent an important building block for regenerative medicine and tissue engineering. Current research focuses on the possible exploitation of stem cells in medicine and their potential to offer a range of effective treatments for various diseases. A variety of stem cells, ranging from embryonic, bone marrow, endogenous, and amniotic fluid have been investigated and may prove useful as novel alternatives for organ regeneration both in vitro and in vivo. ESCs are pluripotent cells derived from the inner cell mass of the early mammalian embryo. Because of their plasticity and potentially unlimited capacity for self-renewal, ESCs have generated tremendous interest both as models for developmental biology and as possible tools for regenerative medicine. This excitement has been attenuated, however, by scientific, political, and ethical considerations. To exploit this potential, it is essential to be able to control ESC differentiation and to direct the development of these cells along specific pathways. Embryology has offered important insights into key pathways regulating ESC differentiation, resulting in advances in modeling gastrulation in vitro and in the efficient induction of endoderm, mesoderm, and ectoderm, and many of their downstream derivatives. This has led to the identification of new multipotential progenitors for the hematopoietic, neural, and cardiovascular lineages and to the development of protocols for the efficient generation of a broad spectrum of cell types including hematopoietic cells, cardiomyocytes, oligodendrocytes, dopamine neurons, and immature pancreatic β- cells. The next challenge will be to demonstrate the functional utility of these cells, both in vitro and in preclinical models of human disease.

The germline stem cells in the mammalian testis form the basis of male fertility. Aberrant germ cell development can result in abnormal gonadal function, incomplete embryogenesis and infertility, or germ cell tumors. Our understanding of the molecular regulation of normal germ cell development in mammals has progressed significantly because of the utility of the mouse as a genetic model system. However, the molecular regulation of human germ cell development is almost completely unknown due to the historical lack of a malleable model. IVF has been an efficient medical treatment for infertility in the past decades. However, conventional IVF approaches may be insufficient when gametes are lacking or nonviable, thus precluding a significant number of patients from treatment. The use of donor gametes may bring legal, ethical, and even social problems of acceptance that can discourage infertile couples from the donor route. Fortunately, emerging reproductive and stem cell technologies, and preliminary results from animal experiments provide some hope for alternative sources of gametes through which these infertile patients can finally conceive their own genetic child.

DEVELOPMENT OF MALE GERM CELLS

Studies in previous years have enormously advanced our understanding of the process of germ cell development. In the mouse, primordial germ cells (PGCs), the progenitor cells of the germline are apparent in the embryo at embryonic day E7.25 (2). They arise from the epiblast, early in gastrulation, and then as they start to proliferate, they migrate from the allantoic base along the hindgut to finally arrive at the genital ridge (3). Formation of the primary testis cords occurs as the somatic-origin Sertoli cells migrate inward and envelope the PGCs. During the formation of the seminiferous tubule, PGCs continue to proliferate and differentiate into gonocytes, and then remain mitotically quiescent until birth. A few days after birth, the gonocytes resume proliferation, move to the basal membrane of the seminiferous tubules, and differentiate into SSCs also known as undifferentiated type-A spermatogonia (4). Upon

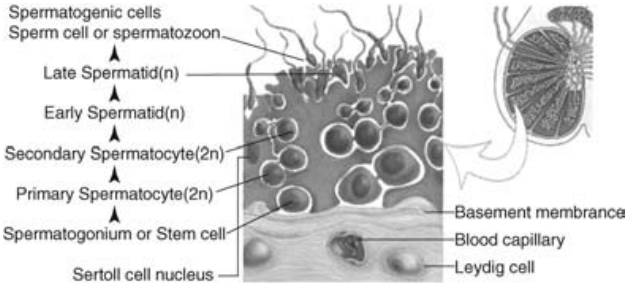

Figure 1 Different stages of development of male germ cells in testis.

division, the SSCs can give rise to two daughter cells which can either separate and lead to stem cell renewal, or can remain connected as a pair of so-called A_{pr} spermatogonia. A_{pr} formation initiates differentiation that ultimately, after a series of maturation steps, leads to the production of spermatozoa (Fig. 1).

