UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

NOTCH SIGNALING, GENITAL REMODELING AND REPRODUCTIVE FUNCTION

Daniel José de Moura Carita Dinis Murta

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TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS

ESPECIALIDADE DE CIÊNCIAS BIOLÓGICAS E BIOMÉDICAS

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To my parents, brothers and Sebastião

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The development of this thesis was a great opportunity. The possibility to join a research team with great expertise in animal reproduction and molecular biology was a major determinant to its final success. This opportunity was given to me by Professor Luís Lopes da Costa. As I knew the project it was easy to guess that it would be accomplished with personal realization. The development of a PhD allowed me to reinforce my personal and professional proficiencies. This experience propelled me to build up new competences and strengthened my personality. The final fulfilment of a PhD was much more than a thesis.

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Title – Notch signaling, genital cellular remodeling and reproductive function

Abstract: The objective of this thesis is to evaluate the association between Notch signaling, genital cellular remodeling and reproductive function, in the mouse model. Five experimental chapters are included in this thesis. In experiments 1 and 2, we evaluated transcription and expression patterns of Notch component and effector genes in testis post-natal development, along the spermatogenic cycle and the epididymis. In experiment 3, we evaluated the male reproductive phenotype of *in vivo* Notch blockade by DAPT. In experiments 4 and 5 we evaluated transcription and expression patterns of Notch component and effector genes in the ovary, oviduct and uterus along the estrous cycle.

Results indicate that Notch signaling is active and associated to male and female genital cellular remodeling. In the male, results prompt for a regulatory role of Notch signaling in spermatogonia pool maintenance, onset of spermatogenesis, in the pace of the spermatogenic cycle, germ cell identity, and epididymis spermatozoa maturation. In the female, results prompt for a regulatory role in ovarian follicle and corpus luteum development, and oviduct and uterus epithelial cell turnover and function. Notch signaling is operating in the testis and ovarian cellular interstitium, and in luminal and glandular epithelia of genital tract, probably regulating intercellular communication.

Keywords: Notch; spermatogenesis; spermatozoa maturation; ovary; oviduct; uterus; mouse.

Título da tese: A via de sinalização Notch, remodelação celular genital e a função reprodutiva.

Resumo : Esta tese avalia a relação entre a via de sinalização Notch, o remodelação celular genital e a função reprodutiva, sendo constituída por cinco capítulo experimentais. Nos dois primeiros, avaliamos o padrão de transcrição e expressão dos componentes e efectores da via Notch no desenvolvimento testicular pós-natal, ao longo do ciclo espermático e no epidídimo. Na experiência 3, avaliamos o fenótipo reprodutivo masculino decorrente do bloqueio *in vivo* da via Notch por DAPT. Nas experiências 4 e 5 analisamos o padrão de transcrição e de expressão dos componentes e efectores da via Notch no ovário, oviduto e no útero ao longo do ciclo éstrico.

Os resultados indicam que a via Notch está activa e associada à remodelação genital masculino e feminino. No macho, os resultados apontam-lhe um papel regulador na manutenção do *pool* de espermatogónias, no início da espermatogénese, na coordenação do ciclo espermático, na identidade celular germinal, e na maturação espermática. Na fêmea, os resultados apontam para um papel regulador no desenvolvimento folicular e do corpo lúteo, e na função e ciclicidade do oviduto e útero. A via Notch opera no interstício celular do testículo e do ovário, e no epitélio luminal e glandular do tracto reprodutivo, regulando a comunicação intercelular.

Palavras-chave: Notch; espermatogénese; maturação espermática; ovário; oviduto; útero; murganho.

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LIST OF ABREVIATIONS

1n – haploid

1M – 1 month

2n – diploid

3β-HSD – 3 beta hydroxysteroid dehydrogenase

4d – 4 days

15d – 15 days

β2mg – beta 2-microglobulin

ADAM – A Disintegrin And Metalloproteinase

ANOVA – analysis of variance

BMP – bone morphogenetic protein

Body D – epididymis body distal segment

Body P – epididymis body proximal segment

CEBEA - Comissão de Ética e Bem-Estar Animal

cDNA – complementary DNA

CIISA – Centro de Investigação Interdisciplinar em Sanidade Animal

CL – corpus luteum

CTRL – control

D1 – Dll1

D4 – Dll4

DAPT – N-S-phenyl-glycine-t-butyl ester

DAZL – deleted in azoospermia-like

DDX4 –DEAD Box Protein 4

DGAV – Direcção Geral de Alimentação e Veterinária

Dll1 – delta-like 1

Dll4 – delta-like 4

DNA – deoxyribonucleic acid

DR – Diário da República

E2 – estrogen

EC – estrous cycle

EDTA – diaminoethane tetraacetic acid

EEC – European Economic Community

FCT – Portuguese Foundation for Science and Technology

FELASA – Federation for Laboratory Animal Science Associations

FGF – fibroblast growth factor

FMV – Faculdade de Medicina Veterinária

FSH – follicle stimulating hormone

GDF9 – growth differentiation factor-9

GFP – Green Fluorescent Protein

H1 – Hes1

H2 – Hes2

H5 – Hes5

hCG – human chorionic gonadotrophin

Head D – epididymis head distal segment

Head P – epididymis head proximal segment

Hes – hairy/enhancer of split

HPRT1 – hypoxanthine phosphoribosyltransferase 1

HRP – research in human reproduction (WHO)

HTF – Human Tubarian Fluid

IgG – immunoglobulin G

IHC – immunohistochemistry

in situ – *in situ* hybridization

IS – Initial segmen

IVF – *in vitro* fertilization

J1 – Jagged1

KSOM - potassium-supplemented simplex optimized medium

mRNA - messenger ribonucleic acid

N1 – Notch1

N1ICD – Notch1 intracellular domain

N2 – Notch2

N2ICD – Notch2 intracellular domain

N3 – Notch3

N3ICD – Notch3 intracellular domain

N4ICD – Notch4 intracellular domain

NICD – Notch intracellular domain

Nrarp – Notch-regulated ankyrin repeat protein

P4 – progesterone

PBS – phosphate buffered saline

PCNA – Proliferating Cell Nuclear Antigen

PCR – polymerase chain reaction

pn – post-natal

qRT-PCR – quantitative real-time PCR

RBP-jk – recombination signal binding protein for immunoglobulin kappa J region

RNA – ribonucleic acid

RPS29 – ribosomal protein S29

RT-PCR – reverse transcription PCR

SEM – standard error mean

SMAD – Mothers against decapentaplegic homolog

SRY – sex-determining region Y

TGFβ – transforming growth factor beta

TUNEL – terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling

VEGF – vascular endothelial growth factor

WHO – World Health Organization

INTRODUCTION

> "In a world that needs vigorous control of population growth, concerns about infertility may seem odd, but the adoption of a small family norm makes the issue of involuntary infertility more pressing."
>
> Mahmoud Fathalla,
> Former Director of the WHO HRP
> (Bull World Health Organ, 2010; 88:881–882)

Reproductive biology gathers increasing interest both in human and animal health. This is driven by the economic and social problems associated with reproductive control. Infertility is a huge concern throughout the world, both in humans and farm animals (Johnson & Gentry, 2000; Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012). New strategies to prevent and control infertility are great demands of modern societies (van der Poel, 2012). On the other hand, control of human and animal overpopulation by means of contraception is also a present big issue. Further knowledge inside the complex regulation of reproductive biology would allow the development of new therapies, with less secondary effects, more controllable and easy to use, that ultimately would increase both the user's compliance and the global final effect in the population.

During the last century, mammalian reproductive function has been intensively studied and major aspects of endocrine regulation of histological/cytological events are well known. However, the molecular mechanisms underneath reproductive function are still poorly understood. The intercellular relations and communication are of great importance in morphogenetic events, such as those occurring in reproduction. Apart from the endocrine control, reproductive events are also regulated by paracrine intercellular signaling, which controls cellular proliferation, apoptosis, differentiation, secretory functions and others. Many of these cellular regulatory mechanisms have not been completely described and several of their aspects are entirely unknown. The gene pathways involved in the control of the highly complex cellular remodeling associated with reproductive function are poorly understood. Deciphering these mechanisms could potentially lead to the development of new therapeutic strategies addressed to male and female infertility and contraception.

Notch is an evolutionarily well-conserved cell signaling pathway that has been implicated in cell fate decisions in several tissues (Artavanis-Tsakonas, Matsuno, & Fortini, 1995; Borggrefe & Oswald, 2009). The research work presented in this thesis aimed to analyze the Notch pathway enrolment in male and female reproductive function, using the mouse model. In the male, the transcription and expression patterns of Notch pathway components and effectors were evaluated during male gonad post-natal (pn) development and along adult

spermatogenesis and spermatozoa maturation along the epididymis. Additionally, Notch pathway blockade during adult spermatogenesis and sperm maturation was accessed through the use of a γ-secretase inhibitor. In the female, the transcription and expression patterns of Notch pathway components and effectors were analyzed in the reproductive tract (ovary, oviduct and uterus) along the estrous cycle (EC), and associated to key cellular events of follicle and corpus luteum (CL) development.

The above studies were converted into five manuscripts, submitted for publication in international journals, and constitute the five chapters of the experimental work include in this thesis, as follows:

1. **Dynamics of Notch pathway expression during mouse testis post-natal development and along the spermatogenic cycle**

Murta, D., Batista, M., Silva, E., Trindade, A., Henrique, D., Duarte, A., Lopes da Costa, L. (2013). *Plos One,* 8 (8).

2. **Notch pathway gene components and effectors are differentially transcribed and expressed along the epididymis epithelium.**

Murta, D., Batista, M., Silva, E., Trindade, A., Henrique, D., Duarte, A., Lopes da Costa, L. Submitted to *Molecular Reproduction and Development*

3. ***In vivo* Notch blockade induces male germ cell fate aberrations and apoptosis, and increases spermatozoa defects.**

Murta, D., Batista, M., Trindade, A., Silva, E., Henrique, D., Duarte, A., Lopes da Costa, L. Submitted to *Fertility & Sterility*

4. **Differential expression of Notch component and effector genes during ovarian follicle and corpus luteum development along the estrous cycle**

Murta, D., Batista, M., Silva, E., Trindade, A., Mateus L., Duarte, A., Lopes-da-Costa L. Submitted to *Reproduction Fertility and Development*

5. **Dynamics of Notch signaling in the oviduct and uterus along the mouse estrous cycle**

Murta, D., Batista, M., Trindade, A., Silva, E., Mateus L., Duarte, A., Lopes da Costa, L. Submitted to *Reproduction*

LITERATURE REVIEW

1. Mammalian reproduction

> "If you press a piece of underwear soiled with sweat together with some wheat in an open jar, after about 21 days the odor changes and the ferment, coming out of the underwear and penetrating the husks of wheat changes the wheat into mice… But what is remarkable is the mice of both sexes emerge, and these mice successfully reproduce with mice born naturally from parents…But what is even more remarkable is that the mice which come out of the wheat and underwear are not small mice, not even miniature adults or aborted mice, but adult mice emerge!"
>
> Jean Baptiste Van Helmont (1577–1644)

Individuals are transient and a population transcends finite life spans only by reproduction, the creation of new individuals from existing ones. Our knowledge on reproductive biology has evolved exponentially during the last century. Presently, the great focus is on how to control reproductive function.

Although, in the animal kingdom both asexual and sexual reproduction occurs, in the case of mammals, there is the need of the fusion of oocyte and sperm, and so the fusion of different pools of genes, to generate new life. This way, sexual reproduction increases genetic variability among offspring by generating unique combinations of genes inherited from two parents.

In sexual reproduction the creation of offspring results from the fusion of haploid (1n) gametes, which then form a diploid (2n) zygote. Mammalian gametes arise by meiosis. The female gamete, the oocyte, is a large immotile cell, whereas the male gamete, the sperm, is a much smaller and motile cell. In the male, spermatozoa develop in the testis, whereas in the female, oocytes develop in the ovary. During mating, sperm are ejaculated in the female reproductive tract. Fertilization depends on mechanisms that help sperm to meet the oocyte.

The male gonads, the testes, consist of highly coiled tubules, the seminiferous tubules, interspersed by interstitial tissue and surrounded by several layers of connective tissue. Leydig cells are scattered in interstitial tissue and secrete testosterone and other androgens. Following release in the seminiferous tubules, spermatozoa pass along the coiled tubules of the epididymis. During this passage, spermatozoa become motile and gain the ability to fertilize. At ejaculation, spermatozoa are propelled through the duct deferens, ending, in case of a successful copulation, in the female reproductive tract.

The female gonads, the ovaries, contain many follicles. Each follicle consists of one oocyte surrounded by one or more layers of granulosa cells, which nourish and protect the developing oocyte. However, throughout life, only a small portion of these oocytes will

develop and ovulate. The granulosa cells are also enrolled in producing female sex steroids, the estrogens (E2). Nevertheless, after ovulation, the remaining granulosa cells grow within the ovary to form the CL. This cyclic transient endocrine gland main function is to secrete progesterone (P4), which plays a central role on the regulation of the EC and on pregnancy establishment and maintenance.

2. The mouse as an experimental model

The relationship between mice and men has always been close and essentially difficult. Mice has long been a problem to man. In huge numbers, mice can extensively destroy crops and become a great economical and nutritional problem for human populations. As a pest, mouse is also a great problem, as a vector of several diseases. However, mice also became of human interest, probably first as a pet, and then as a research model. It was the setting of an inbred strain by Clarence Cook Little (Staats, 1966) that launched mice as a tool for the understanding of the biology and genetics of ourselves. This led the way to the establishment of defined strains with diverse but repeatable genotypes for biomedical study.

The beginning of mice era in research started when Mendel's work was rediscovered, and Lucien Cuénot decided to evaluate whether Mendel's laws applied to animals as well (Cuénot, 1902). Soon after, it was found that tumors transplanted into mice only continued development in the host in some strains, but not in others, and, in 1909, it was reported that at least part of the basis for tumor rejection was heritable (Tyzzer, 1909). This work was continued, and in 1916 it was published "a Mendelian explanation of rejection and susceptibility" (Little & Tyzzer, 1916). These studies convinced Clarence Cook Little that a genetically uniform stock of mice was required to further understanding the genetics of cancer susceptibility. With this goal in mind, he began mice crosses that led to the development of the first inbred strain, DBA.

With large mouse populations under observation, it was soon recognized that the mouse had similar (sometimes nearly identical) diseases to humans and that it could provide a powerful basis for a practical understanding of the human medical condition. Furthermore, the mouse is small, breeds quickly, and is easily and economically maintained.

Since the beginning of the twenty century, the mice, as a research model, gained impact and new technologies and manipulations arouse to respond to specific research needs. Mice have provided invaluable models of normal and aberrant processes affecting metabolism, reproduction, aging, cancer, immunologic function, and many others. Recently developed gene-driven approaches, that complement more established phenotype-driven studies, have profited immeasurably from the unique ability to selectively alter the mouse genome by adding, subtracting, and replacing genes. Indeed, the preeminent status of mouse among research organisms at the beginning of the twenty-first century was marked by its selection as the first to be targeted for complete genomic sequencing (Waterston et al., 2002).

3. The male reproductive function

3.1. Spermatogenesis

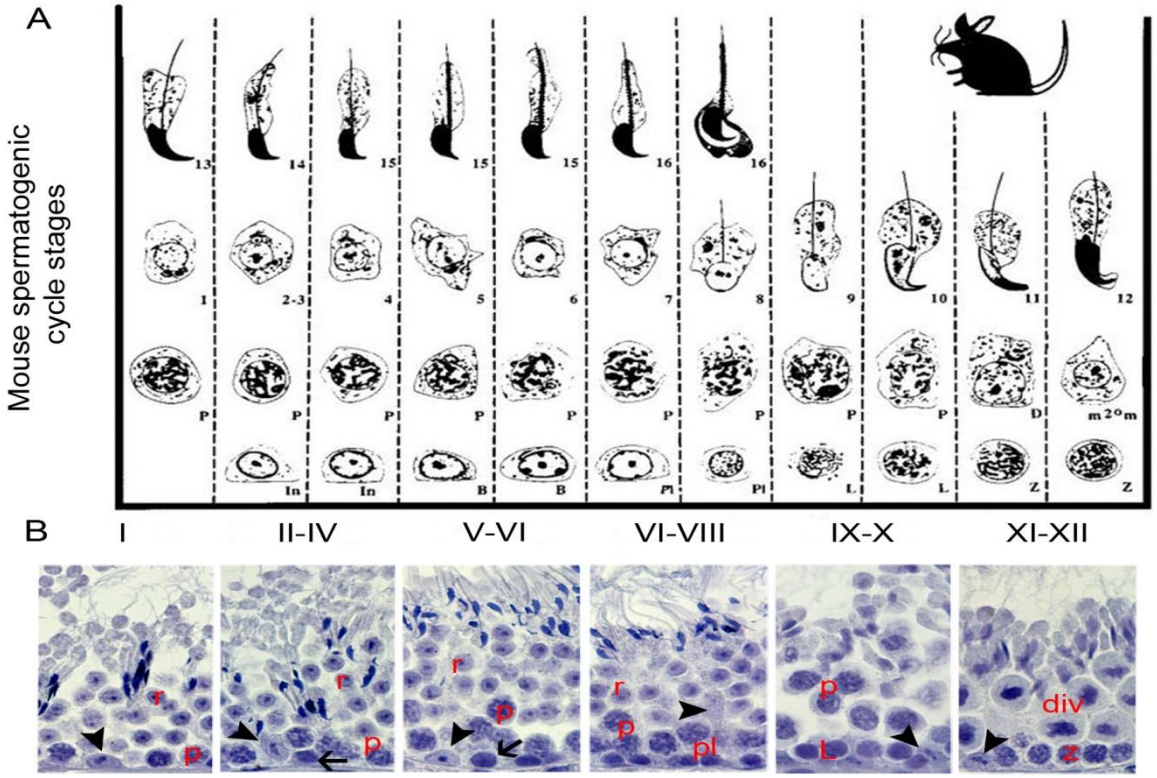
Spermatogenesis is a complex process of cellular transformation in which unspecialized diploid (2n) spermatogonia stem cells gives raise to highly specialized male haploid (1n) spermatozoa. This occurs within the seminiferous tubules. The seminiferous epithelium (Figure 1) consists of germ cells that form numerous concentric layers penetrated by a single type of somatic cell, the Sertoli cells. Germ cells evolve through sequential divisions. At first by repeated mitotic divisions, to maintain germ cell pools, and then by meiosis, that lead to production of haploid gametes. Meiosis involves the duplication of chromosomes, genetic recombination, and then reduction of chromosomes through two cell divisions to produce spherical haploid spermatids that differentiate into spermatozoa, which are then release into the lumen (Hess & Franca, 2008).

This complex and lengthy cellular process was divided in several stages (Figure 1) (Leblond & Clermont, 1952; Hess & Franca, 2008; Hermo, Pelletier, Cyr, & Smith, 2010). These stages are however artificial definitions (Kluin, Kramer, & de Rooij, 1982). The stages cyclically evolve over time and along the seminiferous tubule forming the cycle of the seminiferous epithelium, which has a well-defined duration for a given species. Additionally, the overall time length to spermatogenesis completion (release of spermatozoa within the seminiferous tubule lumen) is specific of each mammalian species, ranging from 30 to 78 days (Clermont & Trott, 1969; França et al., 1998; Hermo et al., 2010). In the mouse, each spermatogenic cycle encompasses 12 stages and takes 8.6 to 8.9 days, the total duration of spermatogenesis being 39 to 40 days (Figure 1) (Clermont & Trott, 1969).

The seminiferous epithelium consists of one somatic cell type, the Sertoli cell, and many different germ cell types (Hess & Franca, 2008). Germ cells within each layer of the seminiferous epithelium change in synchrony with the other layers over time, producing the sequence of stages (Hess & Franca, 2008). These cells do not migrate laterally along the length of the seminiferous tubule. However, at least in rodents, a strict ordering of the stages is observed, whereby sequential stages occur along the length of the tubules, in the so-called ‘wave’ of the seminiferous epithelium. This way, stage I is followed by stage II, and so on until stage XII, which is then followed by stage I (Figure 1) (Hess, 1999). This wave is the result of the synchronous development of germ cells through cellular signaling mechanisms that remain largely unknown.

The mechanism by which spermatogonia stem cells transform into differentiating spermatogonia and simultaneously renew their own population is now a major focus of reproductive biology. Spermatogonia are indeed promising targets for the treatment and control of several reproductive conditions (Brinster, 2007). Spermatogonia give rise to spermatocytes after a fixed number of mitotic divisions that are characteristic of each specie (Clermont, 1972).

Figure 1 – Mouse spermatogenic cycle stages.



A – Draw scheme of the mouse spermatogenic cycle stages showing the histological relationship of cells in the seminiferous tubules of the testis. In the diagram, the lower row of cells rests close to the basement membrane, and the upper row of cells is close to the lumen of the seminiferous tubule. In, intermediate spermatogonia; B, type B spermatogonia; Pl, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes, m2ºm, meiotic divisions. Drawing adapted from (França et al., 1998). **B –** Adult mouse spermatogenic cycle stages. Haematoxylin staining. 400x magnification. spermatogenic stages are aligned with their drawn correspondents in image A. Arrow head point Sertoli cells. Arrows point spermatogonia. p, pachytene spermatocytes; pl, preleptotene spermatocytes; L, leptotene spermatocytes; z, zygotene spermatocytes; div, meiotic divisions; r, round spermatids.

In well-studied laboratory rodents, such as rats and mice, four classes of spermatogonia are present: undifferentiated type A spermatogonia, differentiated type A spermatogonia, intermediate spermatogonia, and type B spermatogonia, identified in light and transmission electron microscopy according to the presence and distribution of heterochromatin (de Rooij & Russell, 2000; Chiarini-Garcia & Russell, 2001; Chiarini-garcia, Hornick, Griswold, & Russell, 2001). Type B spermatogonia divide by mitosis forming two preleptotene spermatocytes, cells representing the beginning of meiotic prophase (Hess & Franca, 2008). Initially, these cells rest on the basement membrane, however, as they progress to leptotene and zygotene spermatocytes they move through the blood-testis-barrier (Russell, 1977).

Spermatocytes are the meiosis cells. These cells are found in all meiotic stages, as meiosis is a prolonged period within spermatogenesis, extending in the mouse for approximately 14 days. However, the meiotic cell division occurs in and defines a single spermatogenic cycle stage (XII). (Hess & Franca, 2008). Round spermatids are formed after the completion of both meiotic divisions. These spherical haploid (1n) cells transform into elongate, highly condensed spermatozoa, which are then released into the seminiferous tubule lumen, a process called spermiogenesis (Hermo et al., 2010). In this process, round spermatids become elongated and pass through a series of four different phases know as Golgi, capping, acrosomal, and maturation. In these phases the acrosome is formed and packed, the nuclear chromatin condensates, and the excess cytoplasm is removed (Hess & Franca, 2008). The excess of cytoplasm is removed in stage VII-VIII and results in the formation of prominent cytoplasmic lobes and residual bodies, which contain unused mitochondria, ribosomes, lipids, vesicles and other cytoplasmic components (Firlit & Davis, 1965; Hess et al., 1993). Compared to many other well-known self-renewing cell systems in the body, spermatogenesis is thought to have the greatest number of cell divisions (Hess & Franca, 2008).

Germ cell apoptosis is a normal event during mammalian spermatogenesis and plays a critical role in determining total sperm output and normal spermatogenesis (Russell, Chiarini-garcia, Korsmeyer, & Knudson, 2002). Although a significant germ cell loss occurs during the spermatogonia phase, apoptosis is also frequent during meiosis, probably related to chromosomal damage (Hess & Franca, 2008).

The Sertoli cells are responsible for the support and nutrition of the developing germ cells and compartmentalization of the seminiferous tubule. This is accomplished by tight junctions, which provides a protected and specialized environment for the developing germ cells. Sertoli cells also control the release of mature spermatids into the tubular lumen, secrete proteins and several growth factors, and phagocyte degenerating germ cells and residual bodies that remain from released spermatozoa (Griswold, 1998). Sertoli cells are the target of hormonal regulation of spermatogenesis (Griswold, 1998; De Gendt et al., 2004). The gene pathways involved in the regulation of the highly complex cellular remodeling associated with spermatogenesis are poorly understood.

3.2 The epididymis and spermatozoa maturation

After leaving the testis through the efferent duct, spermatozoa enter the epididymis. In rodent species the epididymis is subdivided into four major anatomical regions known, from proximal to distal, as the initial segment, the head/caput, the body/corpus, and the tail/cauda (Figure 2) (Abou-Haïla & Fain-Maurel, 1984; Turner, Bomgardner, Jacobs, & Nguyen, 2003). Spermatozoa travel throughout this structure for 10-13 days, depending on the species, and is stored for variable periods in the tail and duct deferens (Turner, 1995; Belleannée, Thimon,

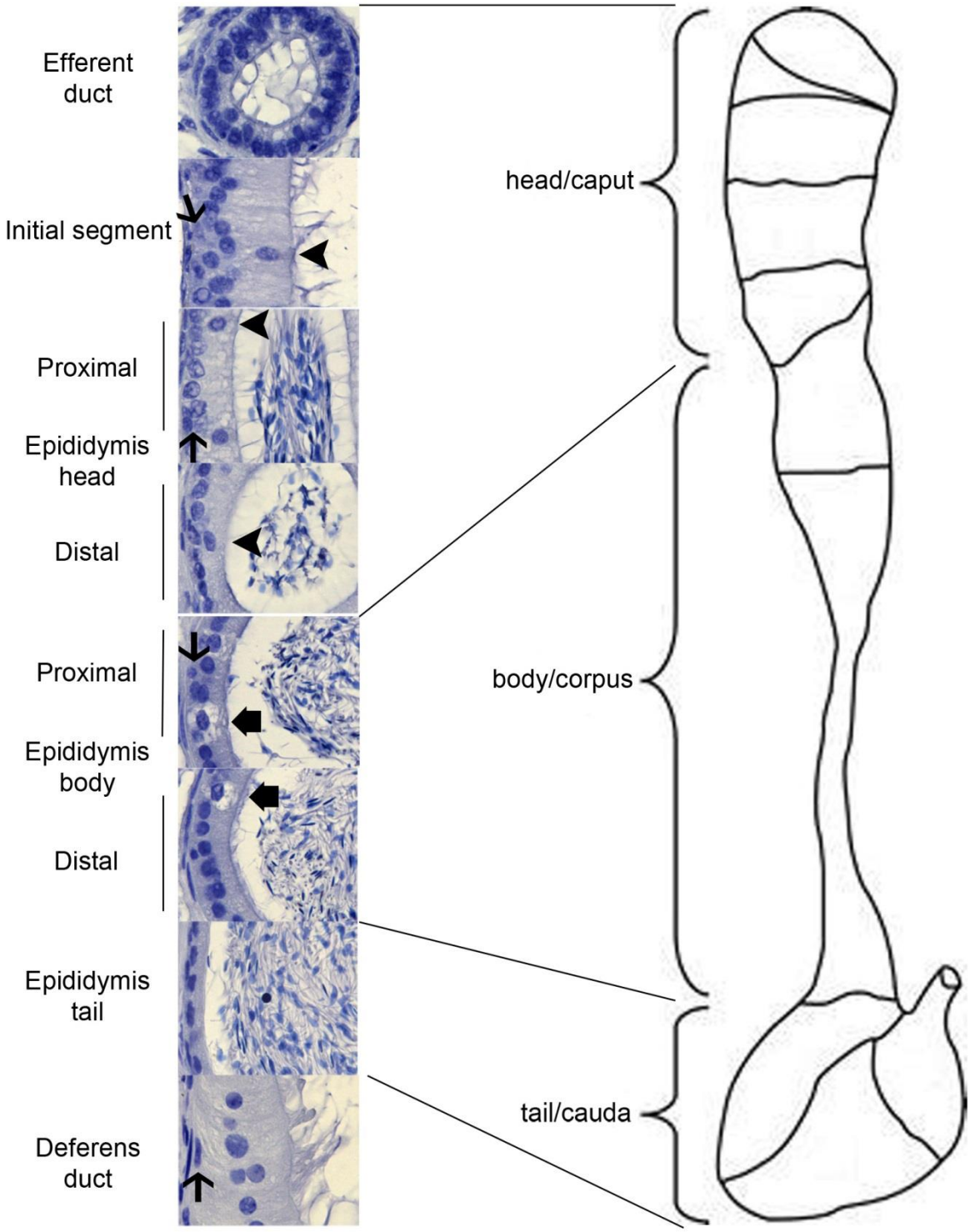
& Sullivan, 2012). Along the epididymis, spermatozoa experience maturational changes that will ultimately enable them to acquire fertilizing capacity. This involves the acquisition of progressive forward motility, the ability to undergo the post ejaculatory events (capacitation and hyperactivation), and the capacity to recognize and to bind to the oocyte (Guyonnet, Dacheux, Dacheux, & Gatti, 2011).

These maturation changes involve biochemical modifications are induced by the continuously evolving epididymal intraluminal composition, which in turn is modulated by the epididymal epithelium (Cuasnicú et al., 1984). Proteins secreted by epididymal epithelial cells interact with maturing spermatozoa to generate fully functional gametes (Sullivan, Saez, Girouard, & Frenette, 2005). The secreted epithelium protein profile changes from one segment to the other, which relies on an fine regulation of epididymis gene expression (Krull, Ivell, Osterhoff, & Kirchhoff, 1993; Gebhardt et al., 1999; Guyonnet et al., 2011). Epithelial gene expression along the epididymis is related with the different steps of sperm maturation (Johnston et al., 2005, 2007; Guyonnet et al., 2009). The acquisition of fertilizing ability occurs at a location in the epididymis that is species-dependent (Dacheux & Paquignon, 1980). In general, whereas luminal secretions from the epididymis head and body are beneficial for the acquisition of sperm motility and fertilizing ability, epididymis tail secretions are important for the proper storage of spermatozoa for several days in conditions that preserve their fertility (Turner, 1995; Jones & Murdoch, 1996).

Epididymal proteins are added to spermatozoa during the maturation process by unusual mechanisms and may have an important role in this process (Saez, Frenette, & Sullivan, 2003). These proteins are transferred to sperm cells in vesicles, named epididymosomes, which have been described in several mammalian species (Légaré et al., 1999; Frenette & Sullivan, 2001; Rejraji et al., 2006). The epididymosome protein composition changes between epididymis segments and between those and the ejaculated semen (Frenette, Girouard, & Sullivan, 2006a), which is relevant for epididymis sperm maturation (Sullivan, Frenette, & Girouard, 2007).

The composition of the luminal milieu is controlled by the surrounding pseudostratified epithelium, which is composed by several cell types displaying distinct functions: principal, narrow, apical, clear and basal cells (Figure 2) (Joseph, Shur, & Hess, 2011; Belleannée et al., 2012). These different cell types need to perform extensive intercommunication in order to regulate the sequential composition of the luminal milieu and the biochemical changes associated with spermatozoa maturation (Shum, Ruan, Da Silva, & Breton, 2013). However, this complex and finely tuned cellular signaling is only poorly understood.

Figure 2 – Segmental structure of the mouse epididymis and deferens duct.



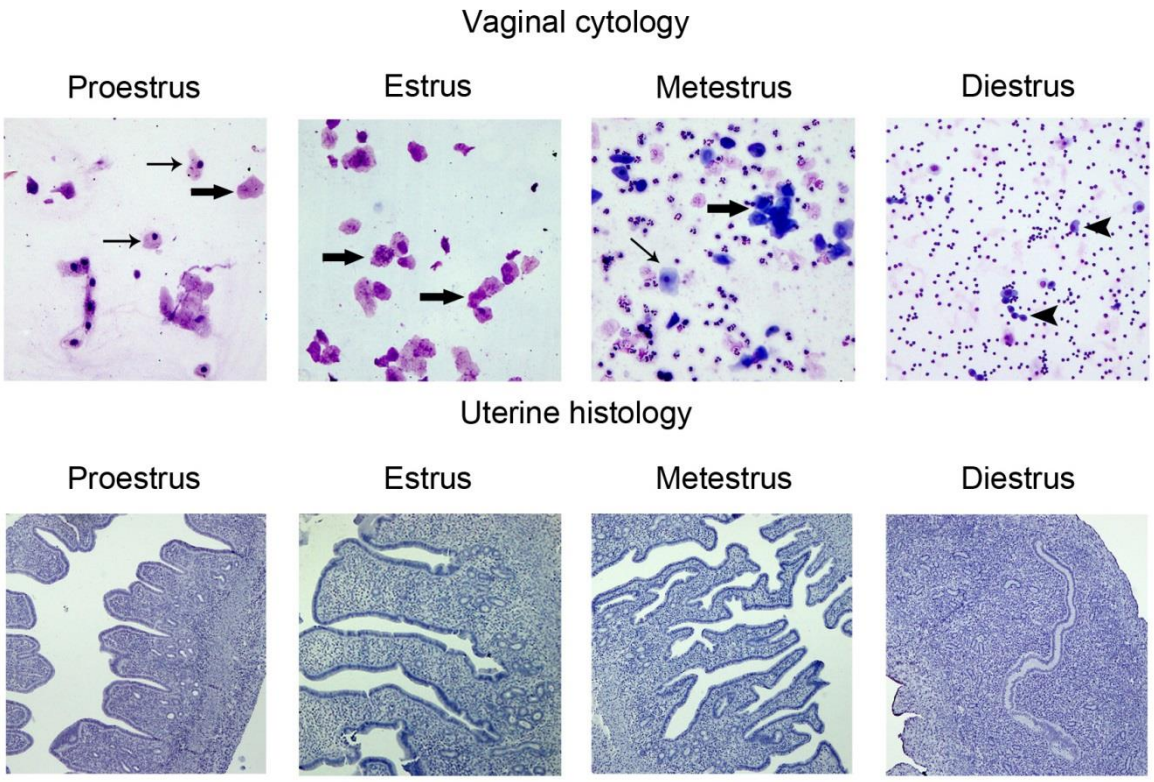
Haematoxylin staining. 400x magnification. Arrow heads point narrow and apical cells. Arrows point basal cells. Bold arrows point clear cells. Principal cells are the majority of epithelial cells and are not marked. Draw scheme is shown with the different epididymis segments separated by fibrous septa. Drawing adapted from (Johnston et al., 2005).

4. The female reproductive cycle

The mouse EC lasts approximately 4 to 5 days and can be divided into four stages: proestrus, estrus, metestrus and diestrus. Cellular remodeling events occurring at the ovary and tubular tract are regulated by changes in circulating hormones levels. Estrogens peak prior to ovulation, which occurs at estrus, while P4 concentrations rise during metestrus and diestrus, and then decline from proestrus to estrus (Walmer, Wrona, Hughes, & Nelson, 1992; Fata, Chaudhary, & Khokha, 2001). These hormonal changes can be used to stage the EC (Walmer et al., 1992; Fata et al., 2001; Wood, Fata, Watson, & Khokha, 2007). Vaginal cytology and uterus histology can also be used to stage the mouse EC (Figure 3) (Wood et al., 2007). Although the main EC cellular events are controlled by hormonal changes, ovarian and uterine functions are not completely dependent on this hormonal regulation. This is the case of postnatal follicle development (Peters, Byskov, & Lintern-Moore, 1973). After initial recruitment from the primordial follicle resting pool, most follicles will develop independently of hormonal stimulus until the follicle stimulating hormone (FSH) dependent early antral stage. Only if a follicle arrives the FSH-dependent stage in the 'window' when FSH is elevated, it can proceed to the next developmental stage (Hirshfield, 1991; Le Nestour, Marraomi, & Lahlou, 1993). This reduces the number of follicles developing to ovulation. Still, the number of follicles in the cohort that continue to develop is greater than the desired ovulatory number, so a further process of reduction occurs. This second phase of follicle selection is known as follicle dominance. Dominant follicles continue to the final stages of development while the remaining subordinate follicles in the cohort ultimately undergo atresia and regress (Baker & Spears, 1999). These larger follicles in a cohort indirectly cause the cessation of growth and development in subordinate members (Zeleznik & Hillier, 1984; Gibbons, Wiltbank, & Ginther, 1997). Follicular dominance is mainly mediated through endocrine regulation. Nevertheless, intra-ovarian interactions are also involved in this regulation. Follicle–follicle interactions have various possible roles both at early stages when follicles are being selected from a cohort or cluster of follicles and/or later, when dominant follicles are affecting the subordinate's growth (Baker & Spears, 1999). However, the cell signaling pathways enrolled in such regulation are still under inquiry.