DERIVATION OF MALE GERM CELLS FROM ES CELLS

Germline cells are unique in their ability to carry the genetic information onto the next generation. Many studies have focused on the cellular and molecular mechanisms underlying the proliferation and differentiation of germline cells. However, germ cell development in humans has not been elucidated in great detail due to practical and ethical difficulties. A cell-based model that could recapitulate the in vivo conditions of spermatogenesis and provide a readily accessible system to examine the genetic and epigenetic mechanisms of germ cell formation would be extremely valuable. Recent advances have shown that ESCs can potentially fill this need. Studies in the past few years have reported that successful differentiation of mouse ESCs into PGCs as well as into mature male and female gametes can be achieved in vitro (5–7).

On the basis of ESCs carrying an Oct4 promoter-driven GFP reporter gene construct, Hubner et al. (5) were able to visualize the initial steps of germ cell formation in vitro. To restrict the expression of the Oct4-GFP gene only in germ cells, upstream regulatory sequences that drive the expression of Oct4 during blastocyst- and epiblast-stage embryo were deleted. Mouse ESCs transfected with this reporter gene were then allowed to differentiate, and PGCs developed in vitro. Further culture showed that these cells were able to give rise to oocytes, form follicle-like structures with adjacent cells, and subsequently, develop into blastocysts, presumably by parthenogenesis. In contrast with the two-dimensional differentiation approach mentioned above, a three-dimensional method was used by Daley and colleagues (7). In their study, PGCs were also tracked by an Oct4 promoter-driven GFP reporter gene and by immunomagnetic sorting of surface antigen SSEA1-positive cells. It was demonstrated that in vitro differentiation of ESCs into embryoid bodies (EBs) could give rise to PGCs, which in the presence of retinoic acid (RA) proliferate and form embryonic germ cell–like clones that show germline epigenetic modifications. Using FE-J1 antibody that specifically recognizes male meiotic germ cells and round spermatids, EB-derived haploid cells were isolated by flow cytometry. These cells were then injected into oocytes to investigate their biological function. About 20% of the injected oocytes progressed to blastocyst stage. Early evidence that ESCs can produce functional germ cells in vitro was shown by a study from Toyooka et al. (8). Mouse ESCs harboring a gene construct with germ cell–specific mouse vasa homolog (*Mvh*) gene and *LacZ* reporter were used to generate germ cells in vitro. Transplantation of the ES-derived MVH-positive cells into reconstituted testicular tubules demonstrated that they could integrate into a somatic epithelium, undergo meiosis, and develop to some extent into early sperm cells in vivo. However, significant unanswered questions about the functionality of these cells remained to be addressed. But recently, it was shown that mouse ESCs in culture can give rise to SSCs, which are capable of undergoing meiosis and forming functional sperm-like cells that can generate viable transgenic offsprings (9).

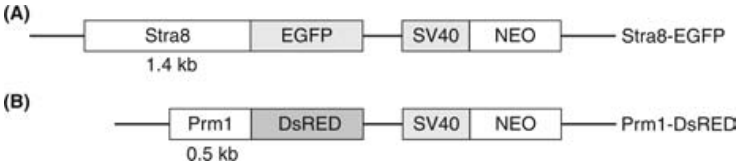

Figure 2 Schematic representation of (**A**) Stra8-EGFP and (**B**) Prm1-DsRED reporter genes harboring a 1.4 and 0.5 kb promoter region of *Stra8* and the mouse protamine 1 (*Prm1*) gene, respectively. These regions are able to direct reporter gene expression specifically in premeiotic and haploid male germ cells, respectively. Both reporter genes contain neomycin phosphotransferase II gene (NEO), which is driven by the SV40 early promoter and enhancer (SV40).

The culture system used by Nayernia and coworkers (9), to demonstrate that ESCs are able to differentiate to mature sperm can fertilize oocytes and produce live animals, involved two stages.

In the first stage, mouse ESCs were transfected with a fusion gene harboring the coding region of enhanced green fluorescent protein (EGFP) under the control of mouse *Stra8* promoter region (10). *Stra8* is an RA-responsive gene that is expressed in premeiotic germ cells and had been shown, in previous studies, that *Stra8* promoter can direct the expression of a reporter gene (Stra8-EGFP) specifically in these cells (11). Transfected mouse Embryonic stem cells (mESCs) were then positively selected using the neomycin phosphotransferase II gene (NEO), which was driven by the SV40 early promoter in the reporter gene (Fig. 2).

Having established mESCs harboring the Stra8-EGFP construct, cells were then cultured in the presence of RA. RA is known to sustain the survival and self-renewal of mouse germ cells in the absence of somatic cell support and also to promote the developmental progression of spermatocytes through early stages of meiosis. After RA induction, EGFP positive cells (later shown by immunohistochemistry and Reverse Transcription - Polymerase Chain Reaction (RT-PCR) to express premeitotic germ cell markers) were isolated by fluorescent activated flow cytometry (FACS).

In the second stage, EGFP-expressing cells were transfected with a second gene construct, which contained the promoter region of protamine 1 gene fused to red fluorescent protein gene (Prm1-DsRED) (Fig. 2). Protamine 1 promoter region is known to be exclusively active in postmeiotic male germ cells (12). Following transfection with Prm1-DsRED construct, positive colonies were then selected by PCR using primers specific for the DsRED-coding region. After a further RA induction for 72 hours, red positive cells that arose from EGFP-expressing cells were released in the medium and could be collected from the supernatant. Isolation and injection of red expressing postmeiotic cells into mouse oocytes gave rise to full-term development of embryos and subsequently to viable animals that carried the transgenic allele.

However, all the animals born were infertile, suffered from severe breathing or walking difficulties, were abnormally large, or had stunted growth. All died within five days to five months of being born, compared with the normal life span of two years for healthy mice. One of the explanations given for the phenotypic abnormalities observed in live animals and the immotile sperm was the impaired establishment of male germline-specific methylation imprints during in vitro ESC-derived gamete formation. This notion was supported by imprinting analysis in the transgenic offspring that showed methylation abnormalities in specific loci. The capacity of the Stra8-EGFP, Prm1-DsRED transfected cells for further development was also investigated in vivo. Cells were transplanted into one of the testes of germ cell–depleted recipient mice. Histological analysis of testes after four months showed the appearance of spermatogenesis-like structures and sperm in the lumen. However, all sperm were immotile or showed reduced motility.

DERIVATION OF MALE GERM CELLS FROM SOMATIC STEM CELLS

Accumulated evidence suggests that in addition to hematopoietic stem cells, bone marrow also harbors endothelial stem cells (EnSCs), mesenchymal stem cells (MSCs), and multipotent adult progenitor cells (MAPCs). Recently, it has also been shown that bone marrow contains a population of stem cells that express early developmental markers such as SSEA and Oct4 (13). These are markers of embryonic stem cells, epiblast stem cells, and PGCs. The presence of these

cells in the bone marrow supports the concept that a population of pluripotent stem cells resides in the adult bone marrow, these pluripotent cells are probably deposited there at the early gastrulation stage during embryo development (13). (During embryonic development, some of the stem cells from germ lineage may go astray on their way to the genital ridges and colonize at fetal level, and subsequently, by the end of the second trimester of gestation, together with fetal liver-derived hematopoietic stem cells, move to bone marrow tissue.) These cells were named very small embryonic-like (VSEL) stem cells (13).

Recently, it has been discovered that bone marrow grafts to female mice, and possibly humans, can produce new follicles and oocytes in the recipient's ovary (14). It has also been reported that these tissues share genes typical of germ cells and proposed that bone marrow stem cells can migrate and colonize the ovaries to maintain a plentiful stock for reproduction (14). Later, Drusenheimer et al. (15) managed to isolate a fraction of bone marrow cells that were able to differentiate to male germ cells. These cells exhibited expression of germ cell and male germ cell–specific markers such as *Oct4*, *Fragilis*, *Stell*, *Vasa*, *c-Kit*, *cyclinA2*, and *Piwil2*. However, Drusenheimer et al. (15) failed to determine whether these male germ cells could undergo meiosis and form functional spermatozoa.

Later, Nayernia et al. (9) used the transgenic mouse line Stra8-EGFP to isolate for the first time male germline stem cells from bone marrow MSCs. Bone marrow stem (BMS) cell-derived germ cells expressed the known molecular markers of PGCs such as Fragilis, Stell, Rnf17, Mvh, and Oct4, as well as molecular markers of SSC and spermatogonia including Rbm, c-Kit, Tex18, Stra-8, Piwil2, Dazl, Hsp90α, β1-, and α6-inegrins (9). Their finding addressed one of the critically important questions in the field of stem cell plasticity: Can adult somatic cells differentiate to germ cells? By showing that BMS cells can differentiate to early germ cells, Nayernia et al. (9) demonstrated that the earlier question is possible. However, a major important and unresolved problem was observed in their study: BMS-GCs stop differentiating at the premeiotic stage of germ cell development, which is an indication of failure in the meiotic programming of BMS-GCs. No EB was observed in their differentiated adherent bone marrow (BM) cell culture, which was present in differentiated ESC cultures suggesting supportive roles of other cell types (Sertoli cells) for differentiation of BMS-derived germ cells.