The formation, maintenance, regression, and steroidogenesis of the CL are among the most closely regulated events in mammalian reproduction (Stocco, Telleria, & Gibori, 2007). The CL has a short life span and is the site of rapid cellular remodeling, growth, differentiation, and death of several cell types, including granulosa, theca, endothelial and mesenchymal cells. The interactions between these cell types are essential in maintaining the development and steroidogenic function of the CL (Stocco et al., 2007).

Figure 3 – Vaginal cytology and uterine histology along mouse EC stages.



EC stage identification through vaginal cytology, giemsa staining (100x magnification). Proestrus is characterized by the presence of few if any neutrophils, some parabasal and intermediate epithelial cells. At this stage there are already some large cornified epithelial cells. In estrus only large cornified epithelial cells are present. Metestrus is characterized by the presence of neutrophils, intermediate and some parabasal cells. Large cornified epithelial cells are present in large agglomerates. During diestrus the smear includes numerous neutrophils and basal and parabasal epithelial cells. Arrow - parabasal epithelial cells. Bold arrow - cornified epithelial cells. Arrow head - basal epithelial cells. Uterine section at each EC stage, haematoxylin staining (40x magnification). Proestrus has a typical undulating endometrium, estrus has a pronounced increase in extracellular fluid throughout the endometrium, at metestrus the luminal surface is very irregular, and at diestrus the uterine lumen is collapsed.

The oviduct is the site of fertilization and its epithelium establishes relationships with both male and female gametes (Myoungkun & Bridges, 2011; Coy, García-Vázquez, Visconti, & Avilés, 2012). Along the EC, the oviduct undergoes cyclic changes involving growth and regression of its epithelial cell layer constituted by both ciliated and secretory luminal epithelial cells.

The endometrium also undergoes extensive cellular remodeling along the EC, in response to systemic changes in steroid hormones, E2 and P4 (Wood et al., 2007). Both in humans and mice, these changes involve a synchronized sequence of cellular proliferation, apoptosis and differentiation events, along with extracellular matrix turnover, angiogenesis, and leukocyte infiltration (Evans et al., 1990). Nevertheless, unlike humans and primates, menstruation, the

physical shedding of the endometrium, is not a feature of the rodent EC. Even so, due to practical aspects regarding laboratory environment, manipulation and genetic tractability, the mouse uterus has been extensively used as a model to understand human and other animal species uterine function. In the mouse, changes from one EC stage to the next occur over a 24 hour period, requiring a rapid cellular response to hormonal fluctuations (Hewitt et al., 2003). This leads to massif changes in epithelial, stroma and gland cell turnover during each EC stage (Wood et al., 2007). Metestrus is also characterized by the migration of neutrophils into the uterine luminal epithelium (Corbeil, Chatterjee, Foresman, & Westfall, 1985). Again, the molecular mechanisms responsible for the regulation of all the cellular remodeling that occurs during the EC are far from being well understood.

5. Molecular and cellular regulation of reproduction

Besides endocrine regulation, several molecular and cellular mechanisms control reproductive function. Gametogenesis involve the interplay of endocrine factors within the hypothalamic-pituitary-gonadal axis, and autocrine, paracrine and juxtracrine interactions between germ cells within the seminiferous tubules/ovaries and neighboring somatic cells. The first genes shown to play a role in reproductive biology were identified on the basis of their abilities to influence sexual differentiation or to alter hormone production or sensitivity. In 1905, the X and Y chromosomes were hypothesized to influence sex determination in insects, however, only in 1959 it was shown that the Y chromosome contains the mammalian male-determining gene (Ford et al., 1959; Jacobs & Strong, 1959). However, several years had to roll until the sex-determining region Y (SRY) gene was identified both in humans and mice (Berta et al., 1990; Koopman et al., 1990; Sinclair et al., 1990). Many other genes and signaling pathways were so far identified as being enrolled in reproductive function regulation. These discoveries historically evolved from the study of cases of infertility and subfertility in male and female human individuals (Matzuk & Lamb, 2002, 2008).

The transforming growth factor β (TGFβ) superfamily is one example of signaling pathway involved in the regulation of reproductive function. Most of TGFβ family proteins signal through a canonical pathway that involves serine threonine kinase receptors, being also related with SMAD and bone morphogenetic proteins (BMPs). Mutations in essentially every major component of these signaling pathways have been created in mice and, along with additional studies in humans and other species, have shown that this signaling pathway is essential to nearly every developmental and physiological process, both in male and female reproductive biology (Chang, Brown, & Matzuk, 2002; Matzuk & Burns, 2012).

Another example are the vascular endothelial growth factor (VEGF) family proteins, which has been implicated in the regulation of both male and female reproductive events, including neoangiogenesis in female genital tract (Gabler, Einspanier, Schams, & Einspanier, 1999; Irusta, Abramovich, Parborell, & Tesone, 2010; Caires, de Avila, Cupp, & McLean, 2012; Kim

et al., 2013). Fibroblast growth factor (FGF) (Sugiura et al., 2008) and growth differentiation factor-9 (GDF9) (Dong et al., 1996) were implicated in follicle growth regulation.

Amazingly, the known number of genes intervening in reproductive function is much higher in the case of the male than of the female. Approximately 1 out of 25 of all mammalian genes are specifically expressed in the male germline (Schultz, Hamra, & Garbers, 2003), illustrating the complexity of the spermatogenic cycle. Several genes have a role both in male and female reproductive biology. Such is the case of the Notch signaling pathway, object of study in this thesis, which has been related to reproductive events occurring in both sexes, from *Drosophila* to humans.

6. Notch signaling enrolment in mammalian reproductive function

6.1. Introduction

Mammalian reproductive function is essentially regulated by hormones. Changes in circulating hormone concentrations trigger a series of cellular responses, including proliferation, apoptosis and differentiation. However, these events require extensive intercellular communication, coordinated through cell signaling pathways. The Notch cell signaling pathway is implicated in cell fate decisions and morphogenetic changes in several tissues (Artavanis-Tsakonas et al., 1995). This cell signaling pathway is also implicated in reproductive function of organisms like *Drosophila* (López-schier & Johnston, 2001; Ward et al., 2006; Assa-Kunik et al., 2007; Song, Call, Kirilly, & Xie, 2007; Kitadate, 2010) and *C. elegans* (Kimble & Crittenden, 2007).

In this literature review we address the enrolment of Notch cell signaling pathway in male and female genital development and reproductive function.

6.2. Notch cell signaling pathway

Notch pathway is a well conserved cell signaling pathway that plays a central regulatory role in the specification of cell fates, determined by local cell interactions, in a wide range of tissues and organisms (Artavanis-Tsakonas et al., 1995). This signaling pathway was first identified in *Drosophila* and since then from *C. elegans* to humans (Borggrefe & Oswald, 2009). In mammals, four receptors (Notch1-4) and five ligands (three delta-like - Dll1, Dll3 and Dll4 - and two serrate-like - Jagged1 and Jagged2) were identified so far (Borggrefe & Oswald, 2009).

This signaling pathway is best understood as a ligand-receptor communication between adjacent cells, whereby both the ligands and receptors are trans-membrane proteins anchored to their respective cell surfaces. Notch signaling is activated through receptor binding with ligands expressed on neighboring cells. Ligand binding promotes two proteolytic cleavages in the receptor. One of them is mediated by a γ-secretase, and other one is

catalyzed by ADAM-family metalloproteases. γ-secretase cleavage of receptors releases their intracellular domain (NICD), which is translocated to the nucleus. Here, together with co-activators like Mastermind/MAML and p300/CBP, NICD converts the transcriptional repressor DNA-binding protein RBPJk into a transcriptional activator, thus activating transcription of Notch target genes (Borggrefe & Oswald, 2009). The more ubiquitous Notch target genes are the hairy/enhancer-of-split (HES) and HES-related with YRPW motif (HEY) families of transcription factors (Ohtsuka et al., 1999; Iso, Sartorelli, Chung, & Shichinohe, 2001; Fischer & Gessler, 2007). Canonically, HES and HEY proteins inhibit expression of other genes by forming complexes with co-repressors (Fischer & Gessler, 2007).

Besides the activation of target genes via RBPJk, referred to as the canonical pathway, additional non-canonical pathways of Notch were described, although are less characterized (Fischer & Gessler, 2007). Notch signaling at distance, without direct cell contact, was also recently reported (Sheldon et al., 2010; Lu et al., 2013). Although there is increasing evidence that signaling through different Notch receptors induce different cellular responses (Shimizu et al., 2002; Yuan et al., 2012), and that different Notch ligands may reveal different affinities to Notch receptors (Benedito et al., 2009), Notch components may be redundant in some scenarios (Zeng, Younger-shepherd, Jan, & Jan, 1998; Kitamoto et al., 2005).

6.3. Notch regulation of cell fate decisions

Notch signaling is enrolled in the regulation of cell fate decisions, including lineage decisions during embryonic development, and homeostasis of adult self-renewing organs (Artavanis-Tsakonas, Rand, & Lake, 1999; Bray, 2006).

Notch can act through lateral inhibition, lateral induction, stem cell maintenance and cell differentiation (Bray, 2006; Borggrefe & Oswald, 2009). Lateral inhibition occurs when in a group of equipotent cells, expressing equal amounts of Notch receptors and ligands, one cell begins to gradually increase ligand expression. This activates a Notch signaling cascade in the neighboring cells, thereby inhibiting their differentiation (Bray, 2006). In lateral induction, Notch ligands are expressed by a different cell type in close proximity, which determines the final cell fate. In the absence of ligands, Notch receptor is not activated and the cell will progress to a different cell fate (Borggrefe & Oswald, 2009). In another way, Notch signaling can either guarantee a stem cell pool maintenance, or drive a terminal differentiation program (Borggrefe & Oswald, 2009).

6.4. Notch pathway enrolment in reproductive function of invertebrate animal models

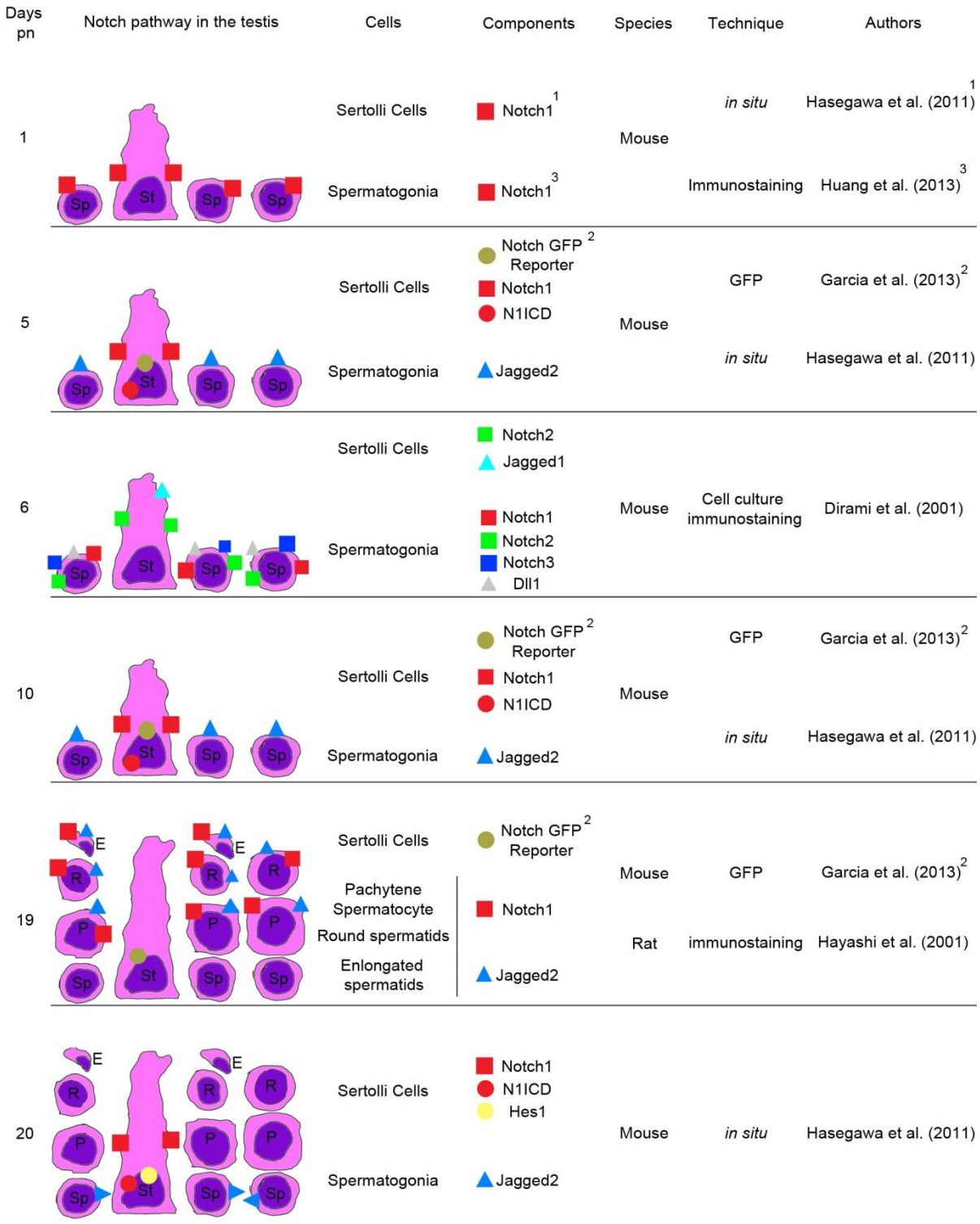
Notch signaling is implicated in reproductive function of organisms as *Drosophila* (López-schier & Johnston, 2001; Ward et al., 2006; Assa-Kunik et al., 2007; Song et al., 2007; Kitadate, 2010) and *C. elegans* (Kimble & Crittenden, 2007). In *Drosophila*, Notch is implicated in ovarian follicular development (López-schier & Johnston, 2001; Assa-Kunik et al., 2007) and germ-line stem cell niche formation in both male and female gonads (Ward et al., 2006; Song et al., 2007; Kitadate, 2010), whereas in *C. elegans* is implicated in germ cell physiology (reviewed by Kimble & Crittenden, 2007).

6.5. Notch signaling in mammalian male reproductive tract post-natal development

Notch is involved in testis embryonic (Tang et al., 2008; K L Hahn et al., 2009; DeFalco et al., 2013) and pn development (Dirami, Ravindranath, Achi, & Dym, 2001; Hayashi et al., 2001; Hasegawa, Okamura, & Saga, 2011; Garcia, Defalco, Capel, & Hofmann, 2013). The present state-of-art regarding involvement of Notch in testis pn development is summarized in Figure 4.

In the mouse, only Sertoli cells and spermatogonia are present in seminiferous tubules until pn day 8 (Nebel, Amarose & Hacket, 1961; Bellvi et al., 1977), being the spermatogonia pool maintained quiescent. At pn day 1 *Notch1* is transcribed in Sertoli cells (Hasegawa et al., 2011) and spermatogonia (Huang, Rivas, & Agoulnik, 2013), at pn day 3, Notch signaling is activated in Sertoli cells (Garcia et al., 2013), and at pn day 5, *Jagged2* is transcribed in spermatogonia and cleaved Notch1 intracellular domain (N1ICD) is detected in Sertoli cells (Hasegawa et al., 2011). Cell cultures obtained from testis at pn day 6 express Notch2 and Jagged1 in Sertoli cells, and express Notch1, Notch2, Notch3 and Dll1 in spermatogonia (Dirami et al., 2001). At pn day 10, when meiosis starts (Nebel et al., 1961; Bellvi et al., 1977), *Jagged2* is transcribed in spermatogonia (Hasegawa et al., 2011). The activation of Notch signaling continues to be detected in Sertoli cells at pn day 12 and 18 (Garcia et al., 2013), when round spermatids first appear (Nebel et al., 1961; Bellvi et al., 1977). Hayashi et al. (2001) analyzed the expression of Notch1 and Jagged2 in pn day 19 rat testis, and detected expression of Notch1 and Jagged2 in pachytene spermatocytes and in round and elongated spermatids. In the mouse, at pn day 20, Hasegawa et al. (2011) detected transcription of *Notch1* in Sertoli cells and of *Jagged2* in spermatogonia. These authors also detected the presence of *Hes1* in Sertoli cells (Hasegawa et al., 2011).

Figure 4 - Notch pathway along testis post-natal development



Notch pathway components and effectors time and cell type specific detection along the testis pn development is represented in shapes and colors according to legend. pn – post-natal; *in situ* - *in situ* hybridization; GFP – Green Fluorescent Protein; N1ICD – Notch1 intracellular domain; Sp – spermatogonia; St – Sertoli cells; P – Pachytene spermatocytes; R – round spermatids; E – elongated spermatids

The involvement of Notch in Leydig cells' embryonic and pn development was evaluated in two studies (Tang et al., 2008; DeFalco et al., 2013). Inhibition of Notch activation in interstitial cells was evaluated through the use of TNR-GFP transgenic Notch reporter mouse (DeFalco et al., 2013). This study showed the enrolment of Jagged1 in maintenance of the Leydig progenitor cells population. Results also lead authors to the suggestion that active Notch signaling may play a role in fetal Leydig cells development and transition into adult Leydig cells prior to puberty, although being not required during adulthood (DeFalco et al., 2013).

Transcription of *Notch1* and *Jagged2* in the epididymis and deferens duct was also analyzed (Hayashi, et al., 2004c). In fact, the first mutant mouse line used to explore Notch function in reproductive events, had the constitutive expression of active Notch1 (Lupien et al., 2006). This mutant developed abnormal duct formation and sterility, demonstrating that the constitutive activation of Notch1 significantly affects development of male reproductive organs. A similar phenotype was latter shown to occur in mice with deficiency of lunatic fringe (Hahn et al., 2009). Involvement of Notch in germ cell pool maintenance was shown through the use of a mutant mouse line with constitutive activation of Notch1 in Sertoli cells (Garcia et al., 2013).

6.6. Notch signaling in male reproductive function

Several Notch pathway components have been identified in the testis during adult spermatogenesis (Mori, Kadokawa, Hoshinaga, & Marunouchi, 2003; Sahin et al., 2005; Hasegawa et al., 2011; Garcia et al., 2013; Huang et al., 2013). The present state-of-the art regarding transcription and expression patterns of Notch components in the testis during adulthood are summarized in Figure 5.

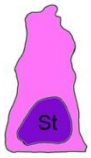
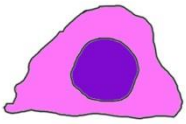
Sertoli cells express Notch1 and Notch2 (Sahin et al., 2005; Hasegawa et al., 2011), and Notch signaling was shown to be active in Sertoli cells, as both N1ICD and *Hes1* were detected in these cells (Hasegawa et al., 2011). Another study, using a mouse TNR-GFP transgenic Notch reporter, confirmed this activation (Garcia et al., 2013).

The presence of Notch components was also described in germ cells. Notch1, Notch2, Notch4, *Jagged2* and the intracellular domains of Notch1, Notch2 and Notch3 were detected in spermatogonia (Mori et al., 2003; Sahin et al., 2005; Hasegawa et al., 2011). Transcription of *Jagged2* (Hasegawa et al., 2011) and expression of Notch1, Notch2, Notch4 and their intracellular domains were detected in spermatocytes (Mori et al., 2003; Sahin et al., 2005; Huang et al., 2013). Round spermatids express Notch receptors 1, 2 and 4, and the presence of their intracellular domains was also detected (Mori et al., 2003; Sahin et al., 2005; Huang et al., 2013). The presence of Notch1 and Nocth2 and the transcription of *Jagged1* was detected in elongated spermatids (Sahin et al., 2005; Hasegawa et al., 2011).

Although transcription and expression of Notch pathway components in the testis were detected by several authors, the enrollment of this signaling pathway in spermatogenesis is still controversial. Recently, two studies reported that Notch signaling was not required for spermatogenesis (Hasegawa et al., 2011; Batista, Lu, Williams, & Stanley, 2012). These authors used *Pofut1* conditional loss-of-function mutant lines, blocking Notch signaling. Pofut1 protein is implied in Notch receptors configuration (Wang et al., 2001). However, in mammals, an unrelated α-glucosidase1 can compensate for Pofut1 in promoting Notch folding and function and thus, Pofut1 is not absolutely required for stable cell surface expression of Notch (Stahl et al., 2008). Other studies, using spermatogonia specific conditional loss-of-function mutant lines showed that Notch1 is not required for spermatogenesis (Batista et al., 2012; Huang et al., 2013). These studies used Notch1 mutant mouse models. Redundancy in Notch receptors may be crucial to normal signaling since paralogues exert redundant or additive effects in maintaining the balance (Zeng et al., 1998). Therefore, blocking Notch1 activation probably do not inhibit overall Notch signaling.

In fact, several studies associated Notch pathway dysfunction with male fertility problems (Hayashi et al., 2001; Hayashi, Yamada, Kageyama, Negishi, & Kihara 2004b; Sahin et al., 2005; Lupien et al., 2006; Hahn et al., 2009; Garcia et al., 2013; Huang et al., 2013). Spermatogenesis was suppressed *in vitro* through the blockade of Notch1 and Jagged2 (Hayashi et al., 2001) and, failure of Notch expression was associated with the pathogenesis of maturation arrest in male human infertility patients (Hayashi, et al., 2004b). A change in the expression pattern of Notch receptors was also related to rat varicocele induced spermatogenesis arrest (Sahin et al., 2005). Both the deficiency of lunatic fringe and the constitutive activation of Notch1 resulted in male reproductive tract defects and fertility problems (Lupien et al., 2006; Hahn et al., 2009). Garcia et al. (2013) reported the enrolment of Notch1 in Sertoli cells and spermatogonia pool maintenance (Garcia et al., 2013), whereas Huang et al. (2013), also using a Notch1 gain of function mouse model, reported the relevance of Notch signaling in the regulation of male germ cells survival and differentiation (Huang et al., 2013). Although DeFalco et al. (2013) showed a decline in Notch activation in Leydig cells throughout adulthood, Sahin et al. (2005) identified expression of Notch receptors 1, 2 and 3 in Leydig cells of adult rats (Sahin et al., 2005).

Figure 5 - Notch pathway in the adult testis

Cell types	Notch components		Species	Technique	Authors
Sertolli cells 	Notch1[2, 3] N1ICD[3] Notch2[2]	Notch GFP Reporter[4] Hes1[3]	Rat[2] Mouse[3, 4]	immunostaining[2] *in situ*[3] GFP[4]	Sahin et al. (2005)[2] Hasegawa et al. (2011)[3] Garcia et al. (2013)[4]
Spermatogonia 	Notch1[1, 2] N1ICD[1] Notch2[1, 2] N2ICD[1] N3ICD[1] Notch4[1]	Jagged2[3]	Mouse[1, 3] Rat[2]	immunostaining[1, 2] *in situ*[3]	Mori et al. (2003)[1] Sahin et al. (2005)[2] Hasegawa et al. (2011)[3]
Spermatocyte 	Notch1[1, 2, 5] N1ICD[1] Notch2[1, 2] N2ICD[1] Notch4[1] N4ICD[1]	Jagged2[3]	Mouse[1, 3, 5] Rat[2]	immunostaining[1, 2, 5] *in situ*[3]	Mori et al. (2013)[1] Sahin et al. (2005)[2] Hasegawa et al. (2011)[3] Huang et al. (2013)[5]
Round spermatid 	Notch1[1, 5] N1ICD[1] Notch2[1, 2] N2ICD[1] Notch4[1] N4ICD[1]		Mouse[1,5] Rat[2]	immunostaining	Mori et al. (2013)[1] Sahin et al. (2005)[2] Huang et al. (2013)[5]
Elongated spermatid 	Notch1[2] Notch2[2]	Jagged1[3]	Rat[2] Mouse[3]	immunostaining[2] *in situ*[3]	Sahin et al. (2005)[2] Hasegawa et al. (2011)[3]
Leydig cells 	Notch1 Notch2 Notch3		Rat	immunostaining	Sahin et al. (2005)

Notch pathway components and effectors cell type specific detection in the testis during adulthood is represented in shapes and colors according to legend. *in situ* - *in situ* hybridization; GFP – Green Fluorescent Protein; N1ICD – Notch1 intracellular domain; N2ICD – Notch2 intracellular domain; N3ICD – Notch3 intracellular domain; N4ICD – Notch4 intracellular domain; Sp – spermatogonia; St – Sertoli cells; P – Pachytene spermatocytes; R – round spermatids; E – elongated spermatids

6.7. Notch pathway enrolment in female reproductive tract post-natal development

At the very first days of pn life (until pn days 4-6), oocytes become surrounded by somatic cells to form primordial follicles. During this process only one-third of the initial number of oocytes survive to form primordial follicles (Pepling, 2012). The genetic mechanisms behind the regulation of these events are still poorly understood. Perturbations during the critical period of primordial follicle formation can significantly affect the size of the primordial follicle pool and follicular phenotypes (Trombly, Woodruff, & Mayo, 2009). Components of Notch pathway were reported to be present in the neonate mammalian ovary (Trombly et al., 2009; Xu & Gridley, 2013). Trombly et al. (2009) identified expression of Notch2 in granulosa cells and Jagged1 in germ cells, and reported that blockade of Notch signaling through the use of a γ-secretase inhibitor resulted in retained germ cell nests and reduced number of primordial follicles (Trombly et al., 2009). Xu & Gridley (2013), using a mouse line with conditional deletion of *Notch2* in granulosa cells, demonstrated that Notch2-mediated signaling regulates oocyte apoptosis non-cell autonomously, and breakdown of germ-cell nests and formation of primordial follicles (Xu & Gridley, 2013).

6.8. Notch pathway in female reproductive function

During adulthood, just a small proportion of the ovarian pool of primordial follicles escape their arrested state and resume growth and development, the major proportion becoming atretic. As follicles reaches maturity, ovulation occurs and the CL is formed. Notch pathway components were identified in the ovary and Notch signaling was implicated in adult mammalian ovarian (Uyttendaele et al., 1996; Baker & Spears, 1999; Johnson et al., 2001; Hahn et al., 2005; Vorontchikhina et al., 2005; Hernandez et al., 2011; Zhang et al., 2011; Fraser et al., 2012; García-Pascual et al., 2013; Jovanovic et al., 2013) and uterine regulation (Cobellis et al., 2008; Mazella, Liang, & Tseng, 2008; Mikhailik, Mazella, Liang, & Tseng, 2009; Afshar, et al., 2012a; Afshar, Miele, & Fazleabas, 2012b; Degaki, Chen, Yamada, & Croy, 2012).

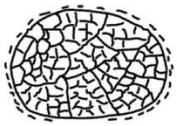
Notch2 and Notch effector Hes1 were identified in granulosa cells of primordial follicles (Trombly et al., 2009), and primary follicles (Trombly et al., 2009; Zhang et al., 2011). Expression of Notch2 and Notch effectors were maintained in granulosa cells when primary follicles progress into secondary follicles; following this follicle developmental stage, Notch3 and Jagged2 also became expressed until the antral follicle stage (Johnson et al., 2001; Zhang et al., 2011; Jovanovic et al., 2013). Notch2, Jagged1 and several Notch pathway effectors were also detected in oocytes (Johnson et al., 2001; Trombly et al., 2009; Zhang et al., 2011). Notch1, Notch3, Notch4, Dll4 and the ICD of Notch1 and Notch4 were detected both in mouse and rat luteal cells (J. Johnson et al., 2001; Hernandez et al., 2011). Notch1, Notch3, Notch4, Jagged1 and Dll4 were also detected in ovarian blood vessels (J. Johnson et al., 2001; Vorontchikhina et al., 2005; Hernandez et al., 2011; García-Pascual et al., 2013;

Jovanovic et al., 2013). All Notch receptors and both Jagged ligands were detected in human cumulus cells (Tanriverdi et al., 2013). The present state-of-the art regarding transcription and expression patterns of Notch pathway components and effectors in the ovary is summarized in Figure 6.

Notch detection in the ovary was associated with follicle selection and dominance (Baker & Spears, 1999) and the implication of this signaling pathway in this phenomenon was straightened by the analysis of its transcription pattern (Johnson et al., 2001). The following studies essentially associated Notch with ovarian neovascularization (Vorontchikhina et al., 2005; Fraser et al., 2012; García-Pascual et al., 2013; Jovanovic et al., 2013), follicle recruitment and CL function (Hernandez et al., 2011; Zhang et al., 2011; García-Pascual et al., 2013; Tanriverdi et al., 2013). Vorontchikhina et al. (2005) reported expression of Notch1, Notch4 and Jagged1 in ovarian blood vessels, and associated their expression with the regulation of ovarian neovascularization during follicular growth and CL formation. In fact, it is well known that neoangiogenesis plays a relevant role in those events (Wulff et al., 2002; Zimmermann et al., 2003; Fraser et al., 2005, 2006). Gonadotropin dependent follicular growth is not depend on Notch ligand Dll4 (Fraser et al., 2012; Jovanovic et al., 2013), but this ligand is central in the regulation of CL angiogenesis and function (Fraser et al., 2012; García-Pascual et al., 2013). Recently, Hernandez et al. (2011) suggested that Dll4-Notch signaling pathway has a luteotropic role by promoting luteal cell viability and steroidogenesis. Notch pathway was also associated to follicle granulosa cell proliferation (Zhang et al., 2011).

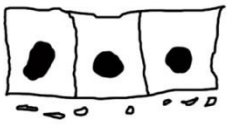
At each reproductive cycle, the female reproductive tract undergoes extensive remodeling, to render the genital tract receptive for the developing embryos. These changes involve a well-orchestrated synchrony of cellular proliferation, apoptosis, and differentiation events, along with extracellular matrix turnover, angiogenesis, and leukocyte infiltration. These events are regulated by sexual steroid hormones, E2 and P4. Notch pathway components were first identified in the human uterus through protein localization (Cobellis et al., 2008; Mazella et al., 2008) and gene transcription (Mikhailik et al., 2009). Later, the use of animal models allowed the evaluation of Notch involvement in uterine physiology (Afshar, et al., 2012a; Degaki et al., 2012). Notch pathway components were identified in the uterus epithelium, glands and stroma. Notch1 and Dll4 were identified in uterus luminal epithelial cells (Mazella et al., 2008; Afshar, et al., 2012b) and Notch1, Notch4, Jagged1 and Dll4 were identified both in uterine glands and stroma cells (Cobellis et al., 2008; Mazella et al., 2008; Afshar, et al., 2012a; Afshar, et al., 2012b). The present state-of-the art regarding the transcription and expression patterns of Notch pathway components in the uterus is summarized in Figure 7.

Figure 6 - Notch pathway components and effectors in the ovary

Structure/cells	Notch components	Species	Technique	Authors
Primordial follicles Granulosa cells 	Notch2 Hes1	Mouse	Immunostaining	Trombly et al. (2009)
Primary follicles Granulosa cells 	Notch2 [3, 5] Notch effectors [5]	Mouse	Immunostaining [3, 5] *in situ* [5]	Trombly et al. (2009) [3] Zhang et al. (2011) [5]
Secondary follicles Granulosa cells 	Notch2 [1, 5, 6] Jagged2 [1] Notch3 [1] Notch effectors [1, 5]	Mouse	*in situ* [1, 5] Immunostaining [5, 6]	Johnson et al. (2001) [1] Zhang et al. (2011) [5] Jovanovic et al. (2013) [6]
Antral follicles Granulosa cells 	Notch2 [1, 5, 6] Jagged2 [1] Notch3 [1] Notch effectors [1, 5]	Mouse	*in situ* [1, 5] Immunostaining [5, 6]	Johnson et al. (2001) [1] Zhang et al. (2011) [5] Jovanovic et al. (2013) [6]
Oocytes 	Notch2 [5] Jagged1 [1, 3, 5] Notch effectors [3, 5]	Mouse	*in situ* [1, 5] Immunostaining [3, 5]	Johnson et al. (2001) [1] Trombly et al. (2009) [3] Zhang et al. (2011) [5]
Corpus luteum cells 	Notch1 [4] N1ICD [4] Notch3 [1] Notch4 [4] N4ICD [4] Dll4 [4]	Mouse [1] Rat [4]	*in situ* [1] Immunostaining [4]	Johnson et al. (2001) [1] Hernandez et al. (2011) [4]
Blood vessels 	Notch1 [1, 2, 4, 6] Jagged1 [2, 6] Notch3 [6] Notch4 [1, 2, 4, 6] Dll4 [4, 6]	Mouse [1, 2, 6] Rat [4]	*in situ* [1] Immunostaining [2, 4, 6]	Johnson et al. (2001) [1] Vorontchikhina et al (2005) [2] Hernandez et al. (2011) [4] Jovanovic et al. (2013) [6]

Notch pathway components and effectors cell type and ovarian structure specific detection in the adult ovary is represented in shapes and colors according to legend. *in situ* - *in situ* hybridization; N4ICD – Notch4 intracellular domain;

Figure 7 - Notch pathway components detection in the uterus

Structure	Notch components	Species	Technique	Authors
Uterus epithelium 	Notch1[4] Dll4[1]	Human[1] Primate[4]	Immunostaining	Mazella et al. (2008)[1] Afshar et al. (2012b)[4]
Uterine stroma 	Notch1[2, 3, 4] Jagged1[2] Notch4[2] Dll4[1]	Human[1, 2] Mouse[3] Primate[4]	Immunostaining	Mazella et al. (2008)[1] Cobellis et al. (2008)[2] Afshar et al. (2012a)[3] Afshar et al. (2012b)[4]
Uterine glands 	Notch1[2, 4] Jagged1[2] Notch4[2] Dll4[1]	Human[1, 2] Primate[4]	Immunostaining	Mazella et al. (2008)[1] Cobellis et al. (2008)[2] Afshar et al. (2012b)[4]

Notch pathway components cell type and uterine structure specific detection in the adult uterus is represented in shapes and colors according to legend.

In the uterus, Notch was associated with uterine angiogenesis (Mazella et al., 2008; Degaki et al., 2012), uterine cyclic cellular events (Cobellis et al., 2008; Mikhailik et al., 2009; Afshar, et al., 2012b) and embryo implantation (Afshar, et al., 2012a; Afshar, et al., 2012b; Degaki et al., 2012). Additionally, Notch1 and Dll1 were implicated in decidualization (Afshar, et al., 2012a; Afshar, et al., 2012b; Degaki et al., 2012).

EXPERIMENTAL WORK

Chapter I - Dynamics of Notch pathway expression during mouse testis post-natal development and along the spermatogenic cycle

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1. Abstract

The transcription and expression patterns of Notch pathway components (Notch 1-3, Dll1, Dll4 and Jagged1) and effectors (Hes1, Hes2, Hes5 and Nrarp) were evaluated (through RT-PCR and IHC) in the mouse testis at key moments of pn development, and along the adult spermatogenic cycle. Notch pathway components and effectors are transcribed in the testis and expressed in germ, Sertoli and Leydig cells, and each Notch component shows a specific cell-type and time-window expression pattern. This expression at key testis developmental events prompt for a role of Notch signaling in pre-pubertal spermatogonia quiescence, onset of spermatogenesis, and regulation of the spermatogenic cycle.

Key Words: Notch pathway; spermatogenesis; testis post-natal development; spermatogenic cycle

2. Introduction

Spermatogenesis, the generation of haploid, highly specialized germ cells (spermatozoa) in the testis, is the result of a complex orchestration initiated at puberty, involving continuous and serial cellular proliferation and differentiation events (Kimmins, Kotaja, Davidson, & Sassone-Corsi, 2004). In the mouse, only Sertoli cells and spermatogonia are present in the seminiferous tubules until pn day 8, meiosis begins on pn day 10, round spermatids appear by pn day 18 after completing meiosis, and another 14 days are required for spermatids to complete their differentiation and to be released from the seminiferous epithelium (Nebel et al., 1961; Bellvi et al., 1977). In this species, the spermatogenic cycle length is about 8.6 days and is divided in 12 stages arranged in order along the length of the seminiferous tubules (Oakberg, 1957; Clermont & Trott, 1969). Leydig cells are interstitial endocrine cells that mainly secrete testosterone. In mammals, two main periods of Leydig cell function occur. A first Leydig cell generation develops during fetal life and the secreted testosterone is responsible for the masculinization of the urogenital system (Habert, Lejeune, & Saez, 2001). These cells regress thereafter, although some fetal Leydig cells persist in adult life (Ariyaratne & Chamindrani Mendis-Handagama, 2000). A second Leydig cell population appears at puberty secreting testosterone required for the onset of spermatogenesis and overall maintenance of male reproductive function (Habert et al., 2001).