THERAPEUTIC APPROACHES

Different approaches of stem cell–based therapy of male infertility are shown in Figure 3. These approaches are based on the following strategies:

- Derivation of gametes from ESCs
- Maturation of germline stem cells in vitro following testicular transplantation
- Heterologous transplantation of germline stem cells
- Derivation of gametes from somatic (stem) cells
- Reconstruction of germline stem cell niche

ESCs to Germ Cells—In Vitro Cell-Based Model

The differentiation of ESCs into germ cells of various stages appears to be a spontaneous and quick process, probably due to the nature of ESCs themselves and the microenvironment of the culture conditions that favor this process. However, to be able to apply this technique to humans, we need to improve this method. A detailed analysis of the in vitro differentiation process and comparison with the in vivo germ cell differentiation will enhance our understanding for the molecular, cellular, and morphological events underlying both processes. Furthermore, the derivation of germ cells from ESCs in vitro provides an invaluable assay both for the genetic dissection of germ cell development and for epigenetic reprogramming. To gain a better comprehension of the molecular mechanisms behind the in vitro formation of germ cells from ESCs, we need a robust cell-based model that will allow step-by-step monitoring of the differentiation process during spermatogenesis.

Formation of the male gamete occurs in sequential mitotic, meiotic, and postmeiotic phases. Many germ cell–specific transcripts are produced during this process. Their expression is developmentally regulated and stage specific (16).

To analyze regulated gene expression of ESC-derived germ cells, a microarray-based kinetic comparison of parental ESCs with ESC-derived germ cells can be performed. In addition,

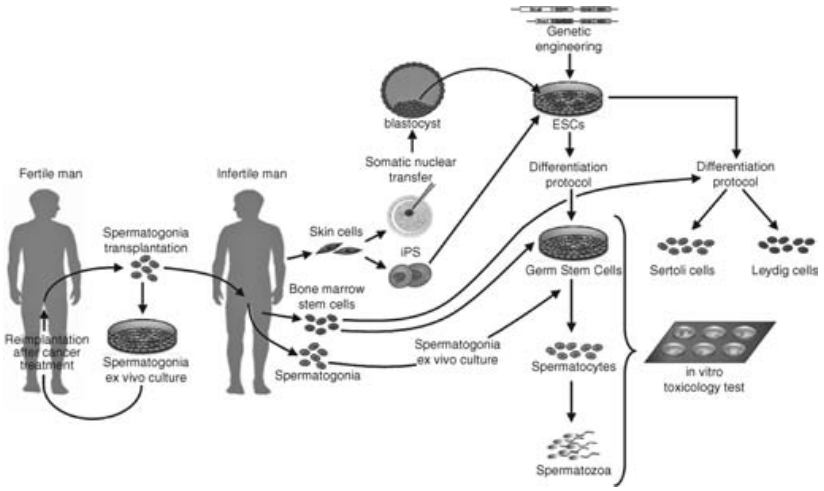

Figure 3 Schematic representation of different stem cell–based approaches for treatment of male infertility.

studying the similarities and differences in gene expression between ESC- and testis-derived germ cells can provide very useful information about the in vitro germ cell differentiation.

Genomic imprinting is the parent-of-origin-specific gene expression, which is a vital mechanism through both development and adult life. One of the key elements of the imprinting mechanism is DNA methylation, controlled by DNA methyltransferase enzymes. Germ cells undergo reprogramming to ensure that sex-specific genomic imprinting is initiated, thus allowing normal embryo development to progress after fertilization. The setting of male-specific epigenetic information is a complex process, which involves a major global reorganization as well as localized changes of the nucleus structure during the premeiotic, meiotic, and postmeiotic stages of the male germ cell differentiation (17). How and exactly when these changes interact, how they affect the epigenetic information, and how the paternal epigenetic marks contribute to the future genome are indeed major issues remaining to be explored. An experimental model that allows the isolation of germ cells at different stages during spermatogenesis (premeiotic, meiotic and postmeiotic) will provide a significant advantage in order to perform a detailed analysis of the imprinting pattern changes exclusively for each developmental stage.

Spermatogenesis is a developmental process that implies drastic changes in cellular morphology and metabolism of germ cells. Many of the changes that occur during this time are essential for the production of fertile sperm. Spermiogenesis includes modifications of the nucleus and perinuclear organelles, formation of the acrosomic system, assembly of the tail structures, topographical arrangement of the cell surface, and cytoplasmic reorganization, the final phase of which results in release of spermatozoa into the lumen of seminiferous tubules (18). An in vitro cell-based model will enable us to study this complex process and understand the sequential changes in the structure and associated molecules of sperm formation.

Taking advantage of the access, at different developmental stages during ESC differentiation to germ cells, provided by a cell-based model like this, the questions that can be addressed are unlimited. Revealing the mechanisms of gamete development can have major implications for treating infertility. ESC differentiation into germ cells can improve our knowledge of the genetic basis of mammalian infertility and can enable analysis of many more genes when an infertile couple enters the clinic. At the moment, the only genetic tests commonly offered to infertile patients are karyotype analysis, sequence analysis of the cystic fibrosis transmembrane conductance regulator gene, and Y chromosome deletion analysis (19).

Although the technological procedures and advances in the fertility clinic have vastly developed, still a lot of controversies and dilemmas remain. One of those with particular importance is that ART for severe male and female factor infertility serves to not only

overcome sterility but also bypasses natural barriers to the inheritance of defective genes. This results in considerable concern that genetic defects will be transmitted to the next generation. In the future, improved protocols for differentiation of ESCs to functional mature male germ cells (sperm) in culture might be used to provide the ability to parents, who are carriers or affected by inherited diseases, to have healthy offsprings without any risk. This can be accomplished by genetic modification of ESCs and subsequent differentiation into germ cells in vitro. However, this prospective application presents many social and ethical challenges, as germline genetic modification could also potentially allow parents to produce a designer baby.

Recent studies have shown that human skin fibroblasts can be directly reprogrammed to pluripotent state cells, so-called human-induced pluripotent stem (iPS) cells, by over-expression of only four proteins (20). These advances provide the potential in the future for treating cases of arrested spermatogenesis by stem cell–based therapeutic approach using cells obtained from skin biopsies.

Ex Vivo Culture of Germ Cells

Isolation of male germ cells from testicular tissue of nonobstructed azoospermic men was recently reported in the literature (21). In this study, it was shown that after six weeks of culturing the male germ cells obtained from the biopsy, haploid cells were generated. These in vitro–produced haploid cells (spermatids) were able to activate human oocytes and induce the initial cleavage. Although the culture system of this study gave rise to haploid cells, these cells were not able to complete the final step of the spermatogenic maturation, that is, spermiogenesis, as sperm was not observed. Investigation of ESC differentiation into germ cells might allow us to define the appropriate culture conditions. Thereafter, having established the culture conditions required for germ cell differentiation in vitro, we could isolate SSCs from patients exhibiting maturation arrest at a specific stage of spermatogenesis, and allowing them to develop in vitro, followed by reimplantation. Providing an ex vivo environment that mimics the normal in vivo conditions present in healthy testis offers a very attractive alternative solution for the therapy of patients who are positive for premeiotic or meiotic germ cells but negative for post-meiotic cells in the seminiferous tubules.

Germline stem cells can be transplanted from the testis of a fertile donor animal to the testis of an infertile recipient (22). These cells can then reestablish spermatogenesis in the recipient testis; however, the sperm produced will transmit the genotype of the donor to the offspring of the recipient. Many patients undergo treatment for cancer by chemotherapy or irradiation. Prior to treatment, a patient undergoes a testicular biopsy to recover stem cells. These cells are then cryopreserved and reimplanted later to restore fertility (23). However, the successful rates for this method are appreciably low.

Development of methods that will allow in vitro differentiation of stem cells to provide mature spermatozoa would be enormously valuable in understanding the complex process of spermatogenesis. It will also allow characterizing factors and signals that support self-renewal and initiate differentiation of human SSCs. This will advance the development of the appropriate culture conditions for human SSCs and will facilitate ex vivo culture, which can potentially improve transplantation and restoration of fertility in patients who underwent cytotoxic treatments for cancer.