The gene pathways involved in the regulation of the highly complex cellular remodeling associated with spermatogenesis and male hormone secretion are poorly understood. Deciphering these mechanisms could potentially lead to the development of new therapeutic strategies addressed to male infertility and male contraception. The Notch pathway is an evolutionarily well-conserved system that has been implicated in cell fate decisions in several tissues (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009). In mammals, four receptors (Notch1-4) and five ligands (three delta-like - Dll1, Dll3 and Dll4 - and two serrate-like - Jagged1 and Jagged2) were identified (Borggrefe & Oswald, 2009). The Notch signaling pathway is activated by binding of the extracellular domain of the receptors with the ligands expressed on neighboring cells. This leads to the cleavage of the Notch intracellular domain by the γ-secretase complex and its translocation to the nucleus, where it associates with other transcriptional factors, thus regulating transcription of Notch target genes (Borggrefe & Oswald, 2009). The hairy/enhancer of split (*Hes*) genes, coding for highly conserved proteins, are the more ubiquitous Notch effector genes (Fischer & Gessler, 2007). In mammals, *Hes1* and *Hes5* are the more represented of these genes (Borggrefe & Oswald, 2009).

Notch signaling was implicated in germ cell development in *Caenorhabditis elegans* (Kimble & Crittenden, 2007) and *Drosophila* (Ward et al., 2006; Assa-Kunik et al., 2007; Song et al., 2007; Kitadate, 2010), and components of the Notch pathway were reported to be present in the neonate and adult mammalian testis (Dirami et al., 2001; Hayashi et al., 2001; Mori et al.,

2003; Hayashi, et al., 2004b; von Schönfeldt, Wistuba, & Schlatt, 2004; Sahin et al., 2005; Hahn et al., 2009; Hasegawa et al., 2011; Garcia et al., 2013). In rodents and humans, aberrant Notch activity was associated with male infertility (Hayashi et al., 2001; Hayashi, 2004b; Sahin et al., 2005; Lupien et al., 2006; Hahn et al., 2009; Garcia et al., 2013). However, other studies (Hasegawa et al., 2011; Batista et al., 2012) reported that most of Notch pathway components are not transcribed in the mouse testis and that Notch blockade in germ and Sertoli cells had no effect on spermatogenesis. Therefore, the overall available information regarding the potential involvement of Notch signaling in spermatogenesis is fragmentary and controversial.

The objective of this work was to identify transcription and perform a detailed evaluation of the expression patterns of Notch pathway components during mouse testis pn development and, at adult life, along the spermatogenic cycle. Additionally, the transcription and expression of Notch effector genes in the adult testis was investigated to allow a conclusion regarding the activation of the pathway during spermatogenesis. Our main hypothesis is that Notch signaling might be involved in the regulation of spermatogenesis and male testis endocrine secretion. As a first approach, we identified transcripts in the adult testis by reverse transcription PCR (RT-PCR) and then performed the immunolocalization (through immunohistochemistry - IHC) of Notch receptors and ligands in the testis at serial moments of the testis pn development (pn day 4; pn day 15; 1 month; 3 months). This was complemented with the identification of transcription of Notch effector genes and their immunolocalization (through IHC) in the testis during adult spermatogenesis.

Altogether, results strongly prompt for a role of Notch signaling in spermatogonia pool maintenance, onset of spermatogenesis, regulation of spermatogenesis pace, germ cells identity and differentiation, and Leydig cells function.

3. Materials and Methods

3.1. Animals

Experiments were conducted in compliance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (Directive n. 86/609/EEC, from the 24th November 1986). Mice manipulation protocols were approved by the national regulatory agency (DGV – Direção Geral de Veterinária) and the Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal). All authors are accredited as FELASA category C scientists or equivalent.

CD1 mice were maintained in a 12-hour light/dark cycle, in ventilated cages with corn cob as bedding, and were given access to standard laboratory diet and water *ad libitum*. The mice health was routinely monitored. Outbred CD1 animals were chosen to introduce normal biological variability within the experiment.

3.2 Experimental design

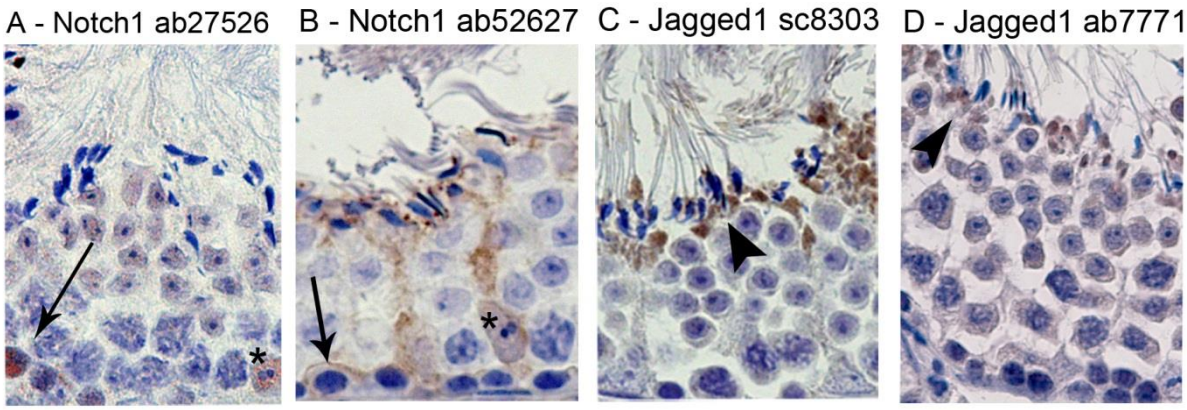
Expression of Notch pathway components in the testis was evaluated at four time-points of pn development: pn day 4; pn day 15; 1 month; 3 months. CD1 male mice were euthanized through cervical dislocation under ketamine (15mg/kg)/xylazine (1mg/kg) anesthesia, followed by exsanguination. Four mice were allocated to each of the above four time-points and their testis were collected and processed for IHC. Dissected testes from another four adult mice were individually processed for RT-PCR.

3.3 Immunohistochemistry

Testes were fixed in 4% neutral phosphate buffered formalin at room temperature for 24h and, after subsequent dehydration in ethanol, were embedded in paraffin. Spatial localization of expression of Notch pathway components (Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1) and effectors (Hes1 and Hes5) was evaluated by IHC, according to a previously described method (Silva et al., 2010). Slices were orientated transversally to the longitudinal axis of the testis, considering sequential sections of 3 µm and sequential twin-slides with each of the three cell markers for each Notch component (minimum of 3 slices per testis for each Notch component in all 16 animals). Tissue sections were stained by haematoxylin and identification of cell types was done through histology (Hess & Franca, 2008) and the use of cell markers in twin slides: The goat anti-3β-HSD antibody was used to identify Leydig cells and the rabbit anti-DAZL and anti-DDX4 antibodies were used to identify pre-meiotic and post-meiotic germ cells, respectively. The antigen retrieval step was performed in citrate buffer (10mM, pH 6.0), except for both anti-Notch1, anti-Hes1, anti-Hes5 and anti-Jagged1 (ab7771) antibodies (Tris-EDTA, pH 9.0). Blocking was performed in PBS with 2% w/v bovine serum albumin (A7906, Sigma-Aldrich, Inc.) for one hour at room temperature. Tissue sections were incubated overnight at 4ºC with the following primary antibodies: rabbit anti-Notch1 (ab27526, Abcam, diluted 1:100), rabbit anti-Notch2 (ab8926, Abcam, diluted 1:100), rabbit anti-Notch3 (ab23426, Abcam, diluted 1:160), rabbit anti-Dll1 (ab10554, Abcam, diluted 1:100), rabbit anti-Dll4 (ab7280; Abcam, diluted 1:200), rabbit anti-Jagged1 (SC-8303, Santacruz Biotechnology, diluted 1:50), rabbit anti-Hes1 (ab71559, Abcam, diluted 1:100), rabbit anti-Hes5 (ab25374, Abcam, diluted 1:100), goat anti-3β-HSD (SC-30820, Santacruz Biotechnology, diluted 1:300), rabbit anti-DAZL (ab34139, Abcam, diluted 1:250), and rabbit anti-DDX4 (ab13840, Abcam, diluted 1:200). The negative controls were performed with the polyclonal rabbit IgG (ab27478, Abcam, diluted 1:100) and, for the 3β-HSD antibody, with the goat control IgG (ab37373, Abcam, diluted 1:300). All primary antibodies were diluted in blocking solution. The peroxidase conjugated monoclonal mouse anti-goat/sheep IgG antibody (A9452, Sigma-Aldrich, Inc., diluted 1:100) and the peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (Dako 410972, diluted 1:100) were used

as secondary antibodies respectively for the anti-3β-HSD antibody, and the remaining primary antibodies. Staining was evaluated in the entire testis slice, considering several seminiferous tubules per slide. Expression patterns were established following the evaluation of a minimum of 24 slices (3 slices/testis x 2 testis x 4 animals) for each Notch component (plus 24 twin-cell marker slides) in each of the four time-points evaluated. In the case of Hes1 and Hes5, only nuclear staining was considered positive, indicating activation of Notch signaling. The antibodies for the Notch components and effectors were previously validated by others in the mouse (anti-Notch1 (Cheng et al., 2007), anti-Notch3 (Feng, Krebs, & Gridley, 2010), anti-Dll1, (Sörensen, Adams, & Gossler, 2009) anti-Dll4, (Sörensen et al., 2009) anti-Jagged1, (Bielesz et al., 2010) anti-Hes1 (Rahman et al., 2013) and anti-Hes5 (Takanaga et al., 2009)) and rat species (anti-Notch2 (Saravanamuthu, Gao, & Zelenka, 2009)). Except for Notch1, all antibodies were polyclonal and the different lots used were originated from different animals (according to manufacturers). To further confirm the specificity of staining, the expression pattern obtained for Notch1 and Jagged1 was evaluated with different antibodies (the anti-Notch1, Ab52627, diluted 1:50 and the anti-Jagged1, Ab7771, diluted 1:100). We selected Notch1 (because all assays were performed with the same antibody lot) and Jagged1 (due to the specific expression pattern) as the targets for these repeats. Evaluation was performed on tissue sections from the same paraffin blocks of the same previously used animals. The two different antibodies against Notch1 and Jagged1 were simultaneously compared in twin slides (Figure 8).

Figure 8 – Evaluation of the specificity of positive staining of Notch1 and Jagged1, using different antibodies.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Notch1: comparison between the anti-Notch1 (Ab27526) (A) and the anti-Notch1 (Ab52627) (B) antibodies; Positive staining was present in spermatogonia, round spermatids and Sertoli cells. Jagged1: comparison between the anti-Jagged1 (sc-8303) (C) and the anti-Jagged1 (Ab7771) (D) antibodies. Positive staining was present in the residual bodies at the sperm head tip. Control was done with rabbit IgG. Arrows point to spermatogonia cells. Asterisks mark Sertoli cells. Arrow heads point to residual bodies containing Jagged1 at the luminal surface of the seminiferous epithelium.

3.4 RT-PCR

Dissected testes of four additional CD1 male mice were individually collected, immediately frozen in liquid nitrogen and stored at −80 ºC until assay. RNA extraction, cDNA synthesis and mRNA transcription was performed as previously described (Silva et al., 2012). The detection of Notch pathway genes (*Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1*) and gene effectors (*Hes1, Hes2, Hes5, Nrarp*) were analyzed with specific primer pair sequences. Transcription of gene *HPRT1* was used as an endogenous control. Primer pair sequences in Annex I.

3.5 Statistical analysis

The relative frequency of pachytene spermatocytes expressing Notch2, Notch3 and Dll4 were determined by counting all marked and unmarked pachytene spermatocytes, in five different seminiferous tubules sections, at each of the spermatogenic cycle stages (I-II; III-IV; V-VI; VII-VIII; IX-X), in all testis. This totalized a mean of 1.005 (range: 812-1172) counted cells per animal. Differences between spermatogenic cycle stages were evaluated through ANOVA. As the Levene test for homogeneity of variances showed unequal variances between stages the Post Hoc test Tamhane's T2 was performed. Significance was assumed at the 0.05 level ($p < 0.05$).

4 Results

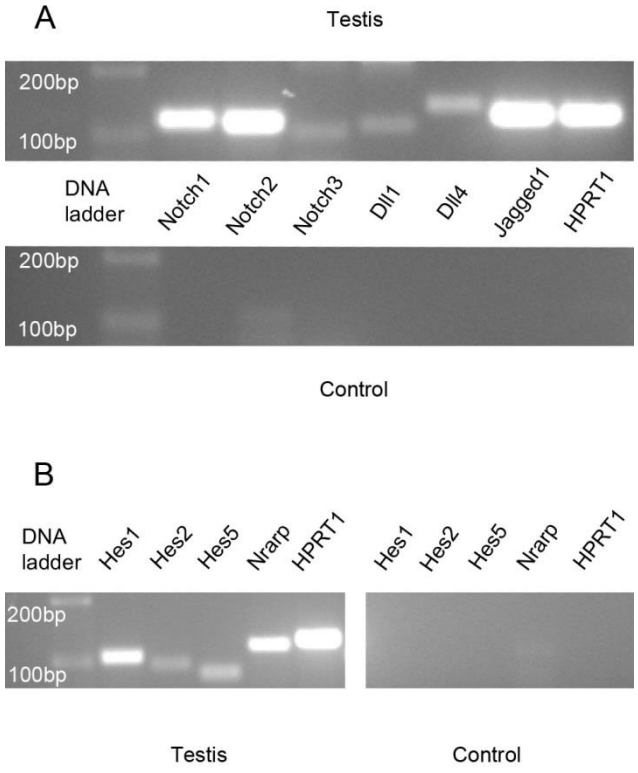
4.1 Notch components and Notch effectors are transcribed in adult mouse testis

As shown in Figure 9, all the analyzed Notch pathway components and Notch pathway effectors are transcribed in the adult mouse testis, as evidenced by RT-PCR.

4.2 Notch3 and Dll4 are expressed in spermatogonia and Sertoli cells in pn day 4 mice testis.

At pn day 4, when seminiferous tubules only present spermatogonia and Sertoli cells, only Notch3 and Dll4 are expressed in these cells (Figure 10 A and B). Spermatogonia were identified with anti-DAZL antibody in twin slides (Figure 10 C).

Figure 9 – Transcription of Notch pathway components and effectors in the adult mouse testis, evidenced by RT-PCR.



Gel electrophoresis RT-PCR results of: (A) Notch pathway gene transcripts - *Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1* and *HPRT1* (endogenous control gene); (B) Notch pathway gene effectors - *Hes1, Hes2, Hes5, Nrarp* and *HPRT1* (endogenous control gene).

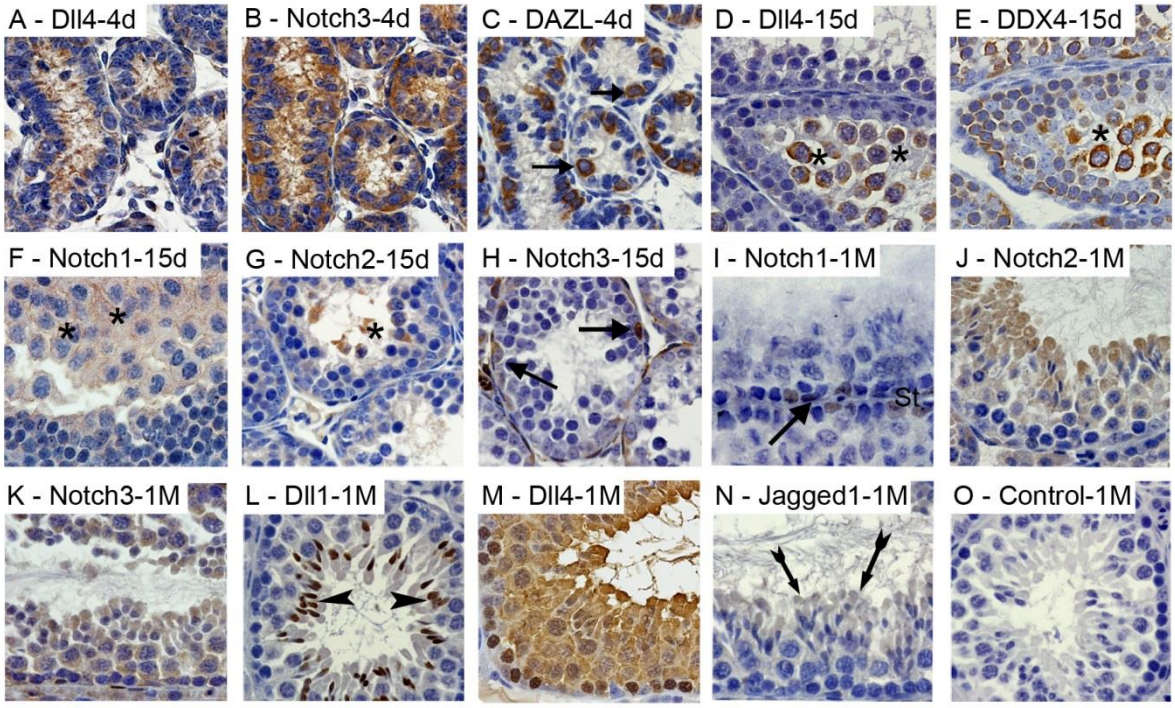
4.3 Notch1, Notch2 and Dll4 are expressed in cells initiating meiosis at puberty, on pn day 15.

At pn day 15, Dll4 expression is no longer observed in spermatogonia and become specific of cells initiating meiosis, which were marked with anti-DDX4 antibody in twin slides (Figure 10 D and E). Notch1 and Notch2 are also expressed in germ cells initiating meiosis (Figure 10 F and G), while Notch3 expression continues to be present in spermatogonia (Figure 10 H) but not in Sertoli cells.

4.4 Dll1 and Jagged1 are first expressed when spermatids start to elongate at 1 month of life.

At 1 month of age, spermatids are about to complete their differentiation and to be released from the seminiferous epithelium. All analyzed Notch components are expressed in the seminiferous tubules (Figure 10 I-N). Notch1 is expressed in spermatogonia and Sertoli cells (Figure 10 I). Notch2, Notch3 and Dll4 (Figure 10 J, K and M, respectively) are expressed in almost all germ cells. The most interesting feature at this time-point is the specific expression of Dll1 (Figure 10 L) and Jagged1 (Figure 10 N) in the elongated spermatids.

Figure 10 – Immunolocalization of Notch pathway components in the post-natal developing testis.

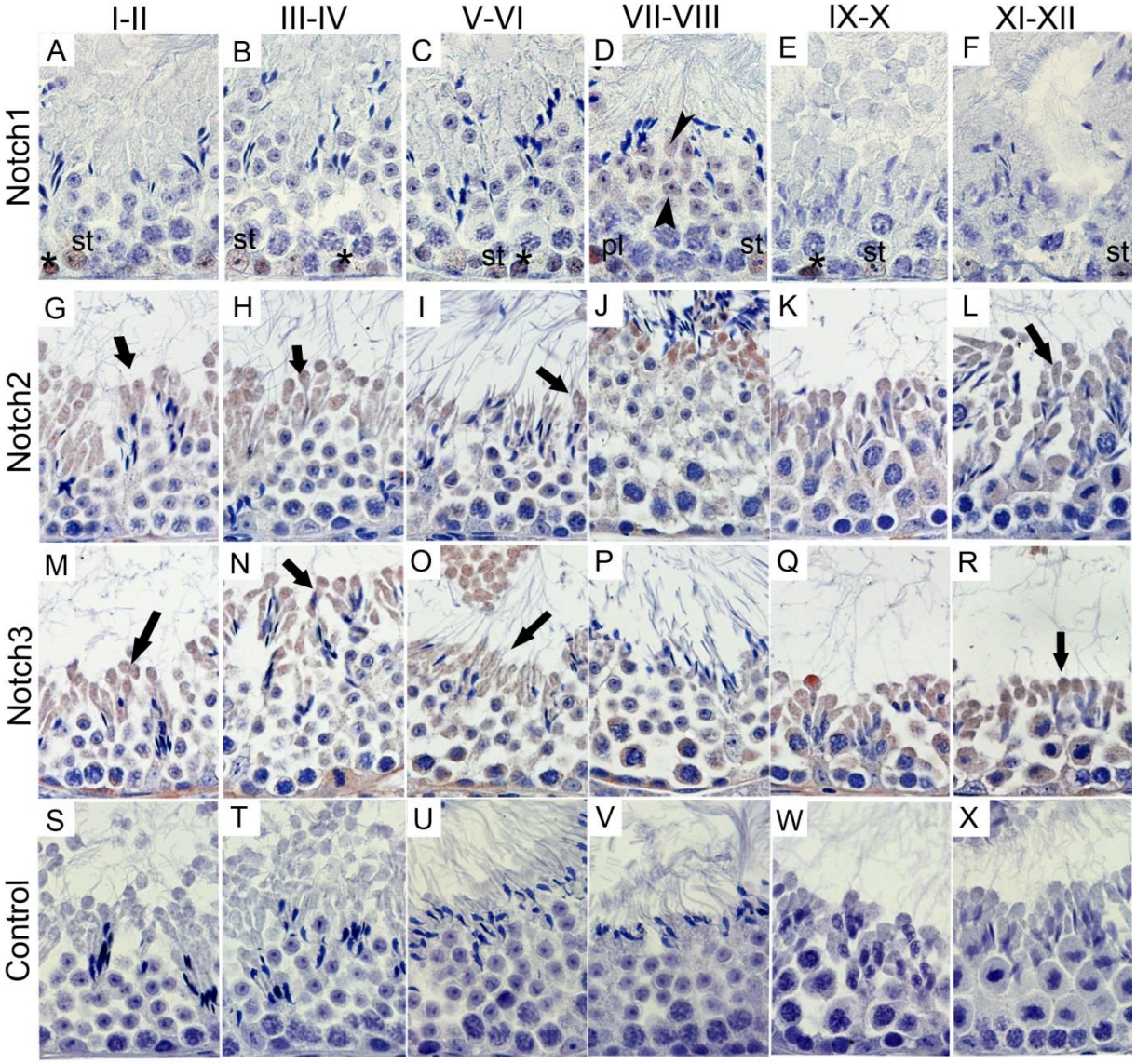


Positive staining in brown, counterstaining with haematoxylin (400x magnification). At pn day 4 (4d), Dll4 (A) and Notch3 (B) are expressed in spermatogonia and Sertoli cells. Spermatogonia cells are marked with anti-DAZL antibody (C). At pn day 15 (15d), Dll4 is expressed in germ cells initiating meiosis (D), marked with anti-DDX4 antibody (E). Notch1 (F) and Notch2 (G) are also expressed in germ cells entering meiosis. Spermatogonia express Notch3 (H). At 1 month pn (1M), spermatogenic cycle stage IX-X (I-O), Notch1 is expressed in undifferentiated germ cells and Sertoli cells (I), while Notch2 and Notch3 are ubiquitously expressed in germ cells (J and K). Dll1 is specifically found in elongated spermatids nucleus (L) and Jagged1 in the elongated spermatids cytoplasm (N), while Dll4 is ubiquitously expressed in germ cells and in some Sertoli cells (M). Controls were done with rabbit IgG (O). Arrows point to spermatogonia; Asterisks mark germ cells entering meiosis; Arrow heads point to elongated spermatids; Tailed arrows point to elongated spermatids' cytoplasm; st - Sertoli cells.

4.5 Notch pathway components have a unique expression pattern along the spermatogenic cycle in adult life.

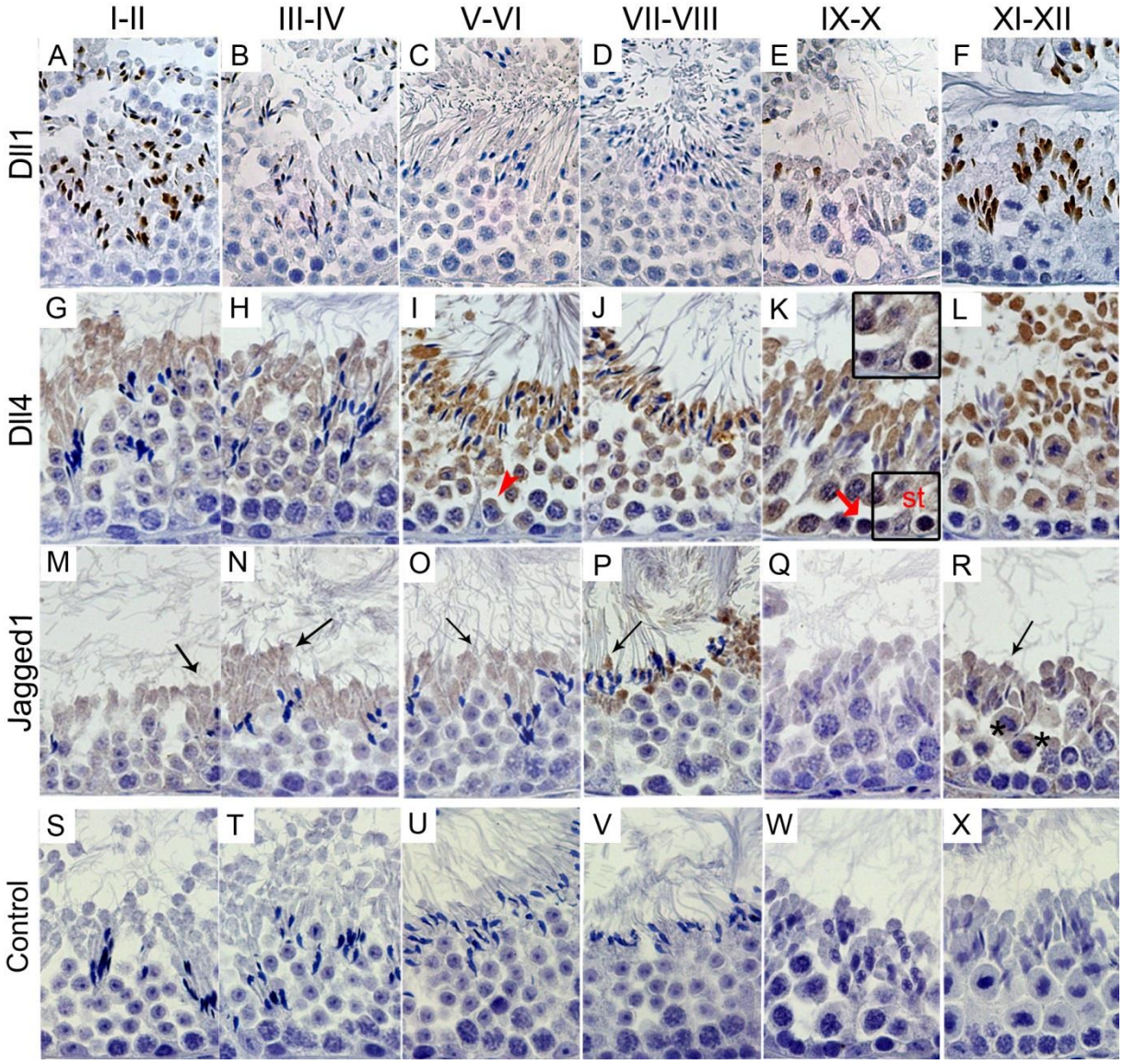
These expression patterns are illustrated in Figure 11 and 12, and resumed schematically in Figure 13. At adulthood (3 months of life), throughout the spermatogenic cycle, spermatogonia express Notch1 and Notch3 (Figure 11 A-C and M-O, respectively). When spermatogonia differentiate into preleptotene spermatocytes, at stage VII-VIII, these latter cells start to express Notch2 (Figure 11 J). Stage IX-X leptotene spermatocytes only express Dll4 (Figure 12 K). However, when leptotene spermatocytes differentiate into zygotene spermatocytes, at stage XI-XII, these latter cells cease to express the above Notch components.

Figure 11 – Expression of Notch pathway receptors during the spermatogenic cycle.



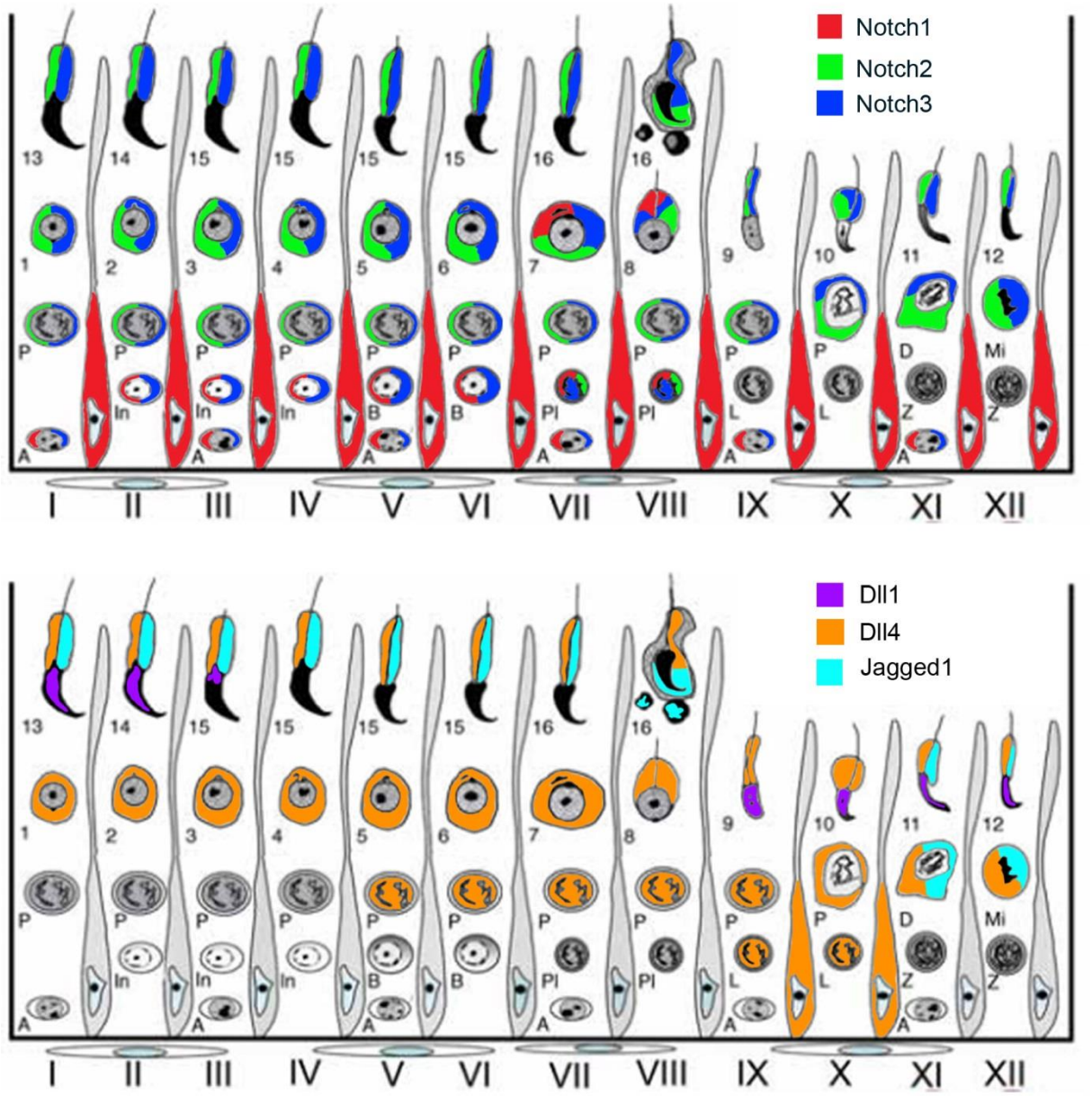
Positive staining in brown, counterstaining with haematoxylin (400x magnification). Notch1 is expressed in spermatogonia and Sertoli cells at all spermatogenic stages (A-F), stage VII-VIII preleptotene spermatocytes and round spermatids (D). Notch2 is expressed in all germ cells, except spermatogonia and leptotene and zygotene spermatocytes (G-L). Notch3 is only absent from leptotene and zygotene spermatocytes (M-R). Notch2 and Notch3 are present in the elongated spermatid cytoplasm (G-R). Control was done with rabbit IgG (S-X). Asterisks mark spermatogonia; pl mark preleptotene spermatocytes; Arrow heads point to round spermatids; Arrows point to elongated spermatids' cytoplasm; st - Sertoli cells.

Figure 12 – Expression of Notch pathway ligands during the spermatogenic cycle.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Dll1 is specifically present in the head of elongated spermatids, between stage IX-X and stage III-IV (A-F). Dll4 show a dynamic expression pattern (G-L), being expressed mainly from stage V-VI pachytene spermatocytes onwards (I), but also in stage IX-X leptotene spermatocytes and Sertoli cells (K). Jagged1 is observed in elongated spermatids' cytoplasm and in diplotene spermatocytes (M-R). Control was done with rabbit IgG (S-X). Arrow head point to stage V-VI pachytene spermatocytes; bold arrow point to stage IX-X leptotene spermatocytes; Arrows point to elongated spermatids' cytoplasm; asterisks mark diplotene spermatocytes; st - Sertoli cells.

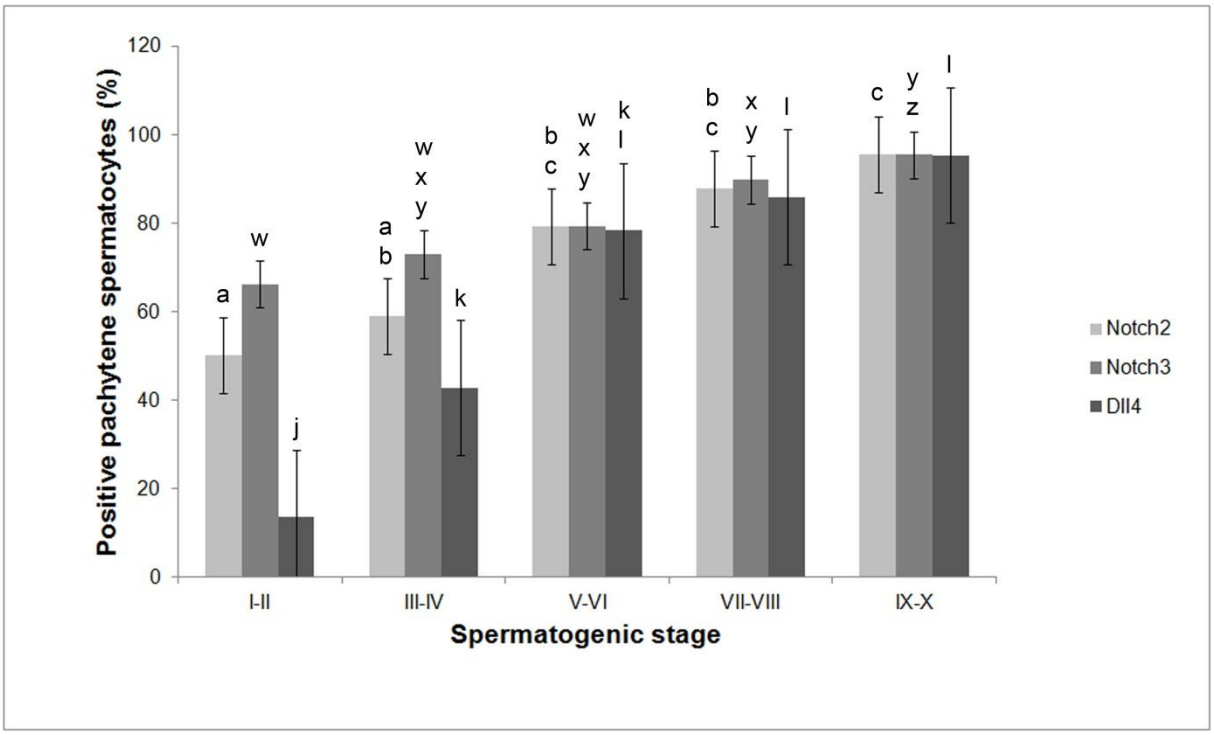
Figure 13 – Schematic illustration of expression patterns of Notch pathway components along the spermatogenic cycle.



Adapted draw-scheme (R. A. Hess & Franca, 2008) representing the mouse stages (I-XII) of the cycle of the seminiferous epithelium in the mouse. Cellular associations of layers of the seminiferous tubules, from the basement membrane to the lumen, are drawn with Sertoli cells separating each stage. Spermatogonia (A, In, B); spermatocytes (Pl- preleptotene, L- leptotene, Z- zygotene, P- pachytene, D- diakinesis, Mi- meiotic division); round spermatids (1-8); elongated spermatids (9-16). Localization of expression of Notch pathway components (top – receptors; bottom – ligands) is drawn in different colors, according to legend.

From stage I-II to stage IX-X, pachytene spermatocytes increasingly express Notch2, Notch3 and Dll4 (Figure 11 G-K, M-Q and Figure 12 G-K, respectively). These data is graphically illustrated in Figure 14: from stage I-II to stage IX-X, the relative frequency of the cellular expression of Notch2, Notch3 and Dll4 significantly and gradually increases, respectively, from 50%, 66% and 14% to 95%. In the final step of meiosis, at stage XI-XII, diplotene spermatocytes also express Jagged1 (Figure 12 R). Following the second meiotic division and the formation of round spermatids, from stage I-II to stage V-VI, only Notch2, Notch3 and Dll4 expression is present (Figure 11 G-J, M-P and Figure 12 G-J, respectively). At stage VII-VIII, Notch1 is also expressed in round spermatids (Figure 11 D).

Figure 14 – Evolution of the relative frequency of pachytene spermatocytes expressing Notch2, Notch3 or Dll4 along the spermatogenic cycle (stages I-II to IX-X).



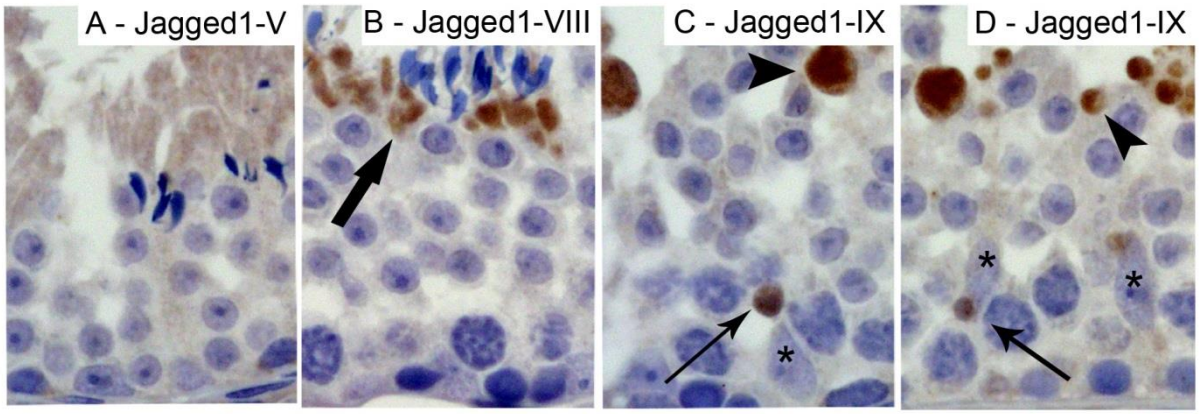
Error bars represent the standard error of the mean (SEM). Columns with different superscript differ significantly. Notch2: abc, $p<0.05$; Notch3: yz, $p<0.05$; Dll4: jkl, $p<0.05$.

Dll1 starts to be expressed in the nuclear part of elongated spermatids when these cells begin to differentiate, at stage IX-X, and is expressed until stage III-IV (Figure 12 E, F, A and B). Notch2, Notch3 and Dll4 are also expressed in elongated spermatids at all stages but only in the cytoplasm (Figure 11 G-L, M-R and Figure 12 G-L, respectively). Jagged1 is located in the residual bodies of elongated spermatids between stage XI-XII and stage VII-VIII (Figure 12 R and M-P). This ligand seems to be translocated from the lumen to the basal region of the seminiferous tubules (Figure 15 A-D). This pattern is associated, following the release of sperm cells within the lumen of seminiferous tubules, with the engulfment of

spermatids' residual bodies by Sertoli cells and their transfer to the basement membrane region of the seminiferous tubule (Figure 15 C-D).

Both Notch1 and Dll4 are expressed in Sertoli cells. However, while Notch1 is present in Sertoli cells at all stages (Figure 11 A-F), Dll4 is only present in some of these cells at stage IX-X (Figure 12 K).

Figure 15 – Jagged1 translocation within the residual bodies.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Jagged1 is located in the elongated spermatids' cytoplasm at stage V (A). At stage VIII, Jagged1 is located in the residual bodies at the sperm head tip (B). Following release of sperm cells from the seminiferous epithelium, at stage IX, residual bodies containing Jagged1 are progressively translocated towards the basement membrane and localized near the Sertoli cells nuclei (C-D). Bold arrow point to residual bodies containing Jagged1 in the sperm head tip. Arrow heads point to residual bodies containing Jagged1 at the luminal surface of the seminiferous epithelium. Arrows point to residual bodies containing Jagged1 near Sertoli cells nuclei. Asterisks mark Sertoli cells.

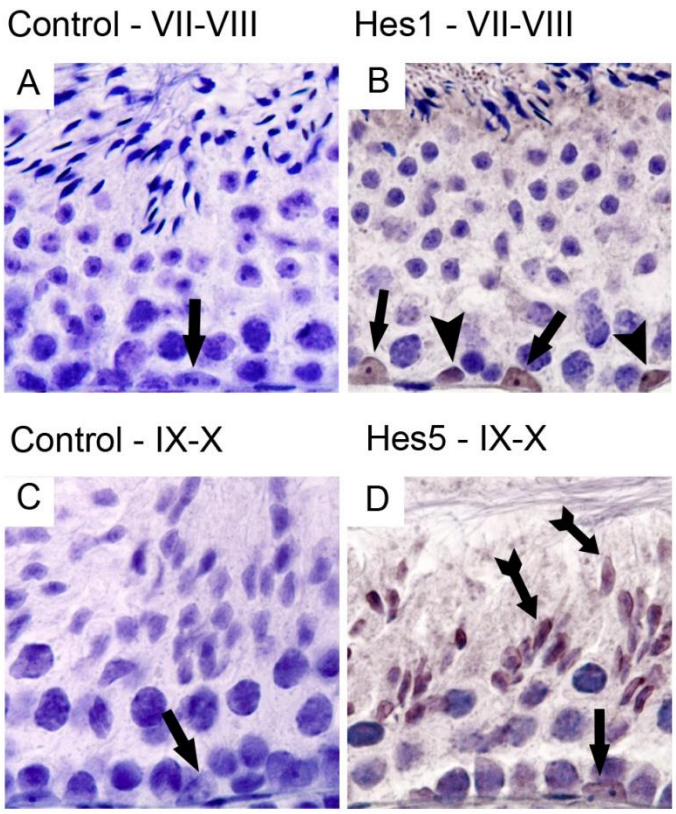
4.6 Notch pathway effectors Hes1 and Hes5 are expressed during adult mice spermatogenesis.

As shown in Figure 16, Hes1 is detected in Sertoli and spermatogonia cells, and Hes5 is detected in Sertoli cells and elongated spermatids.

4.7 Notch pathway components display a dynamic expression pattern in Leydig cells along post-natal testis development

Leydig cells were identified in the interstitial space through the presence of 3β-HSD, an enzyme of the steroidogenic metabolic pathway (Figure 17 E,K,P). At pn day 4, Dll1, Dll4, Notch2 and Notch3 are expressed in these cells (Figure 17 A-D), whereas at pn day 15, expression of Dll1, Notch1, Notch2 and Notch3 is identified (Figure 17 G-J). From 1 month of life onwards only the Notch receptors continue to be expressed in Leydig cells (Figure 17 M-O). However, in adulthood, endothelial cells of blood vessels present in the interstitial space express Jagged1 (Figure 18).

Figure 16 – Expression of Notch pathway effectors Hes1 and Hes5 during adult spermatogenesis.

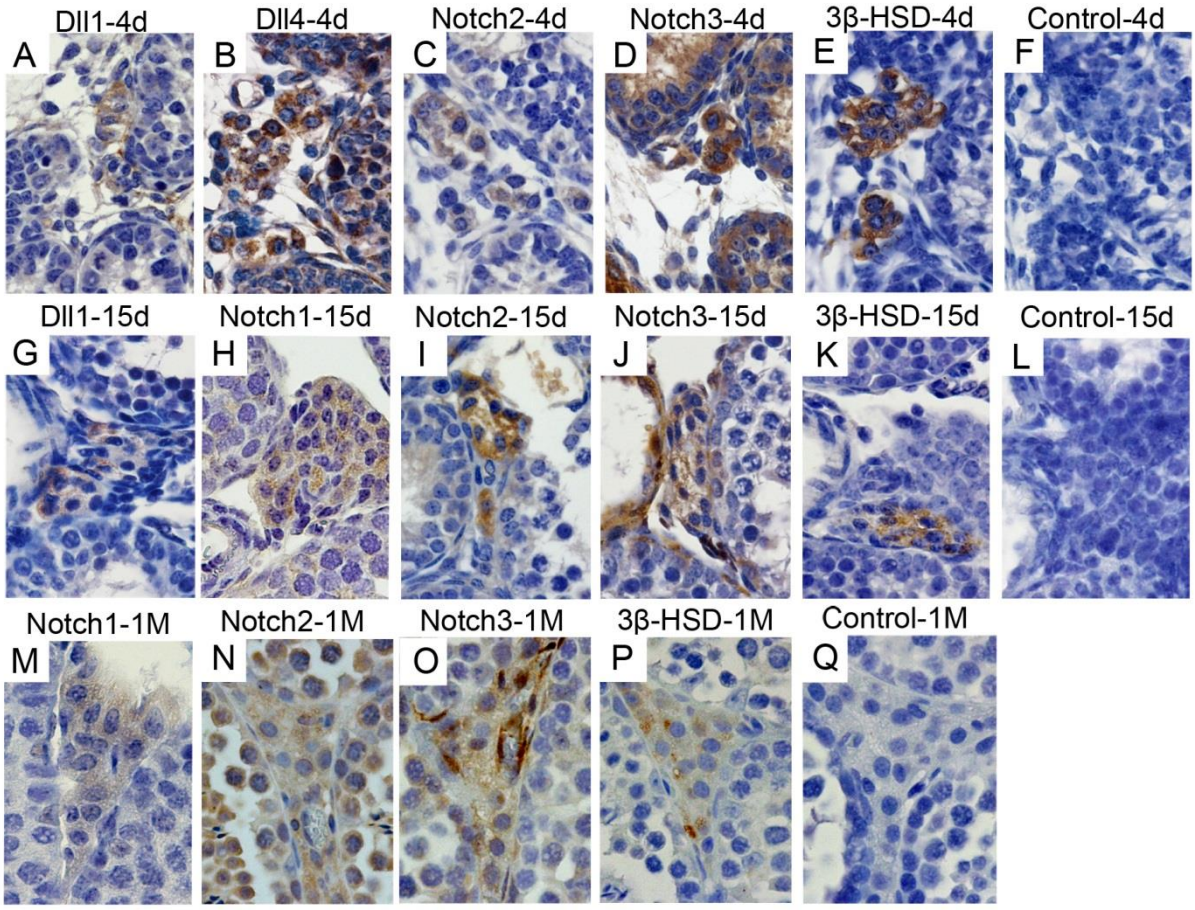


Positive staining in brown, counterstaining with haematoxylin (400x magnification). Only nuclear staining was considered positive. Hes1 is expressed in stage VII-VIII Sertoli and spermatogonia cells (B). Hes5 is expressed in stage IX-X Sertoli cells and elongated spermatids (D). Control was done with rabbit IgG (A, C). Arrow heads point to spermatogonia cells. Arrows point to Sertoli cells. Tailed arrows point to elongated spermatids.

5 Discussion

Transcription of Notch pathway components is present in the adult mouse testis. The use of whole testis tissue for RT-PCR probably originates some background noise resulting from transcription outside the seminiferous tubules (endothelial cells, smooth muscle cells and mesenchymal cells) (Zavadil, Cermak, Soto-Nieves, & Böttinger, 2004; Boucher, Gridley, & Liaw, 2012). Nevertheless, this result strongly leads to the suggestion that Notch pathway genes are transcribed in the seminiferous tubules during adult spermatogenesis. Furthermore, the observed transcription of Notch effector genes may indicate that Notch pathway is active during adult spermatogenesis.

Figure 17 – Dynamic expression of Notch pathway components in Leydig cells during mouse post-natal testis development.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Expression of Dll1, Dll4, Notch2 and Notch3 is observed at pn day 4 (4d) (A-D), while expression of Dll1, Notch1, Notch2 and Notch3 is present at pn day 15 (15d) (G-J). Notch1, Notch2 and Notch3 continue to be expressed at 1 month pn (1M) (M-O). Leydig cells were co-localized in twin slides with the anti-3β-HSD antibody (E,K,P). Control was done with rabbit IgG (F,L,Q).

Notch pathway components show a dynamic expression pattern in the mouse testis along the pn life. Although the expression of this pathway was already reported in the mammalian testis (Dirami et al., 2001; Hayashi et al., 2001; Mori et al., 2003; Hayashi, et al., 2004b; von Schönfeldt et al., 2004; Sahin et al., 2005; Hahn et al., 2009; Hasegawa et al., 2011; Garcia et al., 2013), this is the first integrated evaluation of expression of multiple Notch components in the testis along pn key time-points (pre-puberty, onset of puberty, adulthood). Additionally, our work is the first to describe Notch expression along the spermatogenic cycle. In pre-pubertal male mice, Dll4 and Notch3 are expressed in spermatogonia. As in the reported epithelial canonical function (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009), Notch signaling may be involved in maintaining the undifferentiated quiescent pool of spermatogonia. Dirami et al. (Dirami et al., 2001) immunolocalized Notch 1-3, Dll1 (Dll4 was not screened), Jagged1 and Jagged2 in spermatogonia cells of pre-pubertal mice testis primary cell cultures, obtained as lysates. Inconsistencies between the above results and

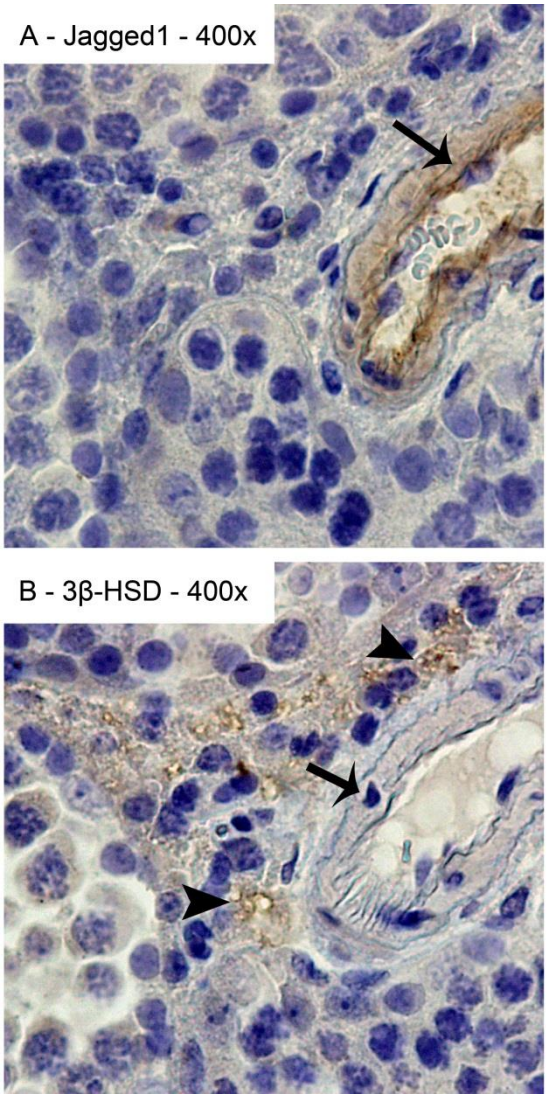
those obtained by us may arise from the use of different models. Disruption of testis architecture and in-vitro culture of testis cells may induce deviations in the gene expression profiles of cells. Hasegawa et al. (Hasegawa et al., 2011), using an *in situ* hybridization approach, only found transcripts of Jagged2 in spermatogonia of pre-pubertal mice. This inconsistency may result from a temporal dissociation between gene transcription and expression.

Dll4 and Notch3 are also expressed in Sertoli cells of pre-pubertal mice. These results contrast with those reported by Dirami et al. (Dirami et al., 2001) using primary cell cultures (Notch2 and Jagged1 immunolocalized) and Hasegawa et al. (Hasegawa et al., 2011) using an *in situ* hybridization approach (Notch1 transcription detected). These inconsistencies may arise from comments addressed above for spermatogonia. Although it was reported that Notch signaling in Sertoli cells (and germ cells) has no effect on spermatogenesis (Hasegawa et al., 2011), a recent study (Garcia et al., 2013), also using genetically modified mice, showed that overexpression of Notch1 in Sertoli cells induces a depletion of the spermatogonia pool caused by an aberrant exit of gonocytes from the mitotic arrest. Therefore, as also prompted by our gene expression results, the above study (Garcia et al., 2013) indicate a role of Notch signaling in the regulation of spermatogonia quiescence.

At onset of puberty Dll4 stops being expressed in spermatogonia and, together with receptors Notch1 and Notch2, undergoes expression in germ cells entering meiosis. Expression of Notch1 in germ cells entering meiosis was also described in rats (Hayashi et al., 2001). These features prompt for a role of Notch signaling in the onset of meiosis, as also reported in *Caenorhabditis elegans* (Lambie & Kimble, 1991).

At adulthood, the specific dynamic expression of Notch pathway components in different cell types at different stages of the spermatogenic cycle suggests a central role of Notch signaling in the regulation of spermatogenesis. At stage VII-VIII of the spermatogenic cycle, when spermatogonia differentiate into pre-leptotene spermatocytes, all three Notch receptors are expressed, which may be related to spermatogonia fate, towards either self-renewal or differentiation. Expression of Notch1-3 in spermatogonia at adulthood was also reported by others (Mori et al., 2003; von Schönfeldt et al., 2004; Sahin et al., 2005), and the involvement of Notch in the regulation of spermatogonia fate was also suggested (Hofmann, Braydich-Stolle, & Dym, 2005). However, *Notch3* mutant mice were not infertile (Krebs et al., 2003), suggesting that *Notch3* function is redundant in spermatogonia.

Figure 18 – Jagged1 expression in endothelial cells of testis interstitial blood vessels.



Positive immunostaining in brown color, counterstaining with haematoxylin (400x magnification). Jagged1 is present in endothelial cells of adult testis interstitial blood vessels (A). Leydig cells are in contact with the testis interstitial blood vessels. Leydig cells were co-localized in twin slides with the anti-3β-HSD antibody (B). Arrows point to endothelial cells. Arrow heads point to Leydig cells.

Stage IX-X leptotene spermatocytes only express the ligand Dll4. This ligand may be signaling back to the pre-leptotene spermatocytes, regulating their progression into the next differentiation step. Intriguingly, at stage XI-XII, when leptotene spermatocytes differentiate into zygotene spermatocytes, no expression of Notch pathway components is observed. The proportion of pachytene spermatocytes expressing Notch2, Notch3 and Dll4 increases from stage I-II to stage IX-X, when almost all these cells express these Notch pathway components. Here again, expression of Notch receptors 2 and 3 and the ligand Dll4 may be associated to cell differentiation progression. At stage XI-XII, at the final step of meiosis, Jagged1 is specifically expressed. Notch pathway expression was previously associated with cell division (Bultje, Castaneda-Castellanos, & Jan, 2009; Das & Storey, 2012). Results here presented, prompt for a role of Jagged1 in the completion of meiosis.

We found all the three analyzed Notch receptors and Dll4 in round spermatids. Notch1 was only identified in stage VII-VIII, when these cells start to elongate. The expression of Notch receptors in round spermatids was already identified in mice (Mori et al., 2003) and rat (Sahin et al., 2005). Dll1 was only observed in the nuclear part of elongated spermatids. This cellular localization was unique among Notch ligands and receptors, as Notch2, Notch3, Dll4 and Jagged1 are also present in elongated spermatids, but in the cytoplasm. Residual bodies result from the elimination of part of the cytoplasm content of elongated spermatids, through an asymmetric cell partition (Firlit & Davis, 1965; Hess et al., 1993). This asymmetric partition of cellular components may lead to changes in the distribution of Notch pathway components inside the cell, which may affect the ability of neighboring cells to deliver and receive Notch signaling.

Jagged1 is mainly expressed in the elongated spermatid cytoplasm. Interestingly, this ligand seems to be transported inside the residual body from the elongated spermatid to the adjacent Sertoli cell. Within Sertoli cells, this structure containing Jagged1 is transported from the luminal position of the seminiferous tubule to near the basement membrane. To the best knowledge of authors, this is the first report of such a Notch ligand translocation from one cell type to another. The effect of this engulfment is unknown. This could be associated with the activation of Notch1 in Sertoli cells or simply may represent a cell phagocytic function. The ectoplasmic specialization, the elongated spermatid anchoring system, maintains adherence between Sertoli cells and elongated spermatids, and also confers cell orientation and polarity within the seminiferous epithelium (Wong, Mruk, & Cheng, 2008). The Par complex proteins were associated to ectoplasmic specialization in spermatid orientation (Wong et al., 2008). Notch signaling was associated to cell polarity decisions in tissues involving Par complex proteins, such as the neural epithelium (Afonso & Henrique, 2006; Bultje et al., 2009). Here, the expression of Notch components near the elongated spermatid anchoring system may be associated with germ cell polarity definition and sperm release. Notch pathway was suggested to be associated with acrosome formation (Hayashi et al., 2001; Sahin et al., 2005). Results here presented regarding Dll1 expression are consistent with a possible role in acrosome formation and overall sperm head shaping.

As RT-PCR detection of Notch effectors in whole testis does not discriminate between somatic and germ cells transcription, and since Notch signaling activation in germ cells remains controversial, we evaluated the activation of Notch pathway in adult spermatogenesis, through the nuclear localization of Notch effector proteins. From the Notch effector genes transcribed in the testis (see above), the more representative effector genes (*Hes1* and *Hes5*) were selected for this evaluation. The detection of these proteins (Hes1 and Hes5) in the nucleus indicates that the pathway is activated during adult spermatogenesis. Overall, the expression patterns identified in this study prompt for a role of Notch signaling in the regulation of spermatogenesis. This is supported by several other

studies relating this pathway with male infertility (Hayashi et al., 2001; Hayashi, 2004b; Sahin et al., 2005; Lupien et al., 2006; Garcia et al., 2013). In mice, treatment with specific antibodies directed against Notch1 and Jagged2 induced a spermatogenic blockade (Hayashi et al., 2001). However, recently Hasegawa et al. (2011) reported that Notch signaling was not required for normal spermatogenesis and Baptista et al. (2012) reported that Notch1 expression was dispensable for spermatogenesis. These studies (Hasegawa et al., 2011; Batista et al., 2012) use genetic engineered mice with conditional deletions in *Pofut1* gene. Pofut1 protein is responsible for transfer of O-fucose to EGF repeats in Notch receptors, which alters receptor configuration (Wang et al., 2001). However, in mammals, an unrelated endoplasmic reticulum α-glucosidase 1 can compensate for Pofut1 in promoting Notch folding and function and thus, Pofut1 is not absolutely required for stable cell surface expression of Notch (Stahl et al., 2008). Batista et al. (2012) also evaluated mutant mice with conditional deletion of *Notch1* in spermatogonia cells and observed no phenotype in spermatogenesis and fertility. However, redundancy in Notch receptors function may be crucial to normal signaling since paralogues exert redundant or additive functions in maintaining the balance (Zeng et al., 1998; Kitamoto & Hayasaka, 2005). Recently, Garcia et al. (2013), using a GFP expression reporter driven by a RBP-J promoter, reported that Notch pathway is not active during spermatogenesis. The discrepancy between our results and those reported in the above study (Garcia et al., 2013) is difficult to explain. In our study, the presence of the Notch pathway effectors (Hes1 and Hes5) in the nucleus was detected in the adult testis, both in Sertoli (as also reported in (Hasegawa et al., 2011)) and germ cells which indicates that the pathway is active during spermatogenesis. The activation of Notch pathway during spermatogenesis was also proposed by others (Hayashi et al., 2001; Mori et al., 2003; Hofmann et al., 2005; Sahin et al., 2005).

Expression of Notch pathway components shows a dynamic pattern in Leydig cells along the pn life. Expression of Notch2, Notch3, Dll1 and Dll4 was observed in the pre-pubertal Leydig cells, while only the three Notch receptors (1-3) were observed in Leydig cells at adulthood. This change in the expression pattern may be related with the turn-over of the Leydig cell population (from embryonic to adult Leydig cells). During embryonic development, evolving blood vessels have a relevant role in testis morphogenesis, and expression of Jagged1 in interstitial cells was associated with maintenance of fetal Leydig progenitor cell populations (DeFalco et al., 2013). In adult mice we observed expression of Jagged1 in endothelial cells of blood vessels surrounding Leydig cells. We hypothesize that a paracrine regulation of Leydig cell function may be in place through the signaling from neighboring endothelial cells, as reported for the endothelial-mesenchymal signaling (Lu et al., 2013).

6 Conclusion

Here are reported the transcription and dynamic expression patterns of Notch pathway components along testis pn development and throughout the adult spermatogenic cycle. Results here described prompt for a role of Notch signaling in spermatogonia pool maintenance, onset of spermatogenesis, pace of the spermatogenic cycle, spermatid differentiation and regulation of pn Leydig cells function. We suggest that similarly to what happens in somitogenesis (Morimoto, Takahashi, Endo, & Saga, 2005; Niwa et al., 2007), Notch pathway components may regulate the pace of spermatogenesis at several points, contributing to the coordination and orchestration of the complex proliferative and differentiation cellular events that take place along the spermatogenic cycle. This can be inferred from Figure 13 that schematically illustrates expression of Notch components along the spermatogenic cycle. As shown, specific combinations of receptor/ligand expression are associated with key events occurring during the spermatogenic cycle, namely differentiation of spermatogonia, onset and completion of meiosis, and spermatid differentiation. Notch signaling results from the specific activation of Notch receptors (Shimizu et al., 2002; Yuan et al., 2012). In the complex cellular syncytium that constitutes the seminiferous epithelium, ligand expression by one cellular type may signal back to the preceding cell types, regulating their progression into the next differentiation step. Additionally, cell-autonomous Notch signaling may trigger forward cell differentiation. The presence of different receptors within a cell-type may be relevant for cell identity and function. The expression patterns observed address the need of future studies involving conditional specific Notch pathway mutant mice to evaluate the role of each Notch component in male reproductive function.

Chapter II - Notch pathway gene components and effectors are differentially transcribed and expressed along the epididymis epithelium.

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1. Abstract

Spermatozoa undergo sequential maturation changes during their transit along the epididymis. These changes, critical for the acquisition of fertilizing ability, are modulated by proteins secreted by epididymal epithelial cells, mainly transferred to spermatozoa within cytoplasmic exocytosis vesicles, the epididymosomes. This dynamic process requires a finely tuned epithelial gene expression. The Notch cell signaling pathway is a major regulator of cell fate decisions in several tissues, including the testis. Here, we evaluated the transcription and expression patterns of Notch components (*Notch1-3*, *Dll1*, *Dll4* and *Jagged1*) and effectors (*Hes1*, *Hes2*, *Hes5* and *Nrarp*) in the adult mouse epididymis.

Notch components and effectors are transcribed and expressed in the epididymis and deferens duct of adult male mouse, and nuclear detection of Notch effectors indicates that Notch signaling is active. Each segment of the epididymis and the deferens duct exhibit a specific combination of epithelial receptor/ligand/effector expression patterns. Notch components (but not effectors) are identified in the cytoplasmic droplet of spermatozoa, in a dynamic and specific pattern along the epididymis. Additionally, Notch components are identified within large and small vesicles in the epididymis lumen. We hypothesize that these vesicles (putative epididymosomes) allow Notch signaling at distance from epididymal epithelial cells to spermatozoa. Altogether, these results prompt for a role of Notch signaling in the regulation of the pace of serial events occurring along the epididymis, through signaling between epithelial cell types and spermatozoa. This may be crucial for the orchestration of epithelial cell secretory function, luminal milieu homeostasis and spermatozoa maturation.

Key Words: deferens duct; spermatozoa; cell signaling; epididymosomes; mouse

2. Introduction

In the mouse, following spermatogenesis, spermatozoa travel along the epididymis for 10-13 days, being then stored in the tail and deferens duct (Turner, 1995; Belleannée et al., 2012). During transit and storage, sperm cells sequentially undergo a series of maturational changes that lead to the generation of fully functional gametes. These changes are modulated by proteins produced by epididymis epithelial cells, liberated into the lumen (Cuasnicú et al., 1984), which interact with spermatozoa (Sullivan et al., 2005). Epididymis epithelial cells also provide instructive signals to spermatozoa by transferring proteins within vesicles named epididymosomes (Saez et al., 2003; Caballero et al., 2013), already described in several mammalian species (Légaré et al., 1999; Frenette & Sullivan, 2001; Rejraji et al., 2006; Caballero et al., 2013). The protein composition of epididymosomes varies along the epididymis, and from this organ to the ejaculated semen (Frenette, et al., 2006a; Girouard et al., 2011). Although it is now consensual that the interaction between epididymosomes and spermatozoa is critical for sperm cells maturation (Sullivan et al., 2007), the complex and finely tuned regulation of this interaction is yet very poorly understood. Deciphering the mechanisms that regulate the maturation of sperm cells will have a relevant impact in the development of new reproductive medicine strategies for fertility control, both in humans and animals.

The epididymis is divided into four segments known, from proximal to distal, as the initial segment, the head/caput, the body/corpus, and the tail/cauda (Abou-Haïla & Fain-Maurel, 1984; Turner et al., 2003). The epithelium contains different cell types with specific functions (Joseph et al., 2011; Belleannée et al., 2012). Principal cells comprise the majority of the epithelial cell population, and synthesize essentially all proteins liberated into the epididymis lumen (Sun & Flickinger, 1979; Joseph et al., 2011), namely within epididymosomes (Sullivan et al., 2007). Narrow and apical cells are mainly present in the initial segment (Adamali & Hermo, 1996; Joseph et al., 2011), and are associated with protein degradation and lumen pH control (Adamali & Hermo, 1996). Clear cells are present throughout the epididymis, but are more frequent in the body and tail. These large cells, characterized by the presence of numerous vesicles and endosomes in their apical region (Hermo, Dworkin, & Oko, 1988), display strong endocytic activity, being responsible for the uptake of proteins excreted by the epididymis epithelium, as well as the contents of spermatozoa's cytoplasmic droplet (Hermo et al., 1988). Basal cells, located against the basement membrane, sense the luminal environment and may regulate function of principal and clear cells (Cheung et al., 2005; Shum et al., 2009). The composition of the intraluminal milieu varies along the epididymis, reflecting a fine regulation of gene expression in epididymis epithelial cells (Krull et al., 1993; Guyonnet et al., 2011).

Notch is an evolutionarily well-conserved cell signaling pathway implicated in cell fate decisions in several tissues (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009).

Our recent work (Murta et al., 2013) showed that Notch signaling is active during testis development and adult spermatogenesis. Although the Notch pathway involvement in epididymis development has been reported (Hayashi et al., 2004c; Lupien et al., 2006; Hahn et al., 2009), it is unknown whether activation of Notch signaling occurs in the adult male. In mammals, four receptors (Notch1-4) and five ligands (three delta-like - Dll1, Dll3 and Dll4 - and two serrate-like - Jagged1 and Jagged2) were identified (Borggrefe & Oswald, 2009). Notch receptors are activated by binding with ligands expressed on neighboring cells. This leads to the cleavage of Notch receptors intracellular domain by a γ-secretase complex and its translocation to the nucleus, thus regulating transcription of Notch target genes (Borggrefe & Oswald, 2009). The more ubiquitous Notch target genes are the hairy/enhancer of split genes (Ohtsuka et al., 1999; Fischer & Gessler, 2007).

Here, we evaluated the transcription of Notch component and effector genes, and their expression patterns along the epididymis of adult male mouse. We found that Notch signaling is active in epididymis epithelial cells, and that Notch proteins are present in the cytoplasmic droplet of spermatozoa and within vesicles in the epididymis lumen. The expression patterns of Notch components and effectors are specific for each epithelial cell type along the epididymis. These results prompt for a role of Notch signaling as a regulator of epididymis epithelial function and spermatozoa maturation.

3. Materials and Methods

3.1. Animals

Experiments were conducted in compliance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (Directive n. 86/609/EEC, from the 24th November 1986). Mice manipulation protocols were approved by the national regulatory agency (DGAV – Direção Geral de Alimentação e Veterinária) and the Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal). All authors are accredited as FELASA category C scientists or equivalent.

CD1 mice were maintained in a 12-hour light/dark cycle, in ventilated cages with corn cob as bedding, and were given access to standard laboratory diet and water *ad libitum*. The mice health was routinely monitored. Outbred CD1 animals (n = 6) with three months of age were chosen to introduce normal biological variability within the experiment. Prior to beginning of the experiment, mice were caged separately with used female bedding, and after four days joined with a female until the observation of a vaginal plug, to grant normal reproductive behavior and the presence of active spermatogenesis and fertility.

3.2. Experimental Design

Mice were euthanized by cervical dislocation under ketamine (15mg/kg)/xylazine (1mg/kg) anesthesia, followed by exsanguination. Reproductive tracts were collected and the epididymis and deferens duct dissected free of the testis. Each epididymis was divided into the following segments: efferent duct, initial segment, proximal head, distal head, proximal body, distal body, tail and deferens duct. All segments from one side were processed for IHC and the contralateral ones processed for RNA extraction and subsequent RT-PCR.

3.3. Immunohistochemistry

Epididymis segments were fixed in 4% neutral phosphate buffered formalin at room temperature for 24h and, after subsequent dehydration in ethanol, were embedded in paraffin. Spatial localization of expression of Notch pathway components (Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1) and effectors (Hes1, Hes2, Hes5) was evaluated by IHC, according to a previously described method (Silva et al., 2010). Slices were orientated in parallel to the longitudinal axis, considering sequential sections of 3 µm and sequential twin-slides for each Notch component (minimum of 3 slices per epididymis for each Notch component and effector in all 6 animals). Tissue sections were counterstained with haematoxylin. The antigen retrieval step was performed in citrate buffer (10mM, pH 6.0), except for anti-Notch1, anti-Hes1, anti-Hes2 and anti-Hes5 antibodies (Tris-EDTA, pH 9.0). Blocking was performed in PBS with 2% w/v bovine serum albumin (A7906, Sigma-Aldrich, Inc.) for one hour at room temperature. Tissue sections were incubated overnight at 4ºC with the following primary antibodies: rabbit anti-Notch1 (ab27526, Abcam, diluted 1:100), rabbit anti-Notch2 (ab8926, Abcam, diluted 1:100), rabbit anti-Notch3 (ab23426, Abcam, diluted 1:160), rabbit anti-Dll1 (ab10554, Abcam, diluted 1:100), rabbit anti-Dll4 (ab7280; Abcam, diluted 1:200), rabbit anti-Jagged1 (SC-8303, SantaCruz Biotechnology, diluted 1:50), rabbit anti-Hes1 (ab71559, Abcam, diluted 1:100), rabbit anti-Hes2 (ab134685, Abcam, diluted 1:100) and rabbit anti-Hes5 (ab25374, Abcam, diluted 1:200). The negative controls were performed with the polyclonal rabbit IgG (ab27478, Abcam, diluted 1:100). All primary antibodies were diluted in blocking solution. The peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (Dako 410972, diluted 1:100) was used as secondary antibody. Staining was evaluated in the entire slice, and expression patterns were established following the evaluation of a minimum of 18 slices (3 slices/epididymis x 6 animals) for each Notch component and effector. Antibodies anti-Notch components and effectors were previously validated by others in the mouse (anti-Notch1 (Cheng et al., 2007), anti-Notch3 (Feng et al., 2010), anti-Dll1, (Sörensen et al., 2009) anti-Dll4, (Sörensen et al., 2009) anti-Jagged1, (Bielesz et al., 2010) anti-Hes1 (Rahman et al., 2013) and anti-Hes5 (Takanaga et al., 2009)) and rat species (anti-Notch2 (Saravanamuthu et al., 2009)). The anti-Hes2 antibody is predicted to work in mouse, according to the manufacturer.