Reconstruction of Germline Stem Cell Niche

Cross-sectionally, the testicle is mainly built up of seminiferous tubules where spermatogenic process takes place in normal condition, producing sperm as a sign of fertility (Fig. 1). Within the seminiferous of the testis, developing germ cells and Sertoli cells are in close association with each other. Through the entire spermatogenesis process, to allow synchronous development of cell stages in each cell division from a spermatogonium to spermatids, the cells remain connected by cytoplasmic bridges. As the spermatogenic cells divide and differentiate, they interact closely and extensively with the adjacent Sertoli cells. These associations between developing male gametes and the Sertoli cells are essential for spermatogenesis. Without physical and metabolic support of Sertoli cells, it is impossible for germ cells' differentiation, meiosis, and transformation into spermatozoa to occur. Impaired development or proliferation of Sertoli cells in early fetal life has been associated with various testicular disorders. Failure of Sertoli cells to mature will result in inability to express functions essential for supporting spermatogenesis.

In a similar way, insufficient spermatozoa production in adulthood is caused by failure of Sertoli cells to proliferate normally at appropriate period in life. Different strategies can be developed to reconstitute defective germline stem cell niche and treat male infertility. One of these approaches is derivation of Sertoli cells from stem cells and transplantation in testis of infertile men.

In Vitro Toxicology Test

Treatment with cytotoxic chemotherapy is associated with significant gonadal damage in men (24), and alkylating agents are the most common agents implicated, which all act to cause damage to the genome. The vast majority of men receiving anticancer regimens for the treatment of lymphomas are rendered permanently infertile. Establishment of stem cell–based assays as innovative platforms for drug screening is a nascent field compared with parallel studies on stem cells for regenerative medicine (25). Preclinical efficacy and toxicity testing are conducted largely in animal models as a means to validate the mechanism of action and predict adverse effects of compounds in human subjects. For developmental toxicity, for example, the most prevalent models for drug investigation are whole animal studies in rabbits and rats. In vivo studies rely on administration of compounds to pregnant animals at different stages of pregnancy and embryonic/fetal development. However, these and other in vivo animal models are limited by a lack of robustness between animal and human responses to chemical compounds. Thus, a human stem cell-oriented predictive in vitro model, such as the in vitro cell-based mode described in this chapter, provides the ability to investigate the effect of various anticancer drugs in the germline. Screening of drugs in in vitro isolated germine cells allows us to examine the pharmacological effects of a drug and to determine the germ cell population where the possible action occurs, as each isolated germ cell population represents a different developmental stage during spermatogenesis.

CONCLUSION AND SUMMARY

ESCs hold a great promise for treating male infertility following recent advances showing differentiation of ESCs to germ cell lineage. Exploiting this potential could increase our understanding of how germ stem cells differentiate and also allow us to dissect and recapitulate the complex process of spermatogenesis in vitro. Furthermore, taking advantage of the ability to generate germ cells from ESCs can provide the starting point for the development of a number of stem cell–based approaches to help patients with problematic spermatogenesis either by genetic and environmental causes or as a result of cytotoxic cancer therapies. These stem cell–based strategies can involve in vitro generation and maturation of germ cells from ESCs or somatic stem cells and ex vivo maturation of germ cells followed by reimplantation in the testis. These models can also support further platforms for investigation of imprinting patterns, chromatin modification, drug toxicity, and molecular profile of germ cells. The increased knowledge about the derivation of gametes in vitro can have a great practical potential in the future for therapeutic approaches to male infertility.

Of the 15% of couples who experience difficulty in conceiving, approximately half involve some degree of male factor infertility, and for 30% to 50% of these men, no cause is identified for the poor sperm characteristics. Although assisted reproductive technologies (ARTs) such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have dramatically improved the prospects for infertile couples, there are some types of male factor infertility that remain untreatable. These include arrested spermatogenesis that may be due to defective germ cells, an abnormal testicular environment, or aberrations in endocrine pathways regulating testis function (1). One of the essential bases for treatment of male infertility is the understanding of the human spermatogenesis by modeling human germ cell development. There has previously been no robust cell-based model for examining the genetic and epigenetic mechanisms of germ cell formation. Embryonic stem cells (ESCs) could potentially fill this need, as all cell types analyzed to date (including mature germ cells) can be identified by marker analysis during ESC differentiation. Furthermore, ESCs could also be used to differentiate mature male germ cells (sperm) in culture as an alternate reprogramming cell for somatic cell nuclear transfer. Another approach is isolating spermatogonial stem cells (SSCs) and allowing them to develop in a more "normal" environment ex vivo, followed by reimplantation. Establishment of human SSCs and investigation of their differentiation to

sperm in vitro might lead to new ways for treating male factor infertility. These techniques could be proven as powerful tools for undertaking new types of reproductive studies and particularly might support the development of new approaches and novel technology in assisted reproductive treatment of male infertility.

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