Notch signaling activation was considered to be present only when nuclear detection of Notch effectors (Hes1, Hes2 and Hes5) was evident.

3.4. RT-PCR

Epididymis segments were individually and immediately frozen in liquid nitrogen and stored at −80 ºC until assay. RNA extraction, cDNA synthesis and mRNA transcription was performed as previously described (Silva et al., 2012). The detection of Notch pathway genes (*Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1*) and gene effectors (*Hes1, Hes2, Hes5, Nrarp*) were analyzed with specific primer pair sequences. Transcription of gene *β2mg* was used as an endogenous control. Primer pair sequences are available in Annex I.

4. Results

4.1. Notch pathway component and effector genes are transcribed along the epididymis and in the deferens duct.

As shown in Figure 19, all Notch component genes (*Notch1-3, Dll1, Dll4, Jagged1*) are transcribed in the three epididymis segments and in the deferens duct. Of the Notch effector genes (*Hes1, Hes2, Hes5* and *Nrarp*), only *Hes1* and *Nrarp* are transcribed in the three epididymis segments and in the deferens duct, while *Hes5* is transcribed in the epididymis body and in the deferens duct, and *Hes2* has a low transcription in the epididymis body and tail.

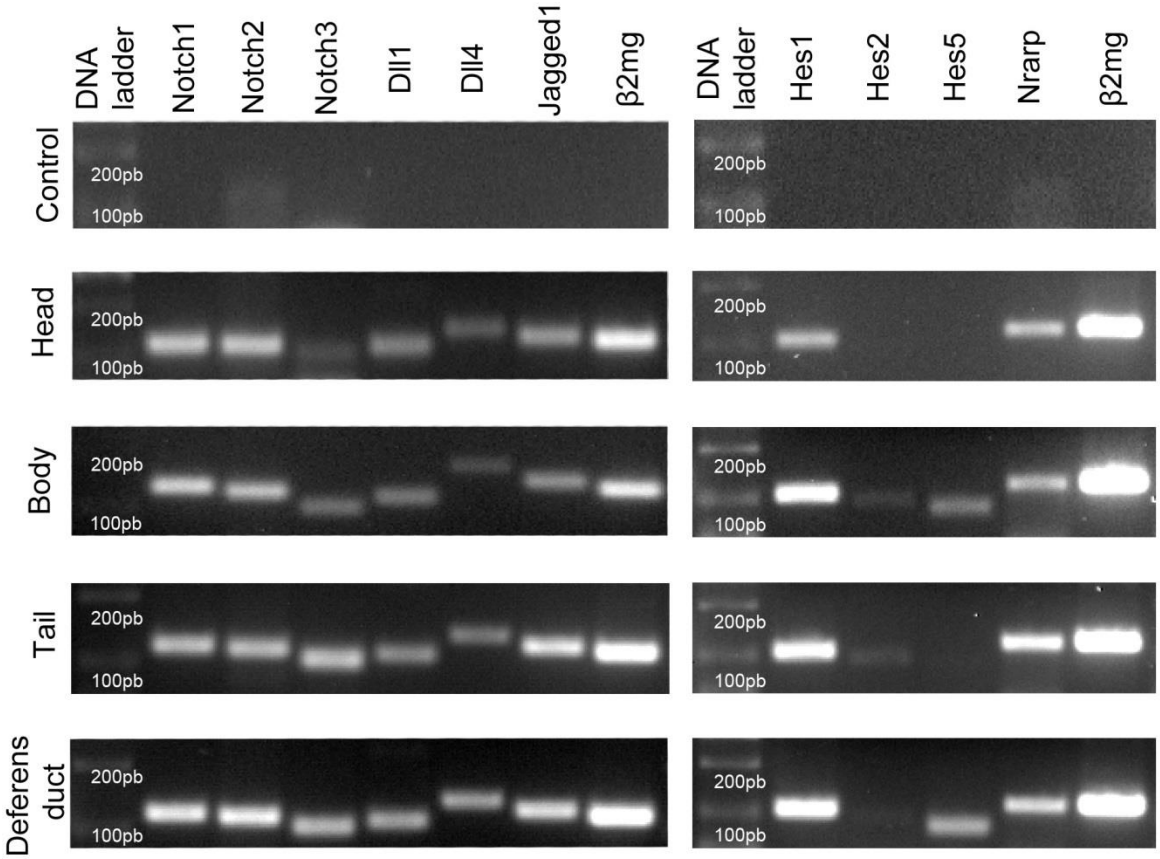
4.2. Notch pathway components and effectors are differentially expressed along the epididymis and in the deferens duct.

Figure 20 shows the expression patterns of Notch components along the epididymis and in the deferens duct. Below follows a brief description of the most notable features. Notch1 is expressed in principal cells of the epididymis and deferens duct, in narrow and apical cells, and in basal cells of the initial segment (A, G, M, S, a, g, m, s). Notch2 is ubiquitous, being expressed along the epididymis and deferens duct (B, H, N, T, b, h, n, t). Notch3 is expressed in most epithelial cell types, although for each cell type expression occurs in specific segments (C, I, O, U, c, i, o, u). Dll1 is expressed in principal cells of most of the epididymis and deferens duct, and in basal cells of the epididymis body, tail and deferens duct (D, J, P, V, d, j, p, v). Dll4 is also expressed in principal cells of most of the epididymis and deferens duct, in narrow and apical cells, and in basal cells along the epididymis (E, K, Q, W, e, k, q, w). Dll4 is the only ligand expressed in clear cells (k). Jagged1 is expressed in principal and basal cells until the epididymis tail and in narrow and apical cells (F, L, R, X, f, l, r, x).

Figure 21 shows the detection of Notch effectors along the epididymis and deferens duct. Hes1 is detected in all epididymis and deferens duct epithelial cells (A, D, G, J, M, P, S, V).

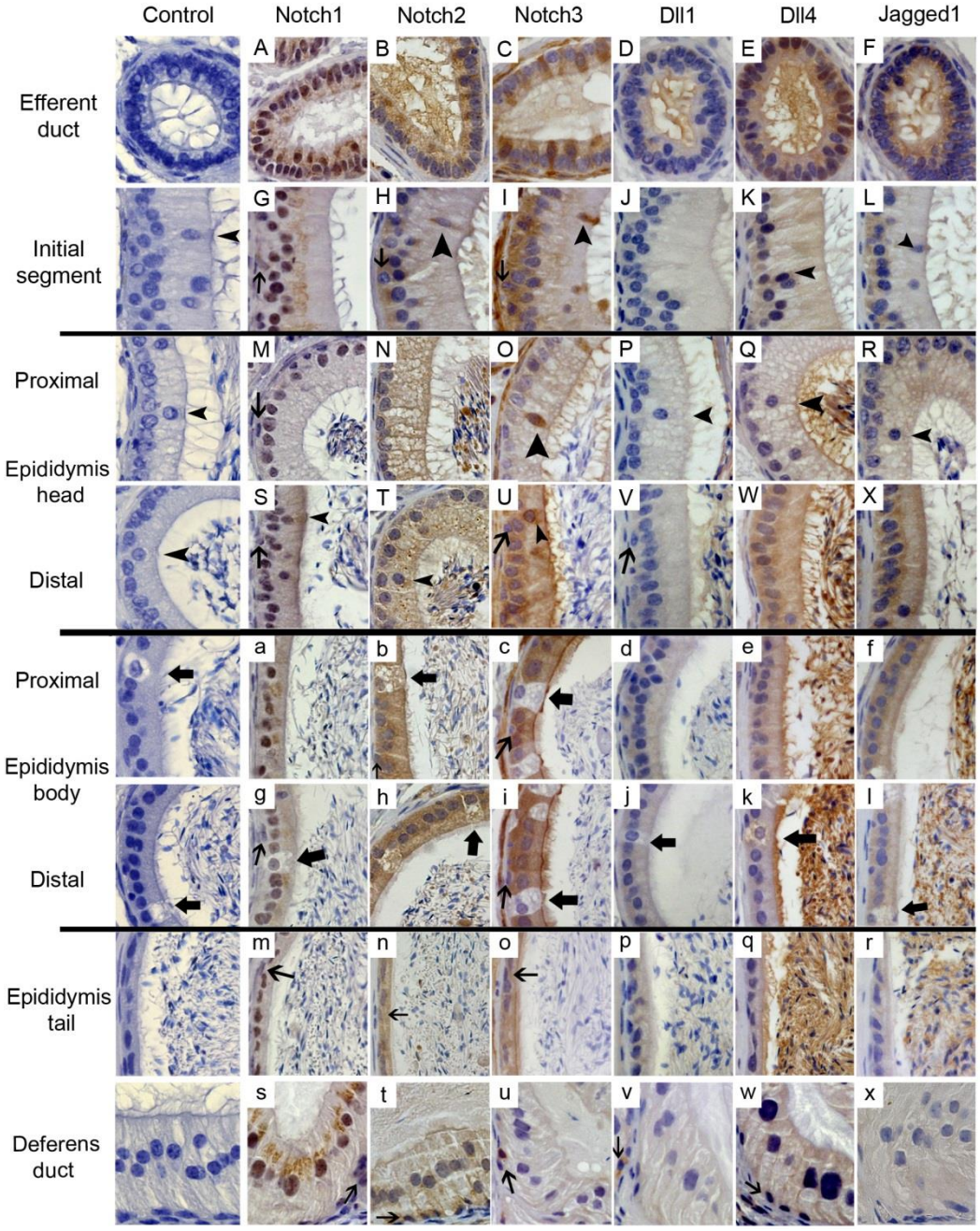
Hes2 is not detected in the nucleus of epididymis head and deferens duct epithelial cells (B, E, H, K, N, Q, T, W), although being detected in the cytoplasm of the deferens duct epithelium (W). Hes5 is only detected in epithelial cells of the body (O, R).
All the above information is schematically illustrated in Figure 22 and summarized in Table 1.

Figure 19 – Transcription of Notch pathway component and effector genes along the epididymis and in the deferens duct.



Gel visualization of Reverse Transcriptase PCR results of samples recovered from different segments of the epididymis (head, body, tail) and from the deferens duct. β2mg: internal control of reaction gene. Notch component genes: *Notch1-3*, *Dll1*, *Dll4*, *Jagged1*; Notch effector genes: *Hes1*, *Hes2*, *Hes5*, *Nrarp*.

Figure 20 – Expression patterns of Notch pathway components along the epididymis and in the deferens duct.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Control was produced with rabbit IgG. Narrow and apical cells were only identified in the initial segment and epididymis head, and clear cells were only identified in the epididymis body. Arrow heads point to narrow and apical cells. Arrows point to basal cells. Bold arrows point to clear cells. Notch1 is expressed in principal cells along the epididymis and deferens duct (A, G, M, S, a, g, m, s), in narrow and apical cells (S), and in basal cells of the initial segment (G). Notch2 is expressed in all epithelial cell types along the epididymis and deferens duct (B, H, N, T, b, h, n, t). Notch3 is expressed in principal cells (except in the proximal head segment), in narrow and apical cells, and in basal cells of the initial segment, distal head segment, proximal body segment, tail and deferens duct (C, I, O, U, c, i, o, u). Dll1 is expressed in efferent duct, body and tail principal cells, and in basal cells of the body, tail and deferens duct (D, J, P, V, d, j, p, v). Dll4 is expressed in principal cells (except in proximal

head, distal body and tail), in initial segment and distal head narrow and apical cells, in clear cells, and in basal cells (except in proximal head, distal body and tail) (E, K, Q, W, e, k, q, w). Jagged1 is expressed in principal (except tail and deferens duct), basal (except tail and deferens duct) and in narrow and apical cells (F, L, R, X, f, l, r, x).

4.3. Notch pathway components, but not Notch effectors, are differentially detected in epididymis spermatozoa and lumen

Figure 23 shows the detection patterns of Notch components in spermatozoa and the lumen along the epididymis. Notch effectors were not detected in spermatozoa and the epididymis lumen. As shown, Notch1 is detected over the sperm head, in spermatozoa present in the epididymis head (A). Notch2, Notch3, Dll1 and Dll4 are detected in spermatozoa's cytoplasmic droplet. However, while Notch2 and Dll4 are detected along the entire epididymis (B, H, N and E, K, Q, respectively), Notch3 is only detected in the epididymis body (I), and Dll1 is only detected in the epididymis body and tail (J, P). Jagged1 is not detected in spermatozoa's cytoplasmic droplet.

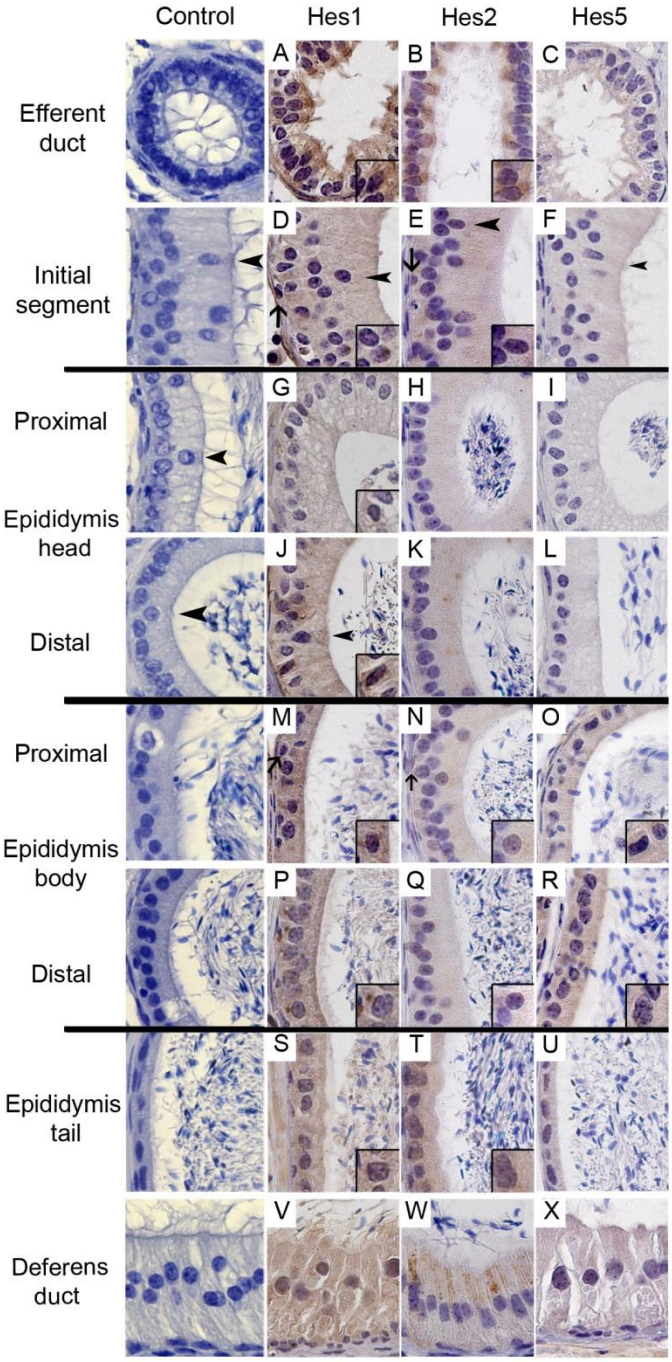
Additionally to the detection in spermatozoa's cytoplasmic droplet, Notch components were also detected within large and small vesicles in the epididymis lumen. Notch2, Notch3 and Dll1 were detected in large vesicles (H, I, J, respectively), Dll4 in large and small vesicles (K, Q), and Jagged1 only in small vesicles in the body and tail segments (L, R).

5 Discussion

Here is reported the dynamic transcription and expression patterns of Notch pathway component and effector genes in the adult mouse epididymis and deferens duct. Although some Notch components were previously identified during epididymis post-natal (Hayashi, 2004c) and pre-natal development (Lupien et al., 2006; Hahn et al., 2009), this study reports for the first time the dynamics of Notch transcription, and expression in different epithelial cell types along the epididymis and deferens duct of adult male mice. Notch pathway components show a dynamic and specific expression pattern in different epithelial cell types along the epididymis and deferens duct. Each epididymis segment shows a specific combination of Notch components' epithelial expression. The nuclear detection of Notch effectors in the epididymis and deferens duct epithelium confirms that Notch signaling is active. Altogether, these results strongly prompt for a role of Notch signaling in epididymis function.

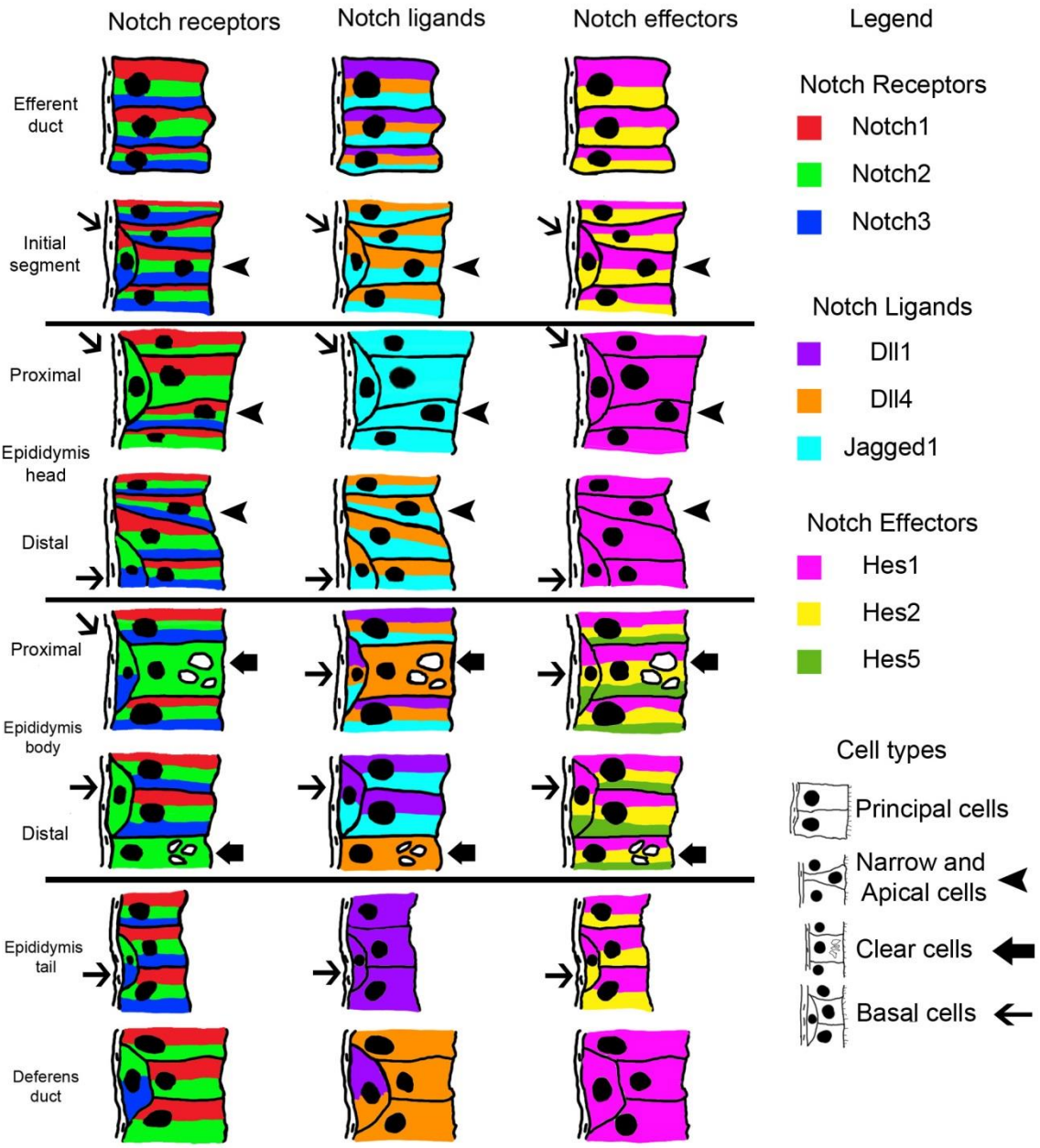
Notch components are expressed and effectors Hes1 and Hes2 are detected in the efferent ducts' epithelium. The efferent ducts allow the transport of spermatozoa from the testis to the epididymis, and their epithelial function is modulated by luminal factors arising from the testis (Joseph et al., 2011). It is possible that Notch signaling is regulating cilium function, as observed in other tissues (Lopes et al., 2010).

Figure 21– Detection of Notch pathway effectors along the epididymis and in the deferens duct.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Notch signaling activation was considered to be present only when nuclear detection of Notch effectors (Hes1, Hes2 and Hes5) was evident. Control was produced with rabbit IgG. Narrow and apical cells were only identified in the initial segment and epididymis head, and clear cells were only identified in the epididymis body. Arrow heads point to narrow and apical cells. Arrows point to basal cells. Hes1 is ubiquitously detected in epithelial cells along the epididymis and deferens duct (A, D, G, J, M, P, S, V). Hes2 is not detected in the epididymis head and deferens duct epithelium (B, E, H, K, N, Q, T, W). Hes5 is only detected in the epididymis body epithelium (O, R).

Figure 22 - Schematic illustration of expression patterns of Notch pathway components and effectors along the epididymis and in the deferens duct.



Draw-scheme representing mouse epididymis epithelium, and the cellular localization of Notch components and effectors expression (from left to right: receptors; ligands; effectors). Arrow heads point to narrow and apical cells. Bold arrows point to clear cells. Arrows point to basal cells. Principal cells are unmarked.

As depicted from Figure 22 and Table 1, each epididymis segment shows a specific combination of Notch receptor/ligand/effector gene expression pattern in their epithelium. This may indicate that Notch signaling is differentially and dynamically regulating epididymis function. These unique combinations of receptor/ligand/effector expression probably elicit specific responses in each cell type in the different epididymis segments. The composition of the epididymis luminal milieu changes along its segments, which relies on a well-

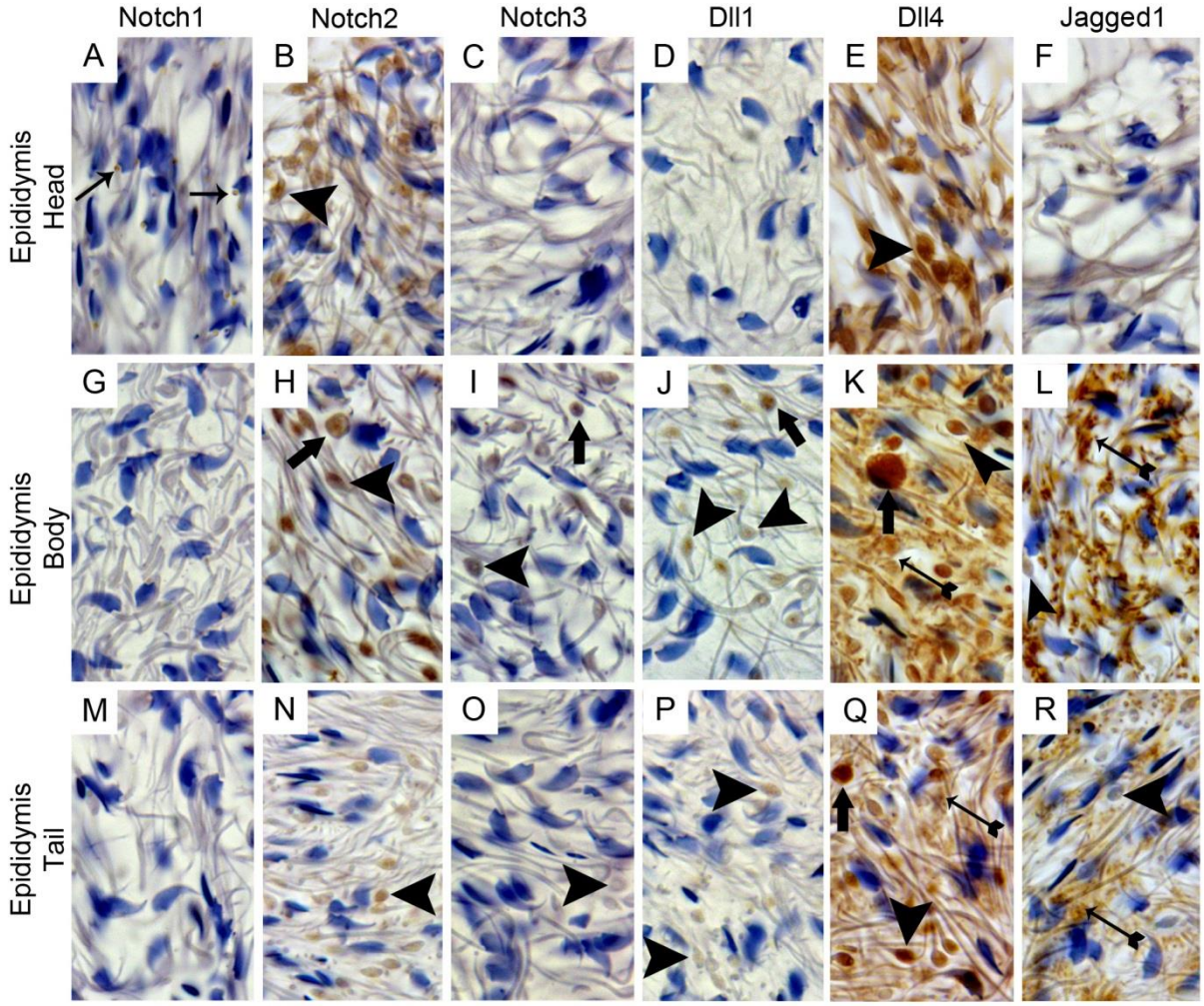
orchestrated and fine regulation of epididymis gene expression (Krull et al., 1993; Guyonnet et al., 2011). Whereas secretions from epididymis head and body are related to sperm motility and fertilizing ability (Turner, 1995), secretions from the tail are related to spermatozoa storage in conditions that preserve their fertilizing ability (Jones & Murdoch, 1996). Thus, Notch signaling along the epididymis may be relevant for the serial regulation of the epithelium's secretory functions, which in turn regulate spermatozoa maturation. This may encompass the regulation of the pace of epithelial secretory and spermatozoa maturation events, as also proposed for spermatogenesis (Murta et al., 2013). Additionally, Notch signaling may be regulating the epithelium homeostasis, controlling the intercellular signaling between different cell types.

Table 1 – Epididymis segment specific expression combinations of Notch pathway component and effector genes in each cell type.

Cell types	Proteins	IS	Head P	Head D	Body P	Body D	Tail	Deferens
Principal	Receptors	N1/N2/N3	N1/N2	N1/N2/N3	N1/N2/N3	N1/N2/N3	N1/N2/N3	N1/N2
	Ligands	D4/J1	J1	D4/J1	D1/D4/J1	D1/J1	D1	D4
	Effectors	H1/H2	H1	H1	H1/H2/H5	H1/H2/H5	H1/H2	H1
Narrow and Apical	Receptors	N1/N2/N3	N1/N2/N3	N1/N2/N3				
	Ligands	D4/J1	J1	D4/J1				
	Effectors	H1/H2	H1	H1				
Clear	Receptors				N2	N2		
	Ligands				D4	D4		
	Effectors				H1/H2/H5	H1/H2/H5		
Basal	Receptors	N1/N2/N3	N2	N2/N3	N2/N3	N2	N2/N3	N2/N3
	Ligands	D4/J1	J1	D4/J1	D1/D4/J1	D1/J1	D1	D1/D4
	Effectors	H1/H2	H1	H1	H1/H2/H5	H1/H2/H5	H1/H2	H1

IS – Initial segment, Head P – epididymis head proximal segment, Head D – epididymis head distal segment, Body P – epididymis body proximal segment, Body D – epididymis body distal segment, Deferens – Deferens duct, N1 – Notch1, N2 – Notch2, N3 – Notch3, D1 - Dll1, D4 – Dll4, J1 – Jagged1, H1 – Hes1, H2 – Hes2, H5 – Hes5.

Figure 23 – Expression patterns of Notch pathway components in epididymis spermatozoa and lumen.



Positive staining in brown, counterstaining with haematoxylin (1000x magnification). Notch1 is detected over the sperm head in the epididymis head (A). Notch2, Notch3, Dll1 and Dll4 are detected in the spermatozoa's cytoplasmic droplet: Notch2 is detected along the epididymis (B, H, N); Notch3 is detected in the epididymis body (I); Dll1 is detected in the epididymis body and tail (J, P); Dll4 is detected along the epididymis (E, K, Q). Notch2 (H), Notch3 (I), Dll1 (J) and Dll4 (K, Q) are detected within large vesicles in the epididymis lumen. Dll4 is also detected within small vesicles (K, Q), as well as Jagged1 in the lumen of the epididymis body and tail (L, R). Dll4 and Jagged1 are detected in the spermatozoa's tail: Dll4 is detected along the epididymis; Jagged1 is only detected in the epididymis body. Arrows point to Notch1. Arrow heads point to cytoplasmic droplets. Bold arrows point to large vesicles. Tailed arrows point to small vesicles.

Notch2, Notch3, Dll1 and Dll4 are detected in the cytoplasmic droplet of spermatozoa, and together with Jagged1 are also detected within large and small vesicles in the epididymis lumen. The presence of these Notch components follows a specific pattern along the epididymis. As Notch effector genes are not detected in spermatozoa and in the epididymis lumen, and expression of both Notch component and effector genes occurs in epididymis epithelial cells, we propose that these proteins are transferred to spermatozoa and to the

lumen within epididymosomes, as reported for other proteins (Légaré et al., 1999; Frenette & Sullivan, 2001; Sullivan et al., 2007). This may allow signaling at distance between epididymis epithelial cells, spermatozoa and oviduct/uterine epithelial cells. Notch signaling at distance was recently reported (Sheldon et al., 2010; Lu et al., 2013). Transfer of Notch components between different testis cell types was recently described by us (Murta et al., 2013). The timely orchestrated transfer of Notch proteins to epididymis spermatozoa and luminal fluid may be relevant for sperm cells' maturation or interaction with the oviduct and oocyte, as also observed with other proteins (Légaré et al., 1999; Frenette et al., 2004; Sullivan et al., 2005; Frenette, Thabet, & Sullivan, 2006b).

All Notch components and effectors are transcribed in the epididymis. The use of whole tissue for RT-PCR probably originates some background noise resulting from transcription outside the epididymis epithelium (endothelial cells, smooth muscle cells and mesenchymal cells) (Zavadil et al., 2004; Boucher et al., 2012). This may explain inconsistencies between transcription and expression.

In conclusion, Notch signaling is active in the epididymis and deferens duct of adult male mouse. Each segment of the epididymis and the deferens duct exhibit a specific combination of epithelial receptor/ligand/effector expression patterns. Notch components are detected in the cytoplasmic droplet of spermatozoa, and within large and small vesicles in the epididymis lumen, in a dynamic and specific fashion along the epididymis. Altogether, these results prompt for a regulatory role of Notch signaling in epididymis function. We hypothesize that Notch signaling may regulate the pace of serial events occurring along the epididymis. This signaling between different epithelial cell types and spermatozoa along the epididymis may be crucial for epithelial differential secretory function, luminal milieu homeostasis and spermatozoa maturation.

Chapter III - *In vivo* Notch blockade induces male germ cell fate aberrations and apoptosis, and increases spermatozoa defects

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1. Abstract

Objective: Evaluate effects of Notch pathway blockade in spermatogenesis

Design: Comparison of spermatogenesis between control untreated and DAPT treated adult mice

Setting: University laboratory

Animals: CD1 laboratory mice

Interventions: DAPT treatment and euthanasia

Main Outcome Measures: Testis samples were collected and processed for immunohistochemistry. Expression patterns of Notch components were evaluated. Germ cells identity, morphology and apoptosis were determined. Spermatozoa were collected from the epididymis tail and assessed for motility, morphology, concentration, and *in vitro* fertilization. Plasma testosterone concentrations were measured. Transcription of Notch effectors was analyzed in epididymis tissue.

Results: *In vivo* Notch pathway blockade by DAPT disrupted the expression patterns of Notch components in the testis, induced male germ cell fate aberrations and apoptosis, mainly in the last stages of the spermatogenic cycle, and increased epididymis spermatozoa defects.

Conclusions: Blockade of Notch signaling in the testis and epididymis induced abnormal spermatogenesis and spermatozoa maturation, which implicates Notch signaling in the regulation of spermatogenesis and epididymis function.

Key Words: Notch pathway, spermatogenesis, germ cells, epididymis, spermatozoa defects

2. Introduction

Mammalian spermatogenesis involves continuous serial cellular proliferation and differentiation events, which occur in the complex cellular syncytium of the seminiferous tubules. These spatial-temporal cellular changes characterize the spermatogenic cycle, which, in the mouse, encompasses 12 stages (Oakberg, 1957). Released spermatozoa sequentially undergo maturation changes during transit along the epididymis, which lead to the acquisition of fertilizing ability (Cooper & Yeung, 2006). These changes are modulated by epididymis epithelial cells, through the sequential modification of the luminal milieu composition, and the transfer of proteins to spermatozoa (Sullivan et al., 2007). Germ cell remodeling occurring at the spermatogenic cycle and epithelial secretion along the epididymis require a finely tuned cellular signaling and a well-orchestrated gene expression. Deciphering the regulatory signaling behind these events could potentially lead to the development of new therapeutic strategies addressed to male infertility and contraception.

Notch is an evolutionarily conserved cell signaling pathway implicated in cell fate decisions in several tissues (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009). In mammals, four receptors (Notch1-4) and five ligands (Dll1, Dll3, Dll4, Jagged1 and Jagged2) were described (Borggrefe & Oswald, 2009). Notch signaling occurs following the coupling of the extracellular domain of receptors with ligands expressed on neighboring cells, which leads to cleavage of Notch intracellular domain (NICD) by a γ-secretase and its translocation to the nucleus. Here, it forms a complex with a transcriptional regulator RBP-jk and other co-regulators, regulating transcription of effector target genes (Borggrefe & Oswald, 2009). From the limited set of Notch target genes so far identified, the hairy/enhancer of split genes are the most ubiquitous (Fischer & Gessler, 2007).

The role of Notch signaling in mammalian spermatogenesis is controversial. Several studies detected expression of Notch proteins in the testis of neonate and adult mice (Dirami et al., 2001; Mori et al., 2003; von Schönfeldt et al., 2004; Garcia & Hofmann, 2013; Garcia et al., 2013), or associated this pathway with male infertility (Hayashi et al., 2001; Hayashi, 2004b; Sahin et al., 2005). However, two studies (Hasegawa et al., 2011; Batista et al., 2012), using genetically engineered mice, reported that the majority of Notch genes are not transcribed in the seminiferous tubules, and that Notch blockade in germ and Sertoli cells has no effects in the normal course of spermatogenesis. Recently, we described the expression patterns of Notch component and effector genes along mouse testis post-natal development and throughout the spermatogenic cycle (Murta et al., 2013). Results prompted for a relevant role of Notch signaling in mammalian testis development and spermatogenesis.

The objective of this study was to evaluate the effects of *in vivo* Notch blockade on spermatogenesis and epididymis function. Based on previous results, our hypothesis was that Notch signaling is a major regulator of germ cell fate decisions occurring during the spermatogenic cycle, and of spermatozoa maturational changes occurring along the

epididymis. To test this hypothesis we blocked *in vivo* Notch signaling using the γ-secretase inhibitor N-S-phenyl-glycine-t-butyl ester (DAPT), and evaluated the expression patterns of Notch component genes, germ cell fate and apoptosis during spermatogenesis, and epididymis spermatozoa characteristics. Validating our hypothesis, blockade of Notch signaling induced abnormal spermatogenesis and spermatozoa maturation. This phenotype indicates a relevant regulatory role of Notch signaling in male reproductive function.

3. Materials and methods

3.1 Animals

All experiments were conducted in accordance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (Directive n. 86/609/EEC, from the 24th November 1986). Mice manipulation protocols were approved by the national regulatory agency (DGAV – Direção Geral de Alimentação e Veterinária) and the Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal). All authors are accredited as FELASA category C scientists or equivalent.

Outbred CD1 mice were chosen to introduce normal biological variability within the experiment. Mice were maintained in a 12-hour light/dark cycle, in ventilated cages with corn cob as bedding, and were given access to standard laboratory diet and water *ad libitum*. Mice health was monitored daily. Prior to the experiment, male mice were caged separately with used female bedding and, after four days, joined with a female until the production of a vaginal plug, to grant normal reproductive behavior and the presence of active spermatogenesis.

3.2 Experimental Design

Male mice (n = 24) with 3 months of age were randomly assigned to 4 groups: i) DAPT (Sigma-Aldrich, Inc., D5942) treatment during 25 days; ii) DAPT treatment during 43 days; iii) Control group (vehicle alone) during 25 days; and iv) Control group (vehicle alone) during 43 days. In adult mice, spermatogenesis (release of spermatozoa in the seminiferous tubule's lumen) takes 43 days (Clermont & Trott, 1969). We evaluated DAPT treatment effects at half the time (25 days) and at the time (43 days) required to accomplish spermatogenesis. Side-effects of Notch blockade by γ-secretase inhibitors are time and dose dependent (Doerfler, Shearman, & Perlmutter, 2001). To reduce toxicity, a low dose regimen of 5mg/Kg/day, 5 days per week was used (Teachey et al., 2008). DAPT powder was reconstituted in 100% ethanol (1 mg/mL) and stored in aliquots as stock solution at -20°C. Each day, fresh treatment solution was prepared, containing a mixture of 90% corn oil and 10% ethanol (Teachey et al., 2008). DAPT and vehicle alone were administered by oral gavage.

At the end of the experiment (25 and 43 days), during last hour of dark, mice were euthanized through cervical dislocation under ketamine (15mg/kg)/xylazine (1mg/kg) anesthesia, followed by exsanguination. A blood sample was collected immediately before cervical dislocation and processed for plasma testosterone analysis. From each mouse, the testis and one epididymis were dissected free and processed for IHC; one epididymis head was processed for RNA extraction, and one epididymis tail used to collect spermatozoa to perform a sperm analysis and an *in vitro* fertilization (IVF) assay.

3.3 Quantitative Gene Transcription Analysis (qRT-PCR)

Epididymis heads were immediately frozen in liquid nitrogen and stored at −80ºC. RNA extraction, cDNA synthesis and mRNA transcription was performed as previously described (Silva et al., 2012). Quantification of Notch effector genes *Hes1, Hes2, Hes5 and Nrarp* transcripts was done using selected primers (pair sequences available in Annex I). Transcription of gene *β2mg* was used as an endogenous control. Real-time PCR was performed in duplicate wells on StepOnePlus™ (Applied Biosystems, Foster City, CA, USA). All PCR reactions were carried out in 96-well optical reaction plates (Applied Biosystems, Warrington, UK) with 6.25µl Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2.5ng of diluted cDNA and 80 nM of each primer in a total reaction volume of 12µl.

3.4 Histological Evaluation and Apoptosis Assay

Paraffin sections were stained with haematoxylin. Spermatogenic cycle stages were identified as previously described (Hess & Franca, 2008). Stages were grouped in two (I-II, III-IV, V-VI, VII-VIII, IX-X, XI-XII) to facilitate description.

Apoptosis was evaluated by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay, for *in situ* visualization of DNA fragmentation, according to manufacturer's instructions (Chemicon, Millipore). The percentage of apoptotic cells was determined for each cell type and spermatogenic cycle stage. This evaluation was performed on a total of 30 seminiferous tubules per animal.

3.5 Immunohistochemistry

The spatial expression patterns of Notch components were determined following IHC, according to a method previously described (Silva et al., 2010). Staining was evaluated in the entire slice. Testis expression patterns were established following the evaluation of a minimum of 36 slices (3 slices/testis x 2 testis x 6 animals) for each Notch component (plus 12 twin-slides with cell marker) in each of the four experimental groups.

The anti-3β-HSD antibody was used to identify Leydig cells and the anti-DAZL antibody was used to identify germ cells. The antibodies against Notch components were previously

validated by others in the mouse (anti-Notch1 (Cheng et al., 2007), anti-Notch3 (Feng et al., 2010), anti-Dll1 (Sörensen et al., 2009), anti-Dll4 (Sörensen et al., 2009), anti-Jagged1 (Bielesz et al., 2010)) and rat species (anti-Notch2 (Saravanamuthu et al., 2009)). The antigen retrieval step was performed in citrate buffer (10mM, pH 6.0), except for the anti-Notch1 antibody (Tris-EDTA, pH 9.0). Blocking was performed in PBS with 2% bovine serum albumin (Sigma-Aldrich, Inc.) for 1 hour at room temperature. Sections were incubated overnight at 4ºC with each primary antibody: Notch1 (ab27526, Abcam), diluted 1:100; Notch2 (ab8926, Abcam), diluted 1:100; Notch3 (ab23426, Abcam), diluted 1:160; Dll1 (ab10554, Abcam), diluted 1:100; Dll4 (ab7280; Abcam), diluted 1:200; Jagged1 (SC-8303, Santa Cruz Biotechnology), diluted 1:50; 3β-HSD (SC-30820, Santa Cruz Biotechnology), diluted 1:300, and DAZL (ab34139; Abcam), diluted 1:250. Negative controls used the polyclonal rabbit IgG (ab27478, Abcam), diluted 1:100 and, for the 3β-HSD antibody, the goat control IgG (ab37373, Abcam), diluted 1:300. All primary antibodies were diluted in blocking solution. The peroxidase conjugated monoclonal mouse anti-goat/sheep IgG antibody (A9452, Sigma-Aldrich, Inc.), diluted 1:100, was used as secondary antibody for the 3β-HSD antibody, and the peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (Dako 410972), diluted 1:100, was used as secondary antibody for the remaining primary antibodies.

3.6 Epididymis Sperm Analysis

Epididymis tails were placed in 200µl of Human Tubarian Fluid (HTF) medium at 37 ºC, and sliced to allow recovery of luminal fluid. After 5 minutes, tissue was removed from medium, and a sample of sperm suspension taken immediately for analysis. Sperm motility (progressive forward motility) was evaluated in a 37ºC heated microscope slide. Sperm concentration was evaluated in a Neubauer chamber. Total viable sperm/mL was calculated (motility x concentration). Sperm morphology was evaluated in eosin-nigrosin stained slides.

3.7 *In Vitro* Fertilization and Embryo Development

Epididymis tail spermatozoa were capacitated through incubation in HTF at 37ºC for 2 hours. Concentration of viable spermatozoa was normalized to 10^6/mL. Cumulus-oocyte complexes were recovered from superovulated CD1 female mice at 13 hours post hCG injection. Oocytes were *in vitro* inseminated and *in vitro* cultured in KSOM for 4 days. Cleavage rate (proportion embryos ≥ 2 cell stage) and blastocyst rate were evaluated at the end-point of culture. Procedures for female superovulation, *in vitro* insemination and *in vitro* embryo culture are described elsewhere (Nagy, Gertsenstein, Vintersten, & Behringer, 2003).

3.8 Measurement of Plasma Testosterone Concentrations

Total plasma testosterone was assayed without extraction by a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 1000, Siemens Healthcare Diagnostics, Lda., Amadora, Portugal), using a commercial kit (Immulite 1000 Total Testosterone kit, Siemens). The inter-assay coefficients of variation were 8.9% and 9.7% for two different controls used in the assay (for concentrations of 279 and 735 ngdL−1, respectively; Multivalent Control Module, Siemens Healthcare Diagnostics). The intra-assay coefficient of variation was 5.1%.

3.9 Statistical Analysis

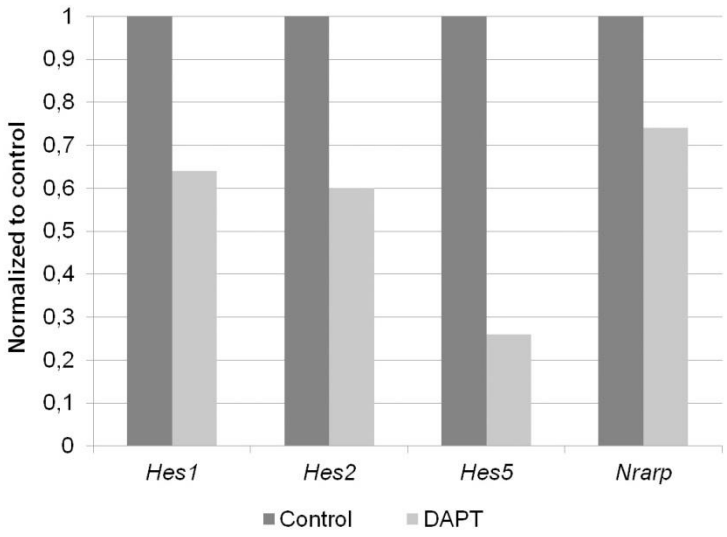
Data analysis was performed with the SPSS version 19.0 statistic software. Comparisons between groups used the Student's t-test or One-way ANOVA followed by Bonferroni or Tamhane's T2 post-hoc tests (assuming homogeneity of variances or not, respectively). Homogeneity of variances was evaluated through the Levene test. Significance was determined at the 5% confidence level (p<0.05).

4 Results

4.1 DAPT Treatment Decreases Transcription of Notch Effector genes *Hes1, Hes2, Hes5* and *Nrarp*

In DAPT treated mice, transcription levels in the epididymis head decreased approximately 30-40% for genes *Hes1*, *Hes2* and *Nrarp*, and 75% for gene *Hes5*, compared with control mice (Figure 24).

Figure 24 - DAPT decreases transcription of downstream Notch effector genes *Hes1*, *Hes2*, *Hes5* and *Nrarp*.



Comparison between a representative animal of each group (Control and DAPT treatment).

4.2 DAPT Treatment Disrupts Expression Patterns of Notch Components in the Testis

Disruption of the normal expression patterns was already foreseeable after 25 days of treatment (data not shown), but became evident after 43 days of DAPT treatment. Figures 25 and 26 schematically illustrate the changes in expression patterns of Notch components in the testis, following 43 days of DAPT treatment. Figure 27 shows the main changes in expression detected in DAPT treated mice. Notch1, Notch2 and Dll1 do not change their expression patterns. Notch3 shows ectopic expression in Sertoli cells at stages VI-IX and in leptotene and zygotene spermatocytes at stages IX-XII. (B and C). Dll4 stops being expressed in leptotene spermatocytes and Sertoli cells at stage IX-X (E and F), and shows ectopic expression in pachytene spermatocytes at stage I-IV and in zygotene spermatocytes at stage XI-XII (H and I). Jagged1 shows the most extensive changes in the expression pattern following DAPT treatment. Ectopic expression is observed in spermatogonia at stages II-VII (K and L), in round and elongated spermatids at stage VII-VIII, in leptotene and pachytene spermatocytes at stages IX-X (N and O), and in zygotene spermatocytes at stages XI-XII (R and S). Additionally, Jagged1 stops being expressed in diplotene spermatocytes and germ cells finalizing meiosis at stages XI-XII (R and S).

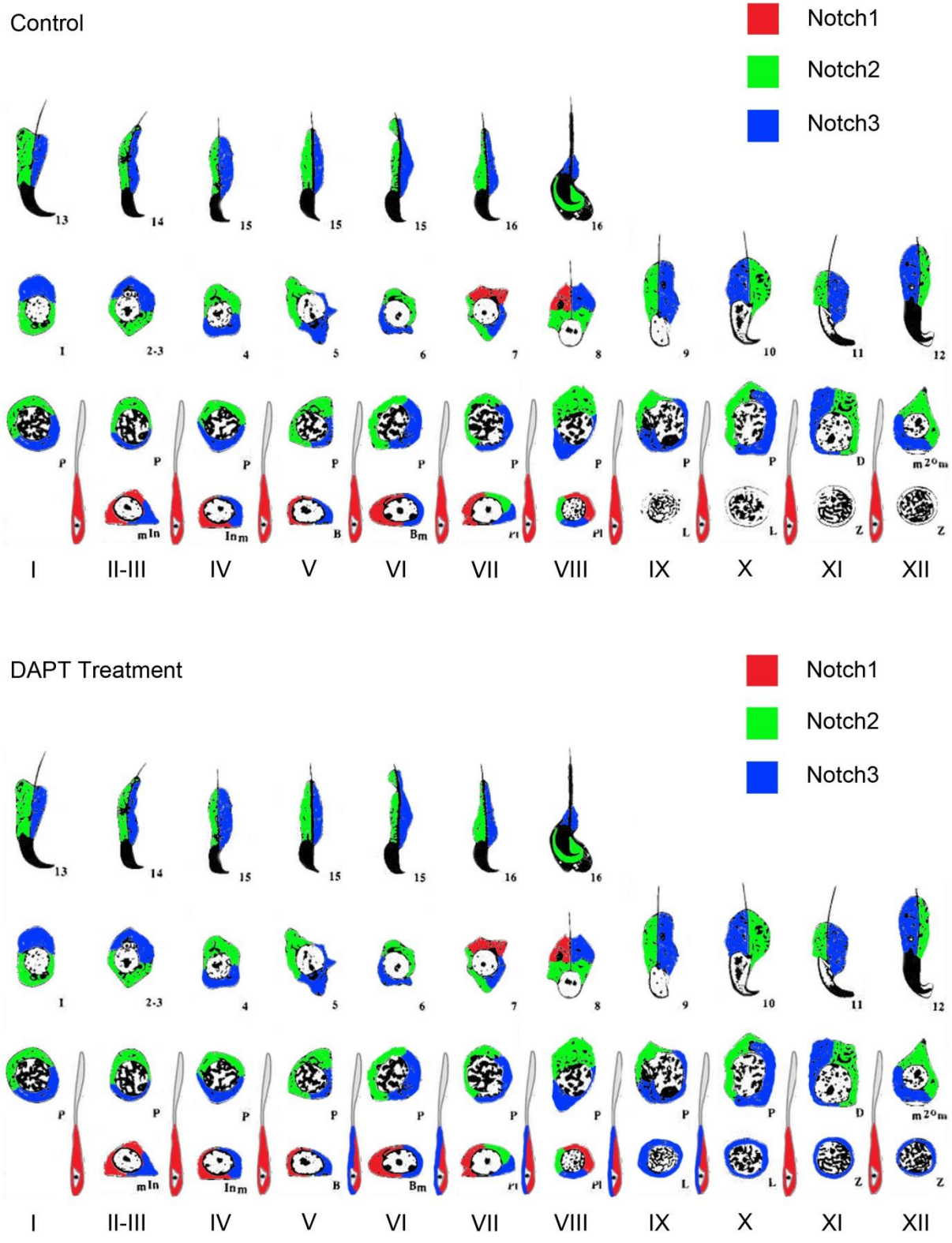
Figure 28A shows the percentage of pachytene spermatocytes expressing Dll4 along the spermatogenic cycle. As can be depicted, DAPT treatment significantly advances expression of this ligand in the spermatogenic cycle. Expression of Notch receptors in pachytene spermatocytes is not altered.

Following DAPT treatment, interstitial Leydig cells gain expression of Jagged1 (Figure 29), whereas all other Notch components do not change their expression pattern.

4.3 DAPT treatment induces germ cell fate aberrations and germ cell abnormal morphology

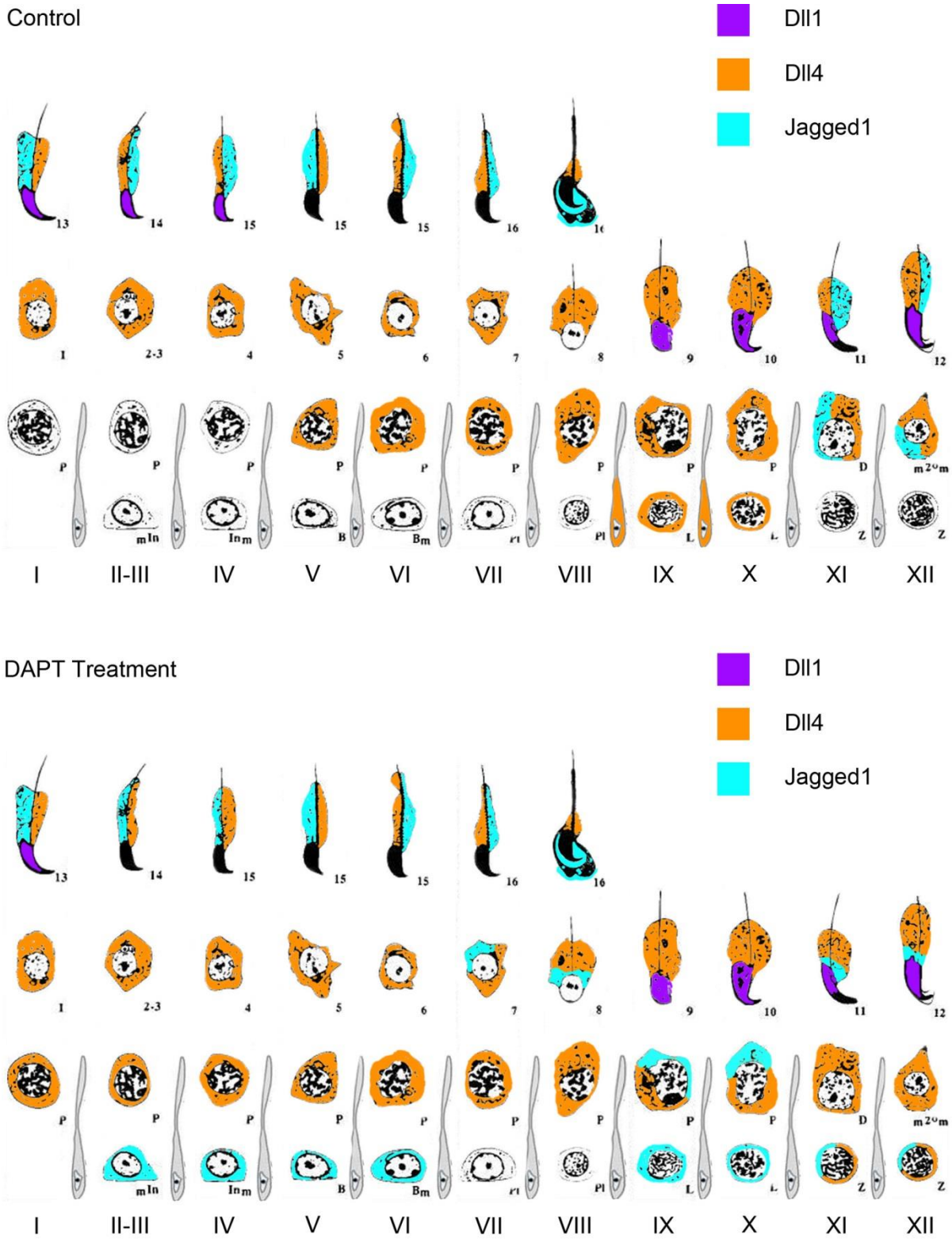
Figure 28B shows the main effects of DAPT treatment on germ cell fate and morphology. Abnormal germ cell fate and morphology are observed in almost all seminiferous tubules, and although the majority of germ cells proceed in differentiation, this largely leads to the production of morphologically abnormal cells. Round spermatids fail to elongate, become round, pyknotic and are released into the lumen (E, H), sometimes displaying a small nucleus (E). Other germ cells fail to enter a normal cell division. These cells become enlarged, with vacuolated cytoplasm and several nuclear fragments (F and G). These anomalous cells express all Notch components, are not marked by DAZL (Figure 30 A-F), are TUNEL negative (Figure 30 G-I), and are released in the lumen, being also identified in the epididymis lumen (Figure 31).

Figure 25 – Schematic illustration of expression patterns of Notch pathway receptors along the spermatogenic cycle: comparison between control and DAPT treated mice.



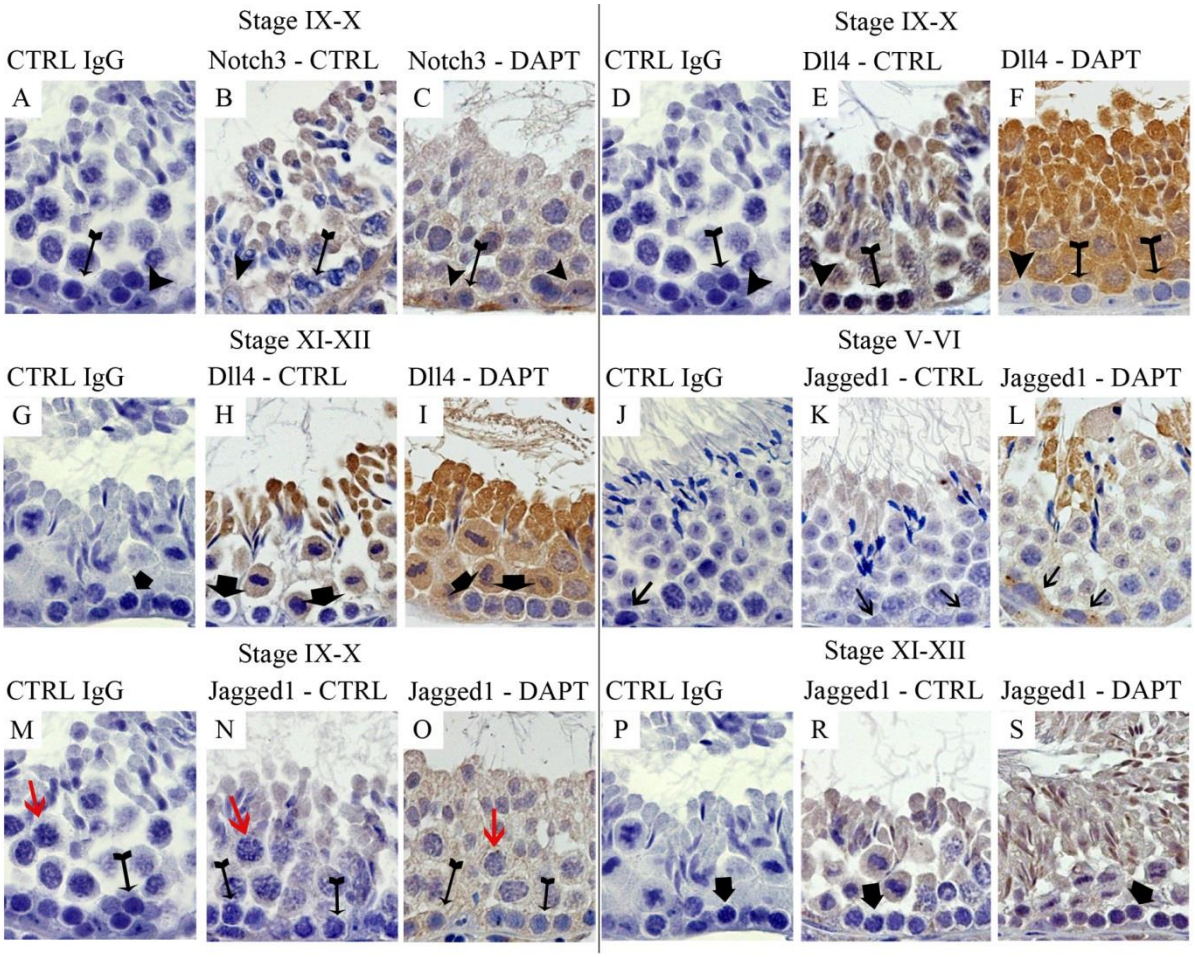
Draw-scheme representing stages (I-XII) of the spermatogenic cycle. Spermatogonia (A, In, B); spermatocytes (Pl- preleptotene, L- leptotene, Z- zygotene, P- pachytene, D- diakinesis, Mi- meiotic division); round spermatids (1-8); elongated spermatids (9-16). Spatial localization of expression of Notch receptors drawn in different colors, according to legend. Drawing adapted from Hess & Franca (2008).

Figure 26 – Schematic illustration of expression patterns of Notch pathway ligands along the spermatogenic cycle: comparison between control and DAPT treated mice.



Draw-scheme representing stages (I-XII) of the spermatogenic cycle. Spermatogonia (A, In, B); spermatocytes (Pl- preleptotene, L- leptotene, Z- zygotene, P- pachytene, D- diakinesis, Mi- meiotic division); round spermatids (1-8); elongated spermatids (9-16). Spatial localization of expression of Notch ligands drawn in different colors, according to legend. Drawing adapted from Hess & Franca (2008)

Figure 27 – Disruption of expression patterns of Notch components along the spermatogenic cycle following DAPT treatment.

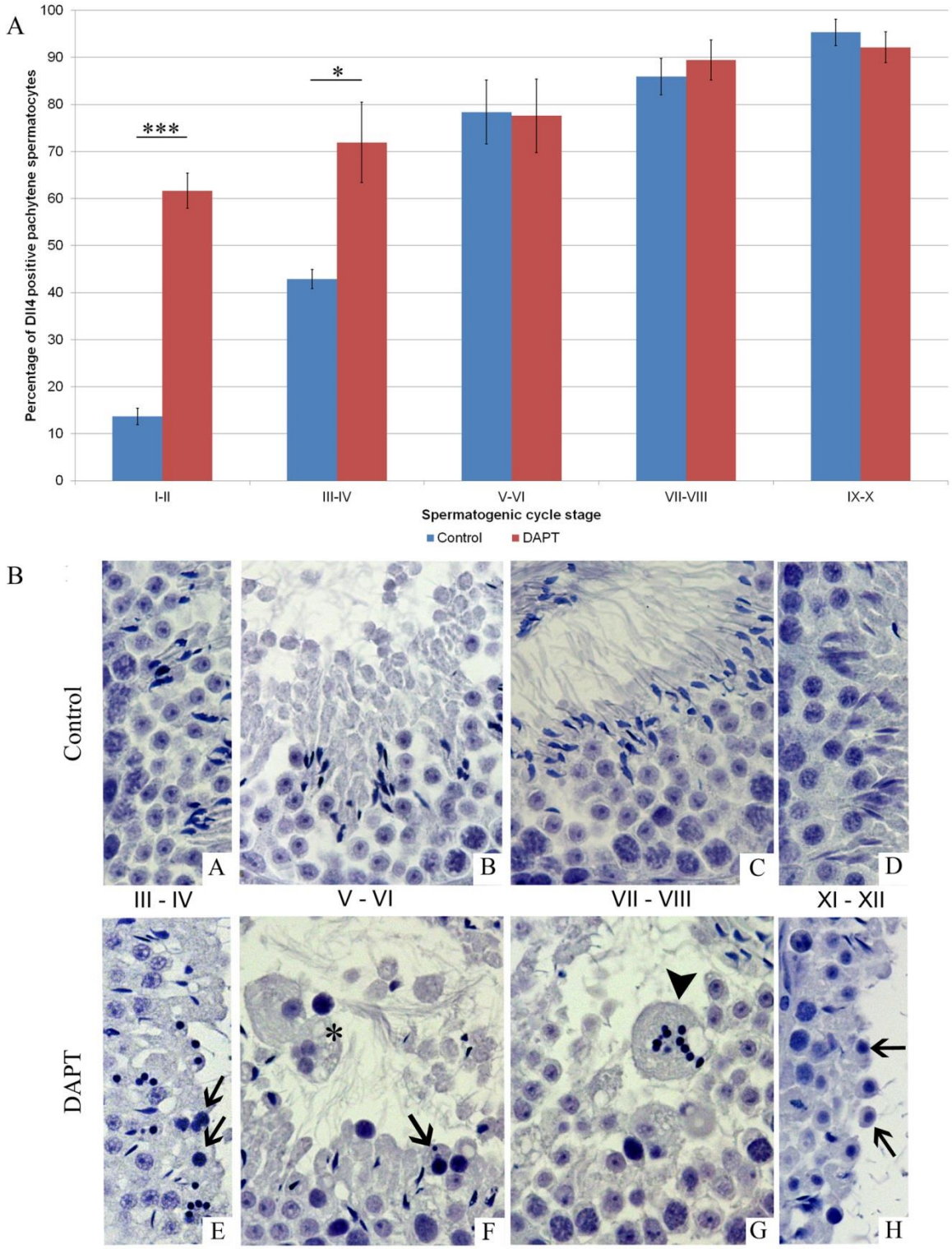


Positive staining in brown, counterstaining with haematoxylin (400x magnification). Arrow heads point Sertoli cells. Tailed arrows point leptotene spermatocytes. Bold arrows point zygotene spermatocytes. Arrows point spermatogonia. Red arrows point pachytene spermatocytes. Control (CTRL) slides used rabbit IgG (A, D, G, J, M, P). Notch3 shows ectopic expression in Sertoli cells and in leptotene spermatocytes at stages IX-X. (B and C). Dll4 stops being expressed in leptotene spermatocytes and Sertoli cells at stage IX-X (E and F), and shows ectopic expression in zygotene spermatocytes at stage XI-XII (H and I). Jagged1 shows ectopic expression in spermatogonia at stages V-VI (K and L); in leptotene and pachytene spermatocytes at stages IX-X, (N, O); and in zygotene spermatocytes at stages XI-XII (R and S).

4.4 DAPT treatment induces increased apoptosis in germ cells

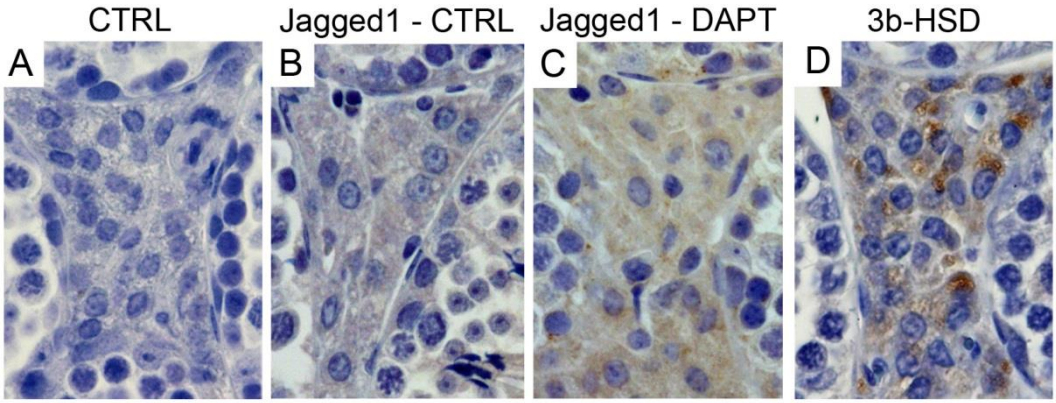
As shown in Figure 32A, compared to control groups, DAPT treatment induces a significantly higher rate of apoptosis in germ cells. However, the apoptotic rate in germ cells is not significantly different following the two DAPT treatment lengths. Apoptosis is mainly observed at the XI-XII stage, and overall differences in the apoptosis rate observed between DAPT treatment and control groups, arise mainly from differences observed at this cycle stage. Zygotene spermatocytes and germ cells undergoing last steps of meiotic divisions are the most affected by DAPT treatment (Figure 32 B and C).

Figure 28 A – DAPT treatment significantly changes the proportion of pachytene spermatocytes expressing Dll4. B - DAPT treatment induces formation of abnormal germ cells.



A - (*) $p < 0.05$; (***) $p < 0.001$. **B** - Haematoxylin staining (400x magnification). Arrows mark spermatids that fail to elongate. Arrow heads mark germ cells with several small nucleus/nuclear fragments. Asterisk marks giant multinucleated germ cell. Seminiferous tubules from control mice (A-D). Elongating spermatids appear with several round small nuclei (E, H). Giant multinucleated germ cells (F). Enlarged germ cells with vacuolated cytoplasm and several nuclear fragments (G). Round spermatids fail to elongate, become round, pyknotic, and are released in the lumen (F, H).

Figure 29– DAPT treatment disrupts Jagged1 expression in testis interstitial Leydig cells.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Control (CTRL) slides used rabbit IgG (A). Jagged1 is not expressed in Leydig cells of control mice (B), but becomes expressed following DAPT treatment (C). Leydig cells are co-localized in twin slides with marker 3β-HSD (D).

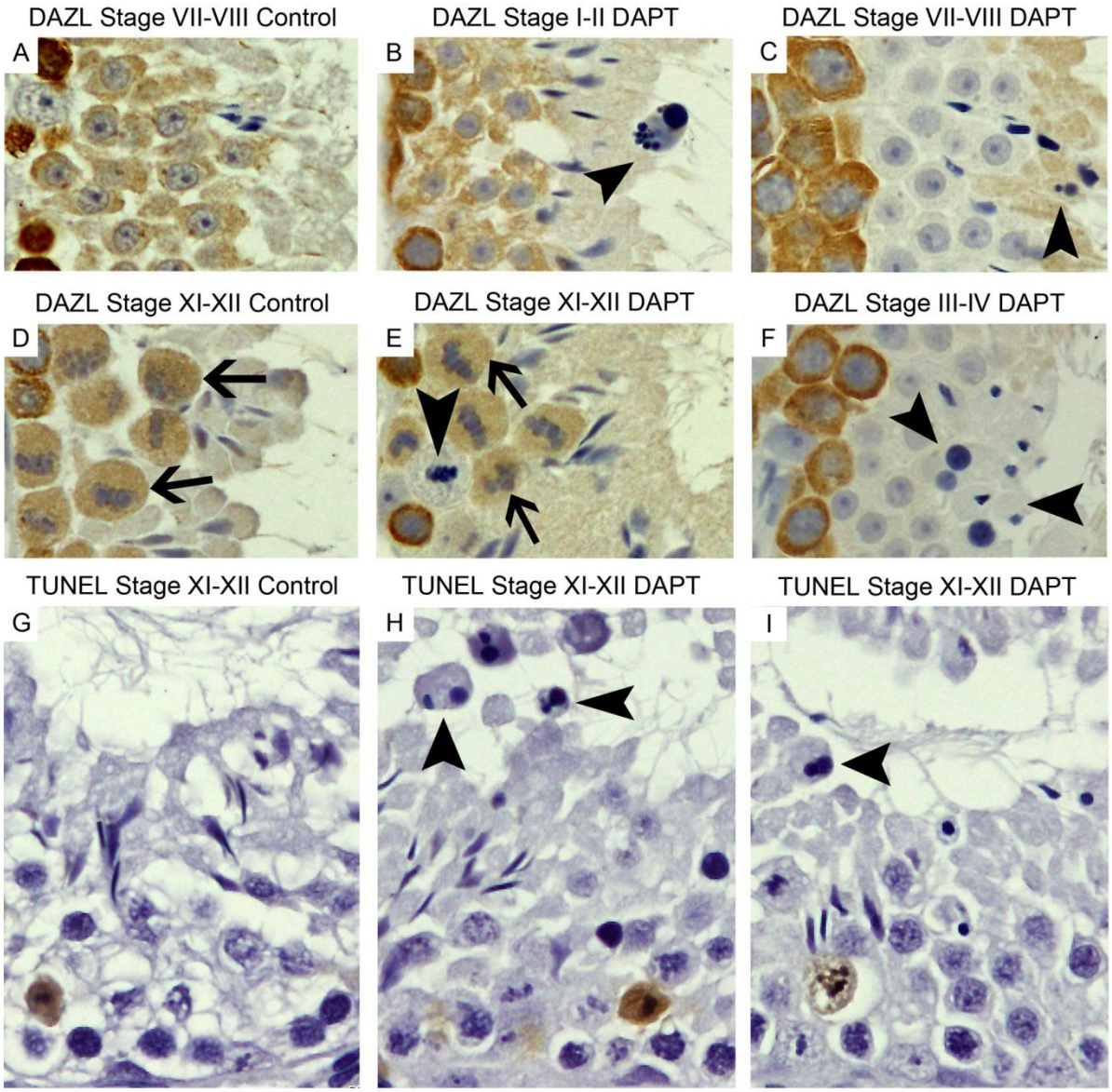
4.5 DAPT treatment disrupts expression patterns of Notch components in spermatozoa.

Expression of Notch2 in the cytoplasmic droplet of spermatozoa along the epididymis is decreased in DAPT treated mice, compared to control mice (Figure 33 A, B, G, H, M, N). In contrast, expression of Dll1 in the cytoplasmic droplet of spermatozoa along the epididymis is increased in DAPT treated mice, compared to control mice (C, D, I, J, O, P). In DAPT treated mice, Jagged1 is now expressed in the cytoplasmic droplet of spermatozoa, contrary to that observed in control mice, where Jagged1 is only detected in the epididymis lumen (E, F, K, L, Q, R).

4.6 DAPT treatment induces decreased motility and increases morphologic defects in epididymis spermatozoa.

As shown in Figure 34A and B, epididymis spermatozoa's progressive forward motility and viable sperm/mL of DAPT treated mice are significantly lower than that of control mice, although sperm concentration is not significantly affected by treatment. As expected from testis histology results, the proportion of spermatozoa with morphological defects is significantly higher in DAPT treated mice than in control mice (Figure 34A). The main morphological defects are primary anomalies of the sperm head and midpiece, including bent, thin and club-shaped heads, coiled, folded and deformed midpieces, and coiled tailpieces (Figure 34C).

Figure 30 – Abnormal germ cells loose identity.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Germ cells of control mice stain with marker DAZL (A and D). Most abnormal germ cells of DAPT treated mice do not stain with marker DAZL (B, C, E and F). TUNEL staining of seminiferous tubules of control (G) and DAPT treated (H and I) mice. Abnormal germ cells are TUNEL negative. Arrow heads point abnormal germ cells. Arrows point normal dividing germ cells.

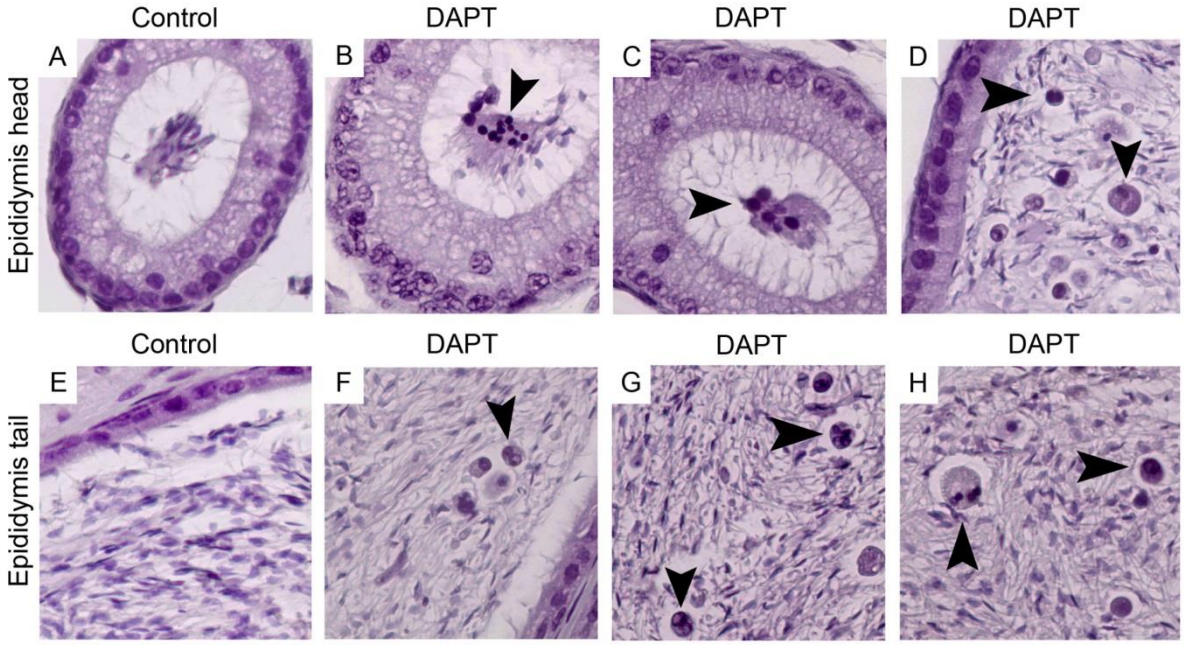
4.7 Plasma testosterone concentrations are not affected by DAPT treatment.

Plasma testosterone concentrations of DAPT treated and control mice are similar (Figure 35A).

4.8 Fertilizing ability of viable spermatozoa is not affected by DAPT treatment.

Viable sperm from DAPT treated and control mice yield similar cleavage and blastocyst rates (Figure 35B).

Figure 31 – Abnormal germ cells are released into the epididymis lumen.



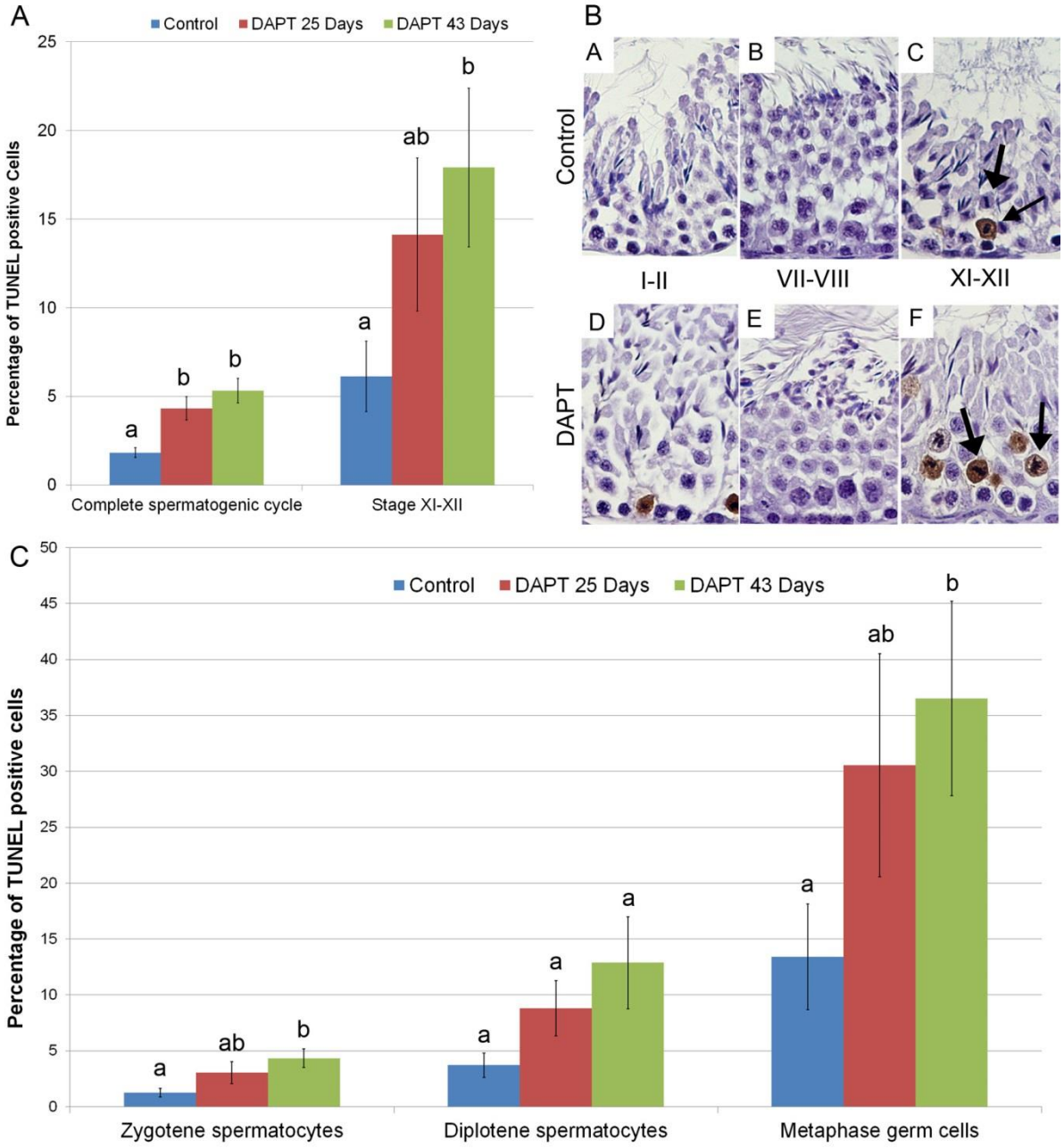
Staining with haematoxylin (400x magnification). Tissue sections from epididymis head and tail lumen of control (A and E) and DAPT treated (B-D and F-H) mice. DAPT treated mice show abnormal germ cells in the epididymis lumen. Arrow heads point abnormal germ cells.

5 Discussion

We recently described the transcription and expression patterns of Notch component and effector genes in mouse testis post-natal development and along the adult spermatogenic cycle (Murta et al., 2013). Results indicated that Notch signaling is active, and prompted for a relevant regulatory role of Notch signaling in testis development and during spermatogenesis. This conducted to the phenotypic evaluation of *in vivo* blockade of Notch signaling using the γ-secretase inhibitor DAPT.

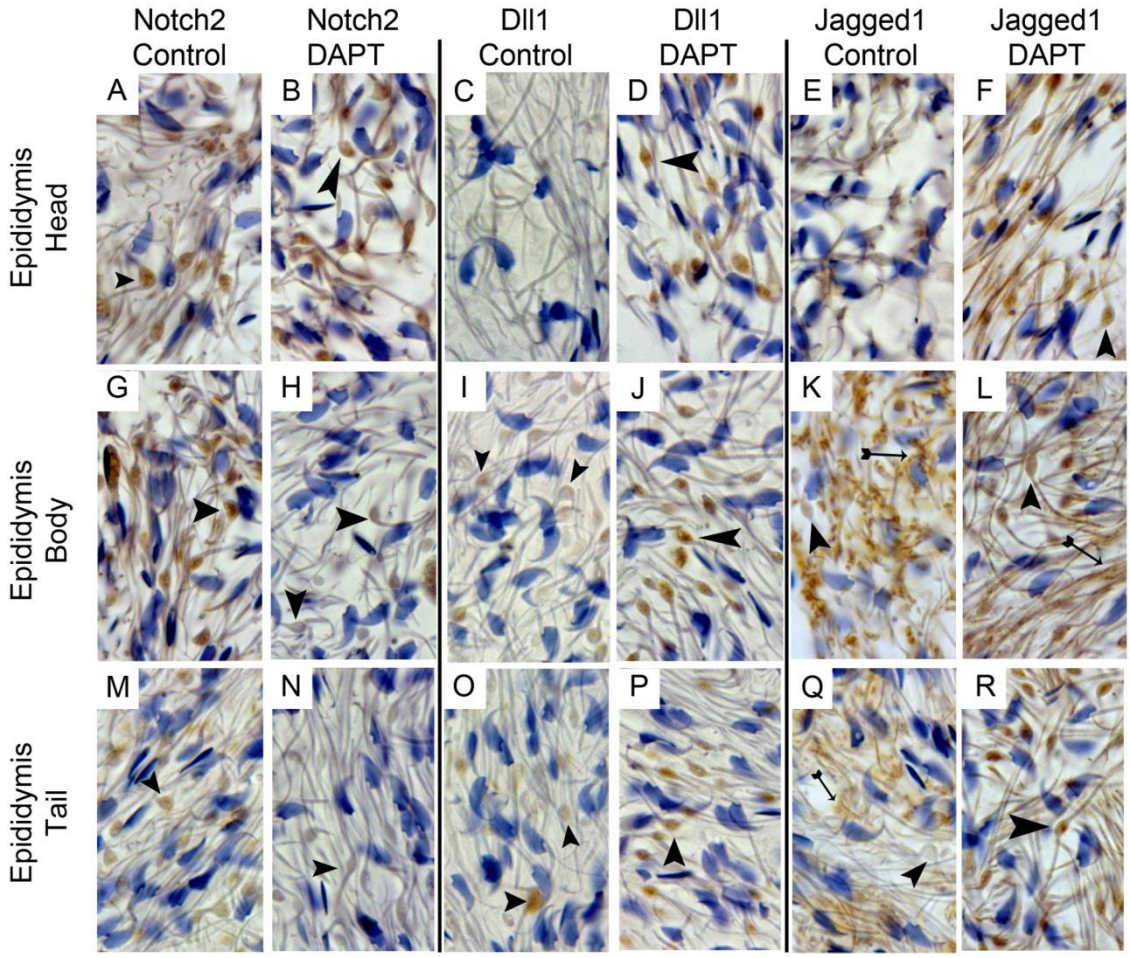
To evaluate the level of Notch signaling blockade, transcription of main Notch effector genes was analyzed in the epididymis head. This tissue was elected based on its strong epithelial expression of Notch component and effector genes (results not shown), and its close anatomical proximity and functional relationship with the testis. DAPT decreased transcription of Notch effector genes, which evidences that treatment attained the expected biological effect. However, the efficacy of treatment was incomplete, probably due to the low drug dosage used. This dosage was chosen to avoid toxic secondary effects associated to the long treatment schedule, as described by others (Teachey et al., 2008). Therefore, results here discussed have to be addressed assuming that most of Notch signaling is still potentially active, and that a more efficacious blockade could induce a more representative picture of the role of Notch signaling in spermatogenesis.

Figure 32 – DAPT treatment significantly increases apoptosis during spermatogenesis



A – Relative frequency of apoptotic germ cells at all spermatogenic cycle stages and at stages XI-XII. Error bars represent the standard error of the mean (SEM). Columns with different superscript differ significantly. ab, $p< 0.05$. **B** – Apoptosis TUNEL evaluation. TUNEL positive staining in brown, counterstaining with haematoxylin (400x magnification). Arrows mark cells at last steps of meiosis. Apoptosis in seminiferous tubules of control (A-C) and DAPT treated (D-F) mice. Cells at last steps of meiosis are more affected (C, F). **C** – Relative frequency of apoptotic germ cell types at the most affected spermatogenic stage (XI-XII). Error bars represent the standard error of the mean (SEM). Columns with different superscript differ significantly: ab, $p< 0.05$.

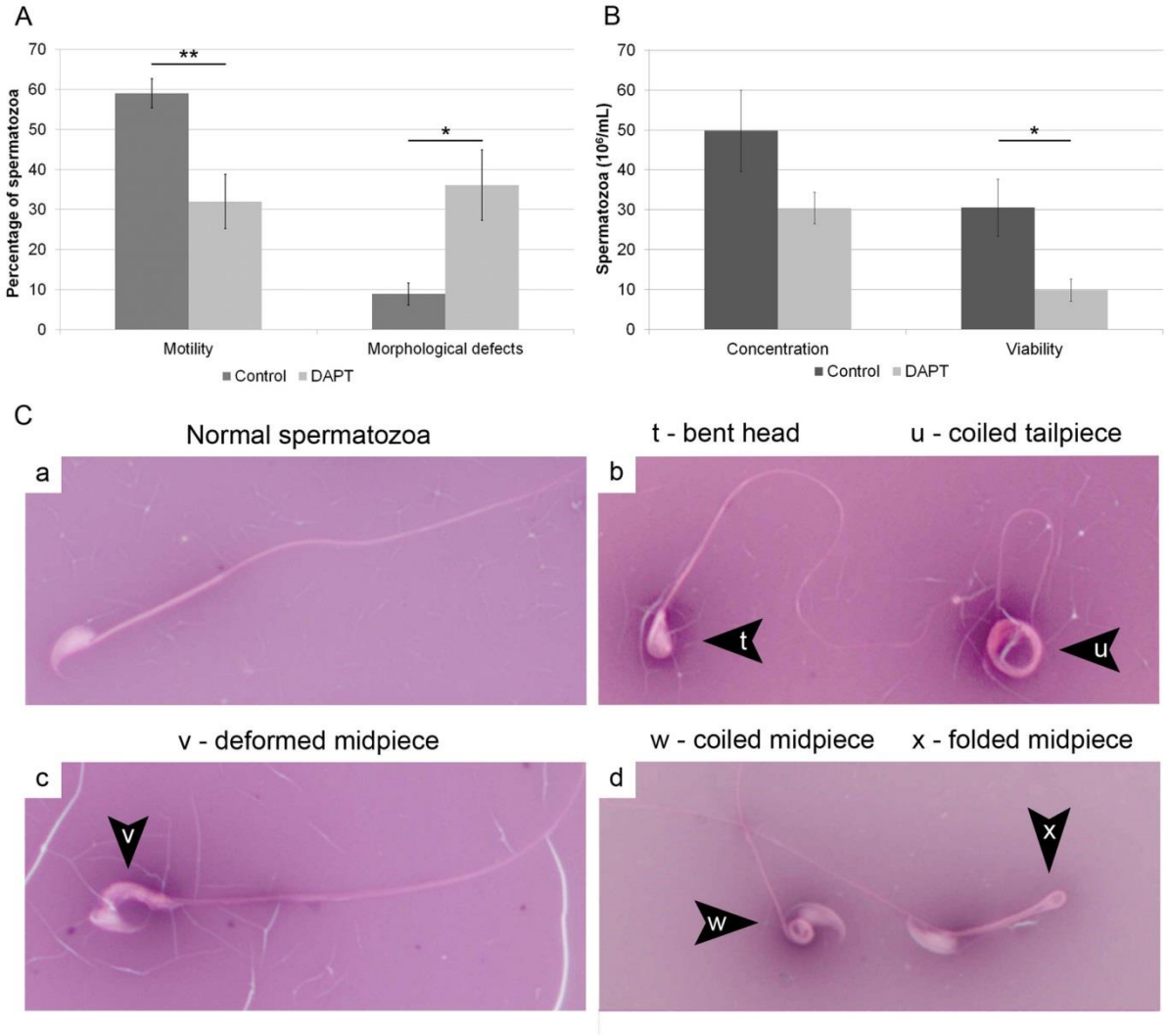
Figure 33 – DAPT treatment disrupts expression patterns of Notch components in epididymis spermatozoa.



Positive staining in brown, counterstaining with haematoxylin (1,000x magnification). Notch2 is expressed in spermatozoa cytoplasmic droplet along the epididymis of control mice (A, G, M), but only in the epididymis head of DAPT treated mice (B). Dll1 is only expressed in a few spermatozoa cytoplasmic droplets in the epididymis body and tail of control mice (I, O), but in most spermatozoa cytoplasmic droplets along the epididymis of DAPT treated mice (D, J, P). Jagged1 is not expressed in spermatozoa but is detected in the lumen of epididymis body and tail of control mice (K, Q). Jagged1 is expressed in spermatozoa cytoplasmic droplets along the epididymis in DAPT treated mice (F, L, R). Arrow heads point to spermatozoa cytoplasmic droplets. Tailed arrows point to Jagged1 in the epididymis lumen.

In vivo Notch blockade disrupts expression patterns of Notch components (Notch3, Dll4 and Jagged1). Enhanced expression of Notch3 may be due to a decrease in the activation of other receptors. This can also lead to the increase in available ligands and justify the enhanced expression of Dll4 and Jagged1, as a compensatory effect. However, as germ cell fate and identity are probably the result of unique expression combinations of receptors and ligands (Murta et al., 2013), ectopic expression of Notch components may induce disturbances in these events, and induce abnormal progression of spermatogenesis, as observed in this study.

Figure 34 – DAPT treatment significantly decreases spermatozoa motility and viability, and increases morphologic defects.

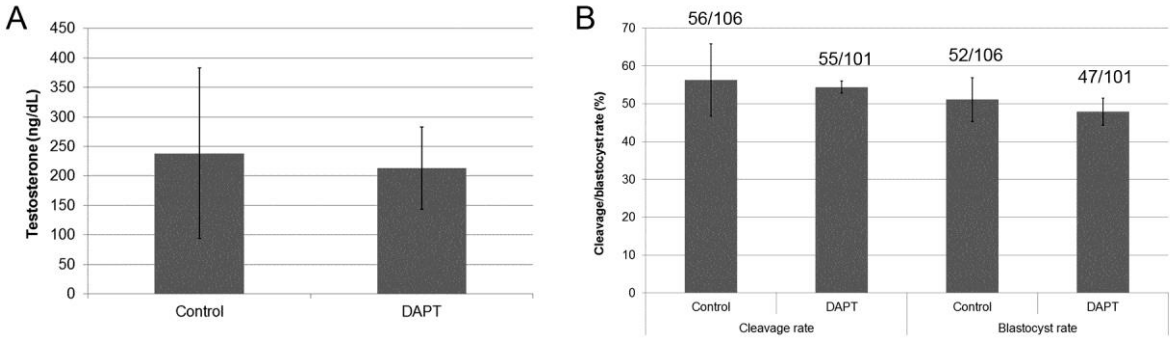


A – Epididymis tail spermatozoa motility (progressive forward motility) and morphology evaluation. (*) $p < 0.05$; (**) $p < 0.01$ **B** – Epididymis tail spermatozoa concentration (number of spermatozoa per mL of collection medium) and viability (number of motile spermatozoa per mL of collection medium) evaluation. (*) $p < 0.05$ **C** – Main spermatozoa morphologic defects found in DAPT treated mice, eosin-nigrosin stain (400x magnification). Normal spermatozoa (a), morphological defects (b-d).

In vivo Notch blockade induces the formation of morphologically abnormal germ cells, including elongated spermatids with a round nucleus and multinucleated vacuolated giant cells. The ectoplasmic specialization is the elongated spermatid anchoring system, which maintains adherence to Sertoli cells, and confers cell orientation and polarity within the seminiferous epithelium (Wong et al., 2008). This spermatid anchoring system is mainly composed by Par complex proteins (Wong et al., 2008). Notch signaling is associated to cell polarity decisions in tissues involving Par complex proteins, such as the neural epithelium (Afonso & Henrique, 2006; Bultje et al., 2009). We have recently reported expression of Notch components in the elongated spermatid anchoring system, and nuclear detection of

Hes5 in Sertoli cells and in elongating spermatids (Murta et al., 2013). Notch blockade induces abnormal spermatid elongation and its premature release into the lumen, and the presence of abnormal cells in the lumen of seminiferous tubules and epididymis. This indicates a regulatory role of Notch signaling in spermatid's elongation and maintenance of the elongated spermatid anchoring system. Notch pathway was associated with cell division (Bultje et al., 2009; Das & Storey, 2012). The formation of multinucleated cells and the increased apoptosis of germ cells undergoing the final steps of the meiotic divisions, following Notch blockade, indicate that Notch signaling is involved in the regulation of germ cell meiosis.

Figure 35 – A - Plasma testosterone concentrations of control and DAPT treated mice. B - Cleavage and blastocyst rates following IVF with viable spermatozoa of control and DAPT treated mice.



In vivo Notch blockade significantly increases the rate of apoptosis in germ cells, mainly at stage XI-XII of the spermatogenic cycle. Zygotene spermatocytes are the germ cells most affected by apoptosis. Interestingly, these germ cells do not normally express Notch components (Murta et al., 2013), but exhibit ectopic expression of Notch3, Dll4 and Jagged1 following DAPT treatment. Apoptosis in these germ cells may follow loss of identity due to blockade of Notch signaling. However, abnormal germ cells stop expressing marker DAZL (which indicates loss of germ cell identity), but do not become apoptotic.

In vivo Notch blockade disrupted the expression patterns of Notch components in Leydig interstitial cells, inducing ectopic expression of Jagged1. However, this had no effect on plasma testosterone concentrations.

In vivo Notch blockade disrupted the expression pattern of Notch components in epididymis spermatozoa, inducing the ectopic expression of Notch2, Dll1 and Jagged1 in the cytoplasmic droplet of spermatozoa. Therefore, Notch blockade might also be affecting epididymis epithelial function, impairing spermatozoa maturation. This may be associated with the significant decrease in epididymis spermatozoa's progressive forward motility observed following DAPT treatment.

In vivo Notch blockade significantly increased the proportion of epididymis spermatozoa with primary morphological defects. This feature probably arises from disturbances in spermatogenesis, as discussed above, and is also probably contributing to the decrease in epididymis spermatozoa progressive forward motility.

Reduced sperm motility and increased sperm morphological defects are associated with reduced fertility (Guzick et al., 2001). Although experimental mice were not allowed to breed following DAPT treatment, we performed IVF using viable spermatozoa of DAPT treated and control mice. Similar cleavage and blastocyst rates were obtained using viable sperm from males of both groups. This indicates that viable spermatozoa arising from non-affected (or less-affected) seminiferous tubules are fertile. However, total number of viable spermatozoa was significantly lower in DAPT treated than in control mice, which may affect fertility. Therefore, the putative effects of abnormal Notch signaling on fertility are probably related with the level of Notch pathway activity. Notch deregulation in the testis was associated with male fertility problems (Hayashi et al., 2001; Hayashi, 2004b; Sahin et al., 2005; Hahn et al., 2009), and potentially linked with maintenance and growth of testicular germ cell tumors (Hayashi, Yamada, Kageyama, & Kihara, 2004a; Garcia & Hofmann, 2013).

In conclusion, *in vivo* Notch blockade disrupts expression patterns of Notch components in the testis and in epididymis spermatozoa, induces germ cell fate aberrations, morphological abnormalities and apoptosis, decreases motility and increases morphological defects in epididymis spermatozoa. This indicates that Notch signaling has a major regulatory role in spermatogenesis, namely in germ cell fate and identity, meiosis, differentiation of spermatids, and on epididymis spermatozoa maturation. This turns the Notch pathway into an attractive fertility therapeutic target.

Chapter IV - Differential expression of Notch component and effector genes during ovarian follicle and corpus luteum development along the estrous cycle

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1. Abstract

Ovarian dynamics along the adult female EC is characterized by cyclic follicle and CL development. These events are tightly regulated, involving extensive cell-to-cell communication. However, the molecular mechanisms behind these cellular interactions are poorly understood. Notch is an evolutionarily well-conserved cell signaling pathway implicated in cell fate decisions in several tissues. Here, we evaluated the expression patterns of Notch component and effector genes during follicle and CL development along the EC.

Five mature CD1 female mice were euthanized at each EC stage. Blood samples were collected for P4 measurement, ovaries were processed for IHC, and expression patterns of Notch components (Notch1, 2 and 3, Jagged1 and Dll1 and Dll4), and effectors (Hes1, Hes2 and Hes5) were characterized. Nuclear detection of Notch effectors indicates that Notch signaling is active in the ovary. Notch components and effectors are differentially expressed during follicle and CL development along the EC. The spatial and temporal specific expression patterns are associated with follicle growth, selection and ovulation/atresia, and CL development and regression.

Key Words: Notch; follicle; corpus luteum; estrous cycle; mouse

2. Introduction

Only a small proportion of the ovarian pool of primordial follicles escapes their arrested state and resumes growth and development, during female adulthood. This cyclic follicle development leads to ovulation and formation of the CL. The follicle and CL, cyclic transient endocrine glands, secrete steroid hormones (E2 and P4) that are major regulators of the EC. The mouse EC sequentially comprises four stages (proestrus, estrus, metestrus, diestrus) and a total length of 4-5 days. Circulating concentrations of E2 peak prior to ovulation, which occurs at estrus, whereas P4 concentrations rise during metestrus and diestrus, and then decline from proestrus to estrus (Wood et al., 2007). Follicle and CL development and steroidogenesis are finely regulated events, involving complex cell-to-cell communication and signaling (Stocco et al., 2007). The molecular mechanisms behind this ovarian cellular remodeling are still poorly understood. Deciphering these mechanisms could potentially lead to the development of new therapeutic strategies addressed to infertility and contraception.

Notch cell signaling is an evolutionarily conserved pathway that is implicated in cell fate decisions in several tissues (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009). In mammals, four receptors (Notch1-4) and five ligands (three delta-like - Dll1, Dll3 and Dll4 - and two serrate-like - Jagged1 and Jagged2) were identified (Borggrefe & Oswald, 2009). Notch signaling is activated by binding of the receptor extracellular domain to ligands expressed on neighboring cells. This leads to cleavage of the Notch intracellular domain by a γ-secretase complex and its translocation to the nucleus, where it associates with other transcriptional factors, thus regulating transcription of Notch effector target genes (Borggrefe & Oswald, 2009). Only a limited set of Notch target genes were identified in various cellular and developmental contexts (Borggrefe & Oswald, 2009), being the hairy/enhancer of split genes the most ubiquitous (Fischer & Gessler, 2007).

Notch signaling was implicated in oocyte and follicle development in *Caenorhabditis elegans* (Kimble & Crittenden, 2007) and *Drosophil*a (López-schier & Johnston, 2001; Ward et al., 2006; Assa-Kunik et al., 2007; Song et al., 2007), and Notch components were identified in the neonate (Trombly et al., 2009; Xu & Gridley, 2013) and adult mammalian ovary (Baker & Spears, 1999; Johnson et al., 2001; Hahn et al., 2005; Vorontchikhina et al., 2005; Hernandez et al., 2011; Zhang et al., 2011; Fraser et al., 2012; García-Pascual et al., 2013; Jovanovic et al., 2013). Additionally, in rodents and primates, aberrant Notch signaling was associated with female reproductive disturbances (Trombly et al., 2009; Hernandez et al., 2011; Fraser et al., 2012; García-Pascual et al., 2013; Xu & Gridley, 2013) and ovarian cancer (Park, Shih, & Wang, 2008; Wang, Wu, Wang, & Xin, 2010). Still, the overall available information regarding the potential involvement of Notch signaling in the regulation of ovarian cellular remodeling is fragmentary.

The objective of this study was to evaluate the expression patterns of Notch component and effector genes in the mouse ovary along the EC, and their relationship with cellular remodeling events occurring during follicle and CL development. Our main hypothesis is that Notch signaling is involved in the regulation of follicle and CL development.

3. Methods

3.1 Animals

Experiments were conducted in compliance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (Directive n. 86/609/EEC, from the 24th November 1986). Experimental protocols were approved by the national regulatory agency (DGAV – Direção Geral de Alimentação e Veterinária) and the Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal). All authors are accredited as FELASA category C scientists or equivalent.

CD1 female mice with 3 months of age were maintained in a 12-hour light/dark cycle, in ventilated cages with corn cob as bedding, and were given access to standard laboratory diet and water *ad libitum*. The mice health was routinely monitored. Blood samples (1 mL) were collected by intracardiac puncture under ketamine (15mg/kg)/xylazine (1mg/kg) anesthesia, just before euthanasia through cervical dislocation followed by exsanguination. Outbred CD1 mice were used to introduce normal biological variability within the experiment.

3.2 Experimental Design

Expression patterns of Notch pathway components and effectors were evaluated at each of the four EC stages (proestrus, estrus, metestrus, and diestrus). Mice were sequentially monitored by vaginal smears and the EC stage determined by cytological evaluation. Five CD1 female mice were euthanized at each EC stage, and the ovaries were processed for IHC. EC stages were confirmed by histological examination of the ovaries and tubular tract, and by circulating P4 concentrations as described by others (Wood et al., 2007).

3.3 Immunohistochemistry

Ovaries were fixed in 4% neutral phosphate buffered formalin at room temperature for 24 hours and, after subsequent dehydration in ethanol, tissues were embedded in paraffin. Samples were sliced towards the center of ovary in several twin slides. Counterstaining was done with haematoxylin. Positive staining (brown) was evaluated in the entire slice. Spatial localization of expression of Notch pathway components (Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1) and effectors (Hes1, Hes2 and Hes5) was evaluated through IHC, as previously described (Silva et al., 2010). Proliferating Cell Nuclear Antigen (PCNA) cell marker was

used to identify proliferating cells (Zheng, Fricke, Reynolds, & Redmer, 1994; Oktay, Schenken, & Nelson, 1995). Only nuclear detection of Notch effectors was considered indicative of Notch signaling activation. The antigen retrieval step was performed in citrate buffer (10mM, pH 6.0), except for the anti-Notch1, anti-Hes1, anti-Hes2 and anti-Hes5 antibodies (Tris-EDTA, pH 9.0). Blocking was performed in PBS with 2% w/v bovine serum albumin (A7906, Sigma-Aldrich, Inc.) for one hour at room temperature. Sequential sections of 3 µm were incubated with primary antibodies anti Notch1 (ab27526, Abcam, diluted 1:100), Notch2 (ab8926, Abcam, diluted 1:100), Notch3 (ab23426, Abcam, diluted 1:160), Dll1 (ab10554, Abcam, diluted 1:100), Dll4 (ab7280; Abcam, diluted 1:200), Jagged1 (SC-8303, Santa Cruz Biotechnology, diluted 1:50), Hes1 (ab71559, Abcam, diluted 1:100), Hes2 (ab134685, Abcam, diluted 1:100), Hes5 (ab25374, Abcam, diluted 1:200) and PCNA (ab18197, Abcam,, diluted 1:400) overnight at 4ºC. The negative controls were performed with a polyclonal rabbit IgG (ab27478, Abcam, diluted 1:100), incubated overnight at 4ºC. All primary antibodies were diluted in blocking solution. The peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (Dako 410972, diluted 1:100) was used as secondary antibody.

The above antibodies were previously validated by others in the mouse (anti-Notch1:(Cheng et al., 2007); anti-Notch3: (Feng et al., 2010); anti-Dll1: (Sörensen et al., 2009); anti-Dll4: (Sörensen et al., 2009); anti-Jagged1: (Bielesz et al., 2010); anti-Hes1: (Rahman et al., 2013); and anti-Hes5: (Takanaga et al., 2009)) and rat species (anti-Notch2: (Saravanamuthu et al., 2009)), but predicted to work in mouse. According to the manufacturer, the anti-Hes2 antibody is predicted to work in the mouse.

3.4 Progesterone Assay

Blood samples (1 mL) were collected by cardiac puncture just prior cervical dislocation, into heparin containing eppendorfs, centrifuged and plasma stored at -20ºC until assay. Plasma progesterone concentrations were measured, without extraction, by a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 1000, Siemens Healthcare Diagnostics, Lda., Amadora, Portugal), using a commercial kit (Immulite 1000 Progesterone kit, Siemens). Concentrations of P4 were used as an additional aid to stage the EC.

4 Results

4.1 Notch pathway component and effector genes are differentially expressed during follicle development

Figure 36 shows the expression patterns of Notch component genes during follicle development. As shown, Notch1 is not expressed in follicles (B-D), whereas Notch2 is expressed at all follicle developmental stages (E-F). Notch3 is not expressed in primordial and early primary follicles, but is expressed in granulosa cells of primary (G), pre-antral and

antral follicles and theca cells (I, K; follicle staging by PCNA staining - H, J). In antral follicles Notch3 is mainly expressed in granulosa cells of the antrum border (I), where PCNA staining is strong (J). Dll1 is not expressed in primordial and primary follicles, but is expressed in granulosa cells of secondary follicles (L-M). This ligand completely surrounds the oocyte in pre-antral and antral follicle stages (O; follicle staging by PCNA staining - N, P). Dll4 is expressed at all follicle developmental stages (Q-R). Jagged1 is expressed in granulosa cells from primordial to pre-antral follicles, but is not expressed in large antral follicles (S, T, V; follicle staging by PCNA staining - U). These results are schematically illustrated in Figure 37.

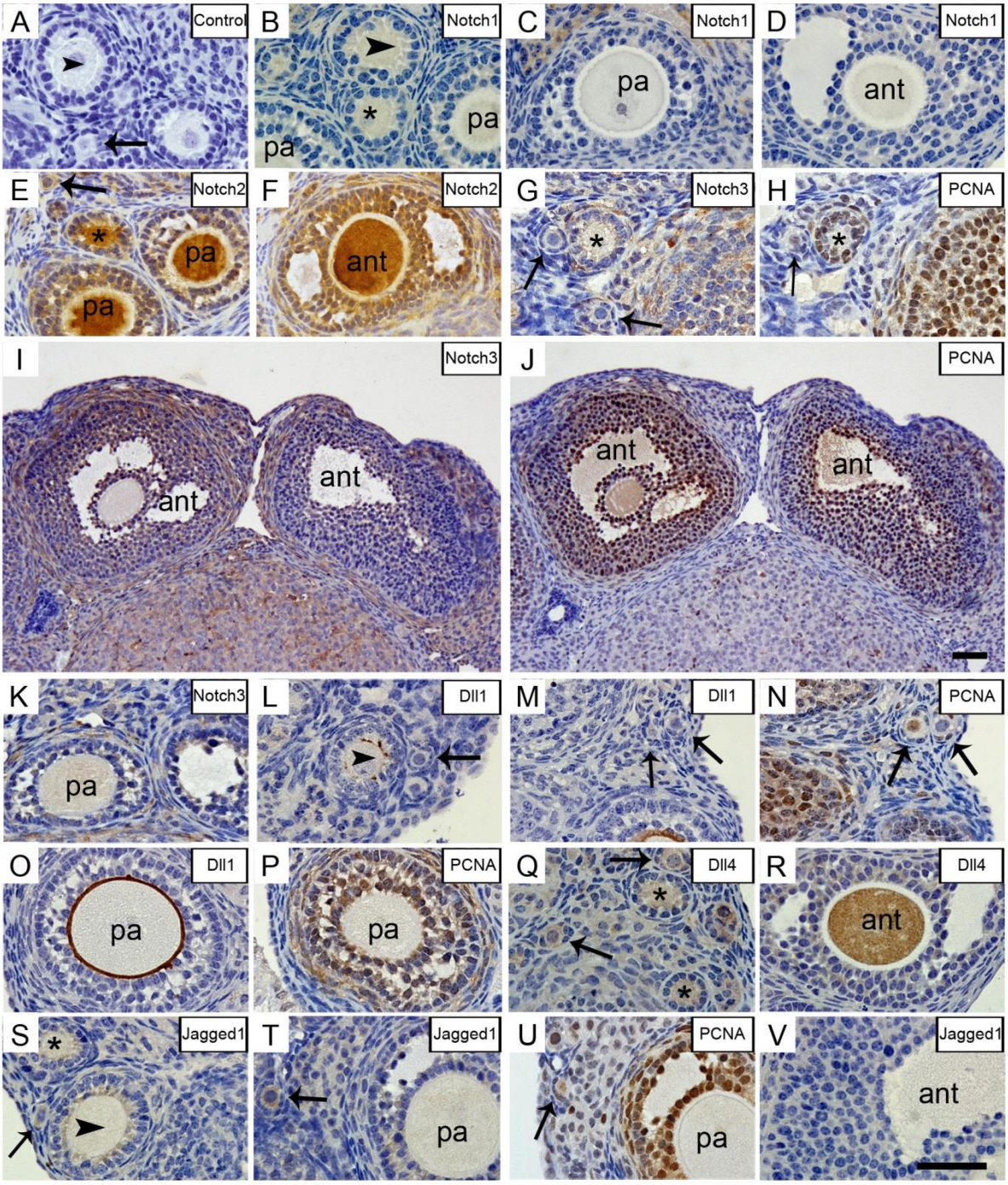
Figure 38 shows the detection of Notch effectors during follicle development. Hes1 is detected in granulosa cells at all follicle stages, except antral follicles (C-H). This effector is also detected in theca cells and in oocytes of developmentally advanced follicle stages (E, H). Hes2 is not detected in follicles (I-K), whereas Hes5 is detected in granulosa cells and oocytes at all follicle developmental stages (L-P).

4.2 Notch pathway component and effector genes are differentially expressed during corpus luteum development.

Figure 39 shows expression patterns of Notch component genes during CL development. Notch1 is expressed in luteal cells at all EC stages (A-D). Notch2 is also expressed in luteal cells at all stages, but staining is increased during diestrus (E-H). Notch3 is expressed in CL capillaries and theca cells at all stages (I-L), but only at estrus in luteal cells, evidencing a strong staining (J). Dll1 is only expressed in small luteal cells during metestrus and diestrus (M-P). Dll4 is expressed in luteal cells at all stages, mainly during proestrus (Q-T). Jagged1 is expressed in a subset of luteal cells at all stages (U-X), but staining is weak at estrus, metaestrus and diestrus (V-X).

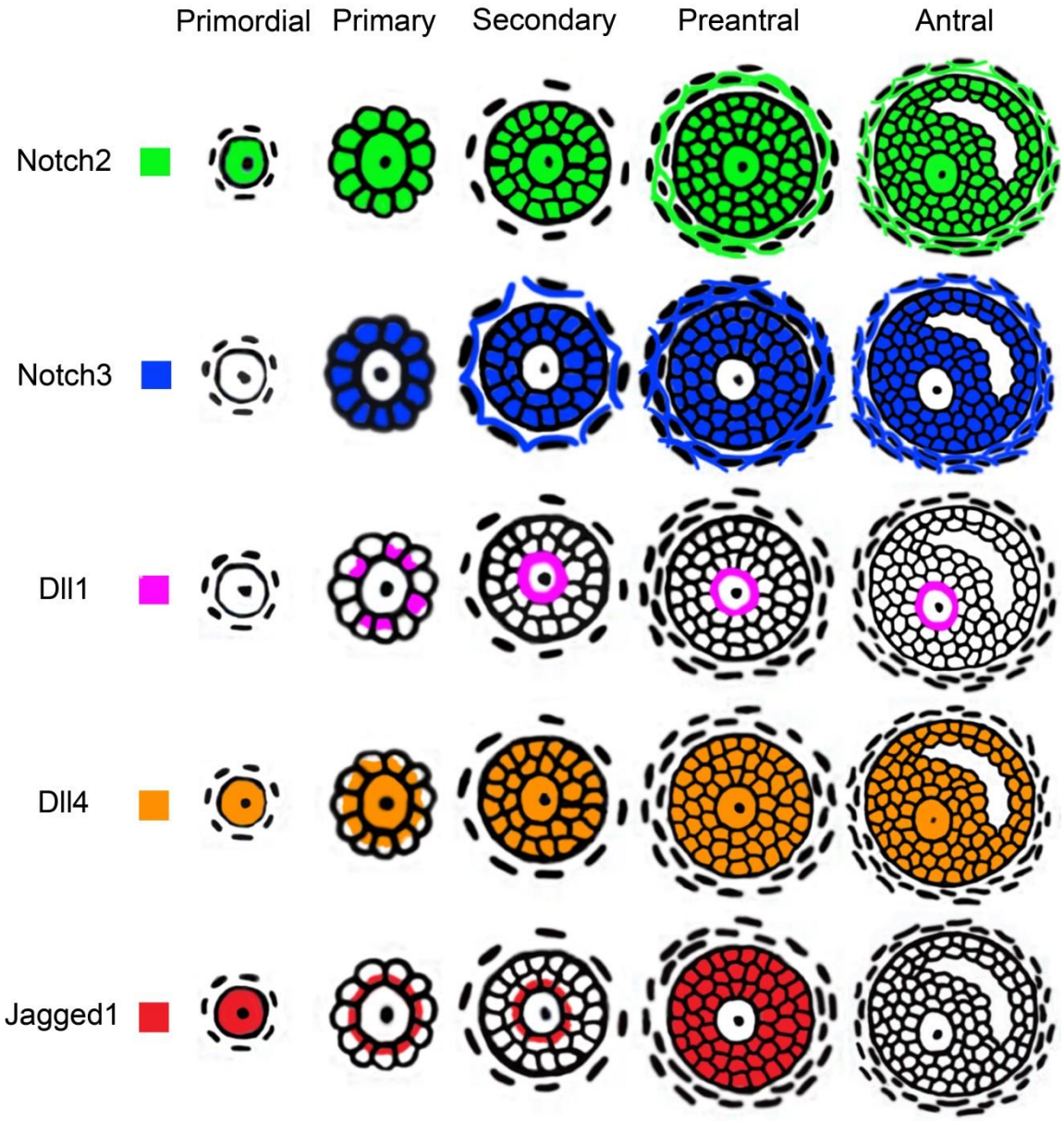
Figure 40 shows the detection of Notch effectors during CL development. Hes1 is detected in luteal cells at all EC stages, evidencing strong staining at metestrus (E-H). Hes2 is only detected in luteal cells at diestrus (I-L). Hes5 is detected in luteal cells at all EC stages, showing a strong staining at proestrus (M-P). Bottom-right corner windows show nuclear detection of Notch effectors at a higher magnification (A-P). PCNA staining was used to confirm CL staging (Q-T).

Figure 36 – Expression patterns of Notch pathway component genes during follicle development



Positive staining in brown, counterstaining with haematoxylin. Magnification 400X (A-H, K-V) or 100X (I, J). Arrows – primordial and early primary follicles; asterisks – primary follicles; Arrow heads – secondary follicles; pa – preantral follicles; ant – antral follicles. Scale bars –50 µm. Negative controls were performed with polyclonal rabbit IgG (A). PCNA in twin slides is used to stage follicle development (Oktay et al., 1995) (H, J, N, P, U). Notch1 is not expressed in follicles (B-D), whereas Notch2 is expressed at all follicle stages (E, F). Notch3 is only not expressed in primordial follicles (G, I, K). Dll1 is expressed in primary follicles, and surrounding the oocyte along follicle development (L, M, O). Dll4 is expressed at all follicle stages (Q, R). Jagged1 is expressed until antrum formation (S, T, V).

Figure 37 – Schematic illustration of expression patterns of Notch pathway components during follicle development

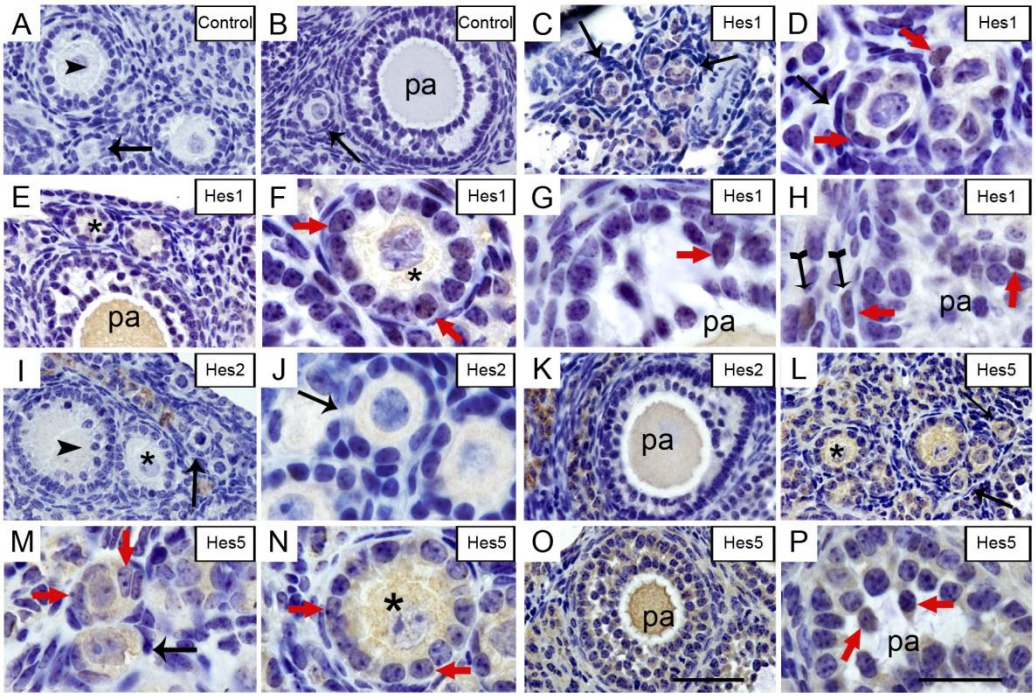


Draw adapted from Orisaka et al. (Orisaka, Tajima, Tsang, & Kotsuji, 2009).

5 Discussion

Here is reported for the first time the dynamic expression of Notch component and effector genes during follicle and CL development along the mouse EC. Although Notch components were already identified in the mouse ovary (Baker & Spears, 1999; Johnson et al., 2001; Vorontchikhina et al., 2005; Hernandez et al., 2011; Zhang et al., 2011; García-Pascual et al., 2013; Jovanovic et al., 2013; Xu & Gridley, 2013), this is the first integrated evaluation of the expression of multiple Notch component and effector genes along the EC, and their association with cellular remodeling events occurring during follicle and CL development.

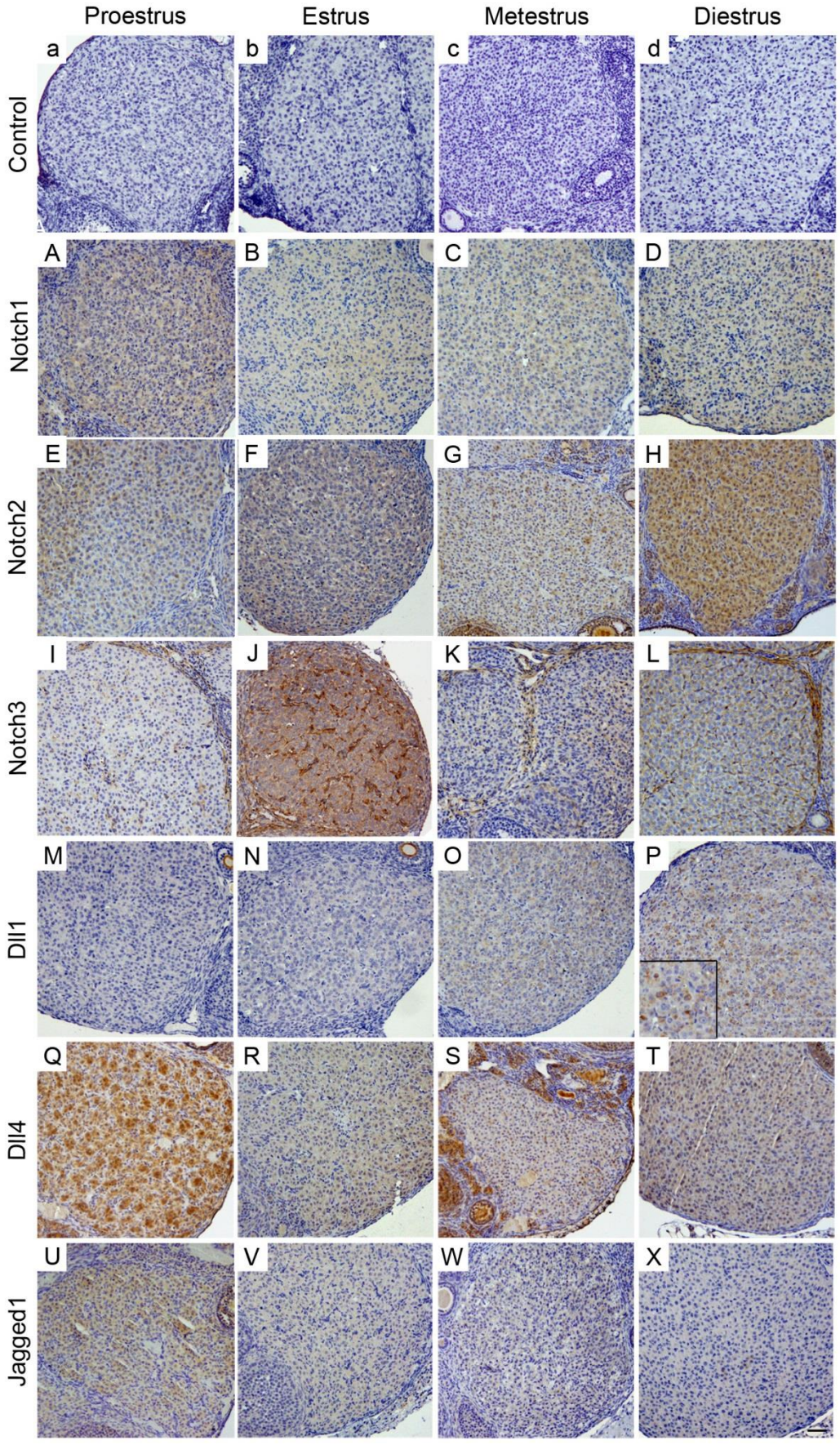
Figure 38 – Detection of Notch pathway effectors during follicle development.



Positive staining in brown, counterstaining with haematoxylin. Magnification 400x (A, B, C, E, I, K, L, O) or 1,000x (D, F, G, H, J, M, N, P). Arrows – primordial and early primary follicles; red arrows - nuclear staining; asterisks – primary follicles; Arrow heads – secondary follicles; tailed arrows – theca cells; pa – preantral follicles. Scale bars – 50 µm. Detection of Notch effector proteins in the nucleus was considered indicative of Notch signaling activation. Negative controls were performed with the polyclonal rabbit IgG (A, B). Hes1 is detected in granulosa cells (C-H), oocytes of advanced stage follicles (E) and theca cells (H). Hes2 is not detected in follicles (I-K). Hes5 is detected in granulosa cells and oocytes of all follicle stages (L-P).

Primordial follicles express Notch2, Dll4, Jagged1, Hes1 and Hes5. Some of these proteins (Notch2, Jagged1 and Hes1) were associated with primordial follicle nest formation and primordial follicle regulation (Trombly et al., 2009; Xu & Gridley, 2013). Additionally, Notch2 was identified in developing follicles (Johnson et al., 2001; Zhang et al., 2011), and its role in granulosa cells' proliferation was demonstrated *in vitro* (Zhang et al., 2011). Notch3 is expressed in follicular theca and granulosa cells of primary and subsequent follicle stages, showing strong expression in antral follicles. In the literature, expression of Notch3 in follicles is controversial. This receptor was only identified in capillary endothelial cells (Jovanovic et al., 2013), or as here reported, also in developing follicles (Johnson et al., 2001). As *Notch3* knockout mice are viable and fertile (Krebs et al., 2003), this receptor may play a redundant role in follicle development. However, Notch3 overexpression is associated with ovarian cancer (Park et al., 2008). Notch1 is not expressed in follicles. This observation is consistent with other studies identifying expression of Notch1 only in capillary endothelial cells (Johnson et al., 2001; Vorontchikhina et al., 2005; Jovanovic et al., 2013).

Figure 39 – Expression patterns of Notch pathway component genes during corpus luteum development.



(Legend in the next page)

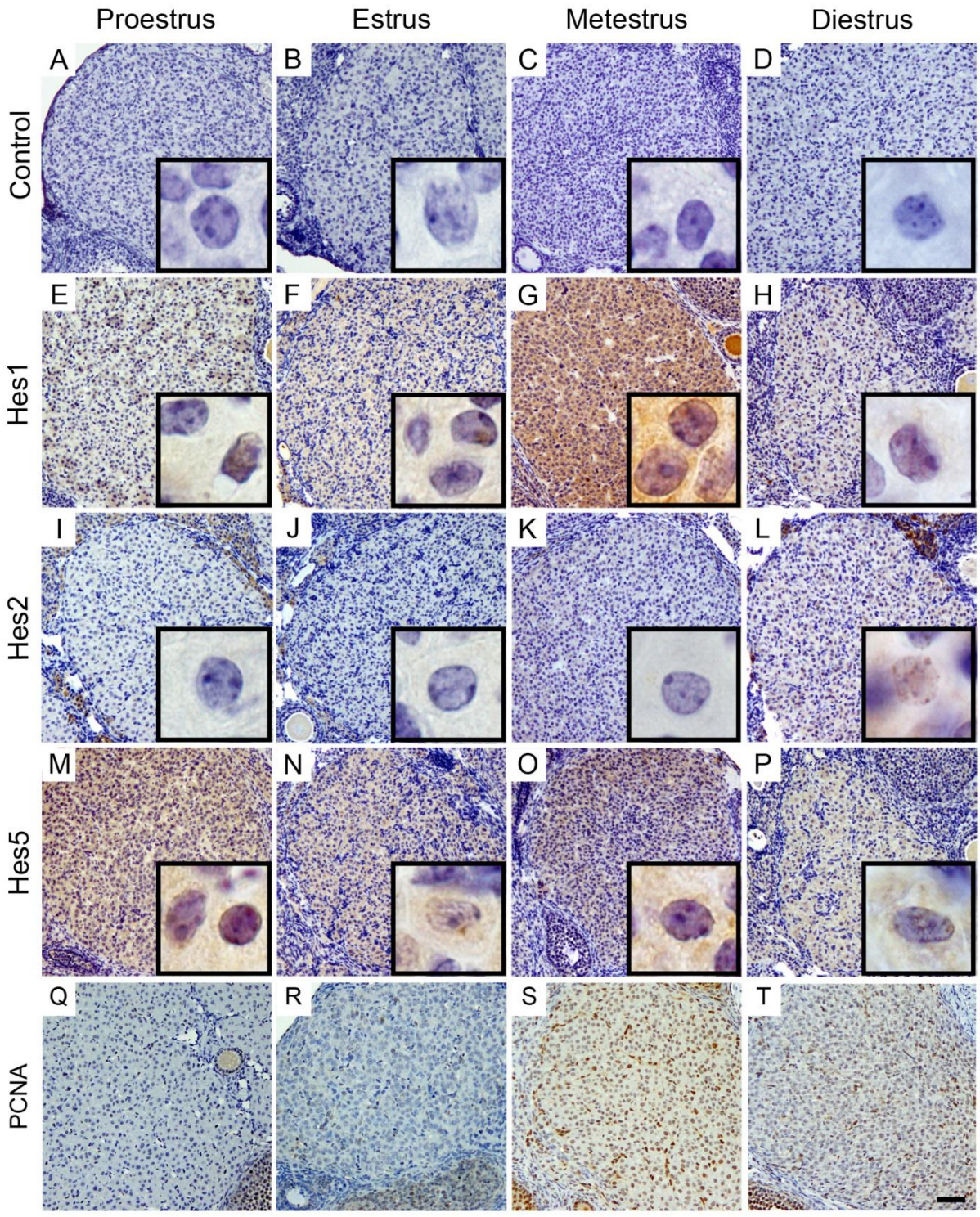
(Figure 39 - Legend) Positive staining in brown, counterstaining with haematoxylin (100x magnification). Scale bar –50 µm. Negative controls were performed with the polyclonal rabbit IgG (a, d). Notch1 (A-D) and Notch2 (E-H) are expressed in luteal cells at all EC stages. Notch3 is expressed in CL endothelial and theca cells at all EC stages (I-L), and in luteal cells during estrus (J). Dll1 is expressed in in small luteal cells during metestrus and diestrus (M-P). Dll4 (Q-T) and Jagged1 (U-X) are expressed in luteal cells at all EC stages.

This is the first study reporting Dll1 expression in the ovary. Dll1 is specifically expressed in a subset of granulosa cells of primary follicles. This observation leads to the suggestion that signaling through Dll1 is regulating follicle development in a canonical Notch signaling mode. This ligand is expressed surrounding the oocyte of secondary follicles and subsequent stages of follicle development, and may play a role in oocyte/granulosa cell signaling. In contrast, Dll4 is ubiquitously expressed during follicle development, and Jagged1 is expressed in primordial and pre-antral follicles. Although Jagged1 was already identified in follicles (Johnson et al., 2001; Zhang et al., 2011), expression of Dll4 and Jagged1 was also reported to occur only in capillary endothelial cells (Vorontchikhina et al., 2005; Jovanovic et al., 2013). Notch effectors are detected in the nucleus of follicular cells, indicating activation of Notch signaling during follicle development. This is in accordance with other studies (Johnson et al., 2001; Trombly et al., 2009). Recently, Hes1 was shown to be necessary to oocyte development and maturation (Manosalva, González, & Kageyama, 2013).

The expression patterns of Notch component and effector genes here described prompt for a regulatory role of Notch signaling in follicle development. Notch2, Dll4 and Jagged1 may be enrolled in primary follicle pool maintenance. Later, receptors Notch2 and Notch3, and ligands Dll1, Dll4 and Jagged1, may be involved in triggering follicle development. Notch2 and Dll4 are always expressed in oocytes and granulosa cells, potentially indicating a role in cell identity. Although Jagged2 expression was not evaluated, this ligand was also associated to follicle development (Johnson et al., 2001).

The CL is the site of extensive cellular remodeling, involving growth, differentiation, and death of granulosa, theca, capillary, and mesenchymal cells. Interactions between these cell types are essential to the steroidogenic competence of the CL (Stocco et al., 2007). In rodents, CL regression occurs in two steps. The early functional regression is associated with a significant decrease in P4 synthesis, and the later structural regression is associated with luteal cells programmed death (Stocco et al., 2007). Regression of previous cycle CL occurs during proestrus (Gaytán et al., 2000). Notch components were identified in CL vasculature (Vorontchikhina et al., 2005) and, more recently, shown to play a regulatory role in CL capillary network establishment (Fraser et al., 2012; García-Pascual et al., 2013). Notch1, Notch4 and Dll4 were also identified in luteal cells during pregnancy in the rat (Hernandez et al., 2011).

Figure 40 – Detection of Notch pathway effectors during corpus luteum development.



Positive staining in brown, counterstaining with haematoxylin (100x magnification – A-T; bottom-right corner windows show nuclear detection at 1,000x magnification – A-P). Negative controls were performed with the polyclonal rabbit IgG (A-D). Scale bar –50 µm.

Detection of Notch effector proteins in the nucleus was considered indicative of Notch signaling activation. Hes1 and Hes5 are detected in luteal cells at all EC stages (E-H and M-P, respectively). Hes2 is only detected in luteal cells during diestrus (I-L). PCNA staining was used to confirm CL stage (Q-T) (Zheng et al., 1994).

We identified active Notch signaling in luteal cells during CL development. To the best knowledge of authors the nuclear detection of Notch effectors in luteal cells is novel. As observed in other tissues, Notch signaling is potentially regulating the cellular remodeling associated with CL development and steroidogenesis. The main changes observed in expression patterns of Notch components in the CL are coincidental with P4 peaking and CL regression. Notch2, Dll1, Hes1 and Hes2 expression are associated with CL increasing steroidogenesis, whereas Notch3, Dll4, Jagged1 and Hes5 are associated with CL regression.

In conclusion, Notch signaling is active during follicle and CL development along the mouse EC. Notch component and effector genes are dynamically and differentially expressed in the ovary along the EC. The spatial and temporal relationship between expression patterns and key cellular events, prompt for a major regulatory role of Notch signaling in follicle and CL development, namely in follicle growth and selection, and CL steroidogenesis and regression.

Chapter V - Dynamics of Notch signaling in the oviduct and uterus along the mouse estrous cycle.

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Submitted to: *Reproduction*

1. Abstract

The oviduct and uterus undergo extensive cellular remodeling along the EC, to establish receptivity for developing embryos. The molecular regulatory mechanisms behind these dynamic cellular events, involving extensive cell-to-cell signaling, are poorly understood. Notch is an evolutionarily conserved cell signaling pathway implicated in cell fate decisions in several tissues. Here, we evaluated the transcription and expression patterns of Notch component and effector genes in the mouse oviduct and uterus along the EC.

Mature CD1 female mice were euthanized at each of the four EC stages. One oviduct and the uterus were processed for IHC and the expression patterns of Notch components (Notch1-3, Jagged1 and Dll1 and Dll4), and effectors (Hes1, Hes2 and Hes5) were evaluated. The other oviduct was processed for RNA extraction and RT-PCR analysis.

All Notch component and effector genes are transcribed in the oviduct. Transcription levels of Notch components and effectors in the oviduct increase from the proliferative to the secretory phase of the EC, and transcription patterns are associated with plasma P4 concentrations along the EC. Notch components (except Dll1), and effectors are expressed in the oviduct, uterine luminal and glandular epithelia. Each component/effector shows a specific expression pattern. Uterine luminal and glandular epithelia show a unique combination of Notch receptors/ligands/effectors expression along the EC. Nuclear detection of Notch effectors indicates that Notch signaling is active. These results prompt for a regulatory role of Notch signaling in oviduct and uterus epithelial function.

Key Words - Notch; epithelium; oviduct; uterus; mouse

2. Introduction

During the EC, the oviduct and uterus undergo extensive cellular remodeling, to allow fertilization and development of embryos. In humans and mice, cyclic cellular proliferation, apoptosis and differentiation, extracellular matrix turnover, angiogenesis and leukocyte infiltration, are regulated by ovarian steroids estradiol and P4. The mouse EC lasts 4–5 days and encompasses four stages (proestrus, estrus, metestrus, and diestrus). Circulating concentrations of E2 peak prior to ovulation, which occurs at estrus, whereas P4 concentrations rise during metestrus and diestrus, and then decline from proestrus to estrus (Wood et al., 2007).

The molecular regulatory mechanisms behind oviduct and uterus' cellular remodeling along the EC are poorly understood. Deciphering these mechanisms could potentially lead to the development of new therapeutic strategies addressed to infertility and contraception. Notch is an evolutionarily conserved cell signaling pathway implicated in cell fate decisions in several tissues (Artavanis-Tsakonas et al., 1995)(Borggrefe & Oswald, 2009). In mammals, four receptors (Notch1-4) and five ligands (three delta-like - Dll1, Dll3 and Dll4 - and two serrate-like - Jagged1 and Jagged2) were identified (Borggrefe & Oswald, 2009). Notch signaling is activated by binding of the extracellular domain of receptors with ligands expressed on neighboring cells. This leads to cleavage of Notch intracellular domain by a γ-secretase and its translocation to the nucleus, where it associates with other transcriptional factors, thus regulating transcription of Notch effector target genes (Borggrefe & Oswald, 2009). Only a limited set of Notch effector genes were identified, being the hairy/enhancer of split genes the most ubiquitous (Fischer & Gessler, 2007).

Notch components were detected in the mammalian uterus (Cobellis et al., 2008; Mazella et al., 2008; Mikhailik et al., 2009; Afshar, et al., 2012a; Afshar, et al., 2012b; Degaki et al., 2012) and Notch1 was reported as a relevant regulator of uterine function (Afshar, et al., 2012a; Afshar, et al., 2012b). However, the potential involvement of Notch signaling in the regulation of cyclic oviduct and uterine cellular changes occurring along the EC is unknown. Our main hypothesis is that Notch signaling is a major regulator of oviduct and uterine epithelial cyclic changes. As a first approach to test this hypothesis, we evaluated the transcription and expression patterns of Notch component and effector genes in the oviduct and uterus of mice along the EC. Results indicate that Notch signaling is active, and prompt for a major regulatory role of Notch signaling in oviduct and uterine epithelial function.

3. Materials and Methods

3.1. Animals

Experiments were conducted in compliance with the Portuguese legislation for the use of animals for experimental purposes (Decreto-Lei nº 129/92 and Portaria nº 1005/92, DR nº 245, série I-B, 4930-42) and with the European Union legislation (Directive n. 86/609/EEC, from the 24th November 1986). Experimental protocols were approved by the national regulatory agency (DGAV – Direção Geral de Alimentação e Veterinária) and the Institutional Animal Care and Use Committee (CEBEA – Comissão de Ética e Bem-Estar Animal). All authors are accredited as FELASA category C scientists or equivalent.

CD1 female mice with 3 months of age were maintained in a 12-hour light/dark cycle, in ventilated cages with corn cob as bedding, and were given access to standard laboratory diet and water *ad libitum*. The mice health was routinely monitored. Blood samples (1 mL) were collected by intracardiac puncture under ketamine (15mg/kg)/xylazine (1mg/kg) anesthesia, just before euthanasia through cervical dislocation followed by exsanguination.

3.2. Experimental Design

Female EC was monitored by cytological evaluation of vaginal smears. Females were euthanized at each of the EC stages (proestrus, estrus, metestrus, diestrus) and one oviduct and the uterus collected and processed for IHC. Paraffin embedded sections were stained with haematoxylin, and the EC stage confirmed by histological analysis (Wood et al., 2007). The other oviduct was processed for RNA extraction and RT-PCR analysis. Blood samples were processed for measurement of plasma P4 concentrations by a chemiluminescence assay. Only mice undergoing normal estrous cycles, with expected vaginal cytology, uterus histology and P4 concentrations for each EC stage, were included in the study. Overall, 20 female mice (5 per each EC stage) were used.

3.3. Immunohistochemistry

Oviduct and uterus samples were fixed in 4% neutral phosphate buffered formalin at room temperature for 24 hours and, after subsequent dehydration in ethanol, tissues were embedded in paraffin. Spatial localization of expression of Notch pathway components (Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1) and effectors (Hes1, Hes2 and Hes5) was evaluated through IHC, as previously described (Silva et al., 2010). The antigen retrieval step was performed in citrate buffer (10mM, pH 6.0), except for the anti-Notch1, anti-Hes1, anti-Hes2 and anti-Hes5 antibodies (Tris-EDTA, pH 9.0). Blocking was performed in PBS with 2% w/v bovine serum albumin (A7906, Sigma-Aldrich, Inc.) for one hour at room temperature. Sequential sections of 3 µm were incubated with primary antibodies for Notch1 (ab27526, Abcam, diluted 1:100), Notch2 (ab8926, Abcam, diluted 1:100), Notch3 (ab23426, Abcam, diluted 1:160), Dll1 (ab10554, Abcam, diluted 1:100), Dll4 (ab7280; Abcam, diluted 1:200), Jagged1 (SC-8303, Santacruz Biotechnology, diluted 1:50), Hes1 (ab71559, Abcam, diluted

1:100), Hes2 (ab134685, Abcam, diluted 1:100) and Hes5 (ab25374, Abcam, diluted 1:200) overnight at 4ºC. The negative controls were performed with the polyclonal rabbit IgG (ab27478, abcam, diluted 1:100), incubated overnight at 4ºC. All primary antibodies were diluted in blocking solution. The peroxidase conjugated goat anti-rabbit IgG polyclonal antibody (Dako 410972, diluted 1:100) was used as secondary antibody. Counterstaining was done with haematoxylin. Staining was evaluated in the entire slice.

The antibodies against Notch components and effectors were previously validated by others in the mouse (anti-Notch1(Cheng et al., 2007), anti-Notch3 (Feng et al., 2010), anti-Dll1, (Sörensen et al., 2009) anti-Dll4, (Sörensen et al., 2009), anti-Jagged1 (Bielesz et al., 2010), anti-Hes1 (Rahman et al., 2013) and anti-Hes5 (Takanaga et al., 2009)) and rat species (anti-Notch2 (Saravanamuthu et al., 2009)), but predicted to work in mouse. According to the manufacturer the anti-Hes2 antibody reacts with mouse.

3.4. Quantitative Gene Transcription Analysis (qRT-PCR).

Oviducts were immediately frozen in liquid nitrogen and stored at −80 ºC. RNA extraction, cDNA synthesis and mRNA transcription was performed as previously described (Silva et al., 2012). Quantification of *Notch1, Notch2, Notch3, Dll1, Dll4, Jagged1, Hes1, Hes2*, *Hes5* and *Nrarp* transcripts was done using selected primers (pair sequences are available in Annex I). Transcription of gene *RPS29* was used as an endogenous control. Real-time PCR was performed in duplicate wells on StepOnePlus™ (Applied Biosystems, Foster City, CA, USA). All PCR reactions were carried out in 96-well optical reaction plates (Applied Biosystems,Warrington, UK) with 6.25µl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), 2.5ng of diluted cDNA, 80 nM of each primer in a total reaction volume of 12µl. Relative transcription levels were obtained for each animal and for each gene, comparing transcription levels at each EC stage with proestrus stage mean. Mean transcription levels were then calculated for each gene at each EC stage.

3.5. Progesterone Assay

Blood samples (1 mL) were collected by cardiac puncture just prior cervical dislocation, into heparin containing eppendorfs, centrifuged and plasma stored at -20ºC until assay. Plasma P4 concentrations were measured, without extraction, by a solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite 1000, Siemens Healthcare Diagnostics, Lda., Amadora, Portugal), using a commercial kit (Immulite 1000 Progesterone kit, Siemens). The inter-assay coefficients of variation were 9,7, 9.2% and 6.9% for three different controls used in the assay (for concentrations of 1.8, 3.4 and 15.3 ngmL−1, respectively; Multivalent Control Module, Siemens Healthcare Diagnostics). The intra-assay coefficient of variation was 5.5%. Mean plasma P4 concentrations were (mean ± SD; ng/mL) 5.4 ± 0.6, 3.9 ± 0.6, 6.3 ± 1.2 and 14.7 ± 5.1 for proestrus, estrus, metestrus and diestrus, respectively.

3.6. Statistical Analysis

Data analysis was performed with the SPSS version 19.0 statistic software. Linear associations between gene transcription levels in the oviduct and plasma P4 concentrations were analyzed through Pearson's correlation coefficient. Significance was determined at the 5% confidence level ($p< 0.05$).

4. Results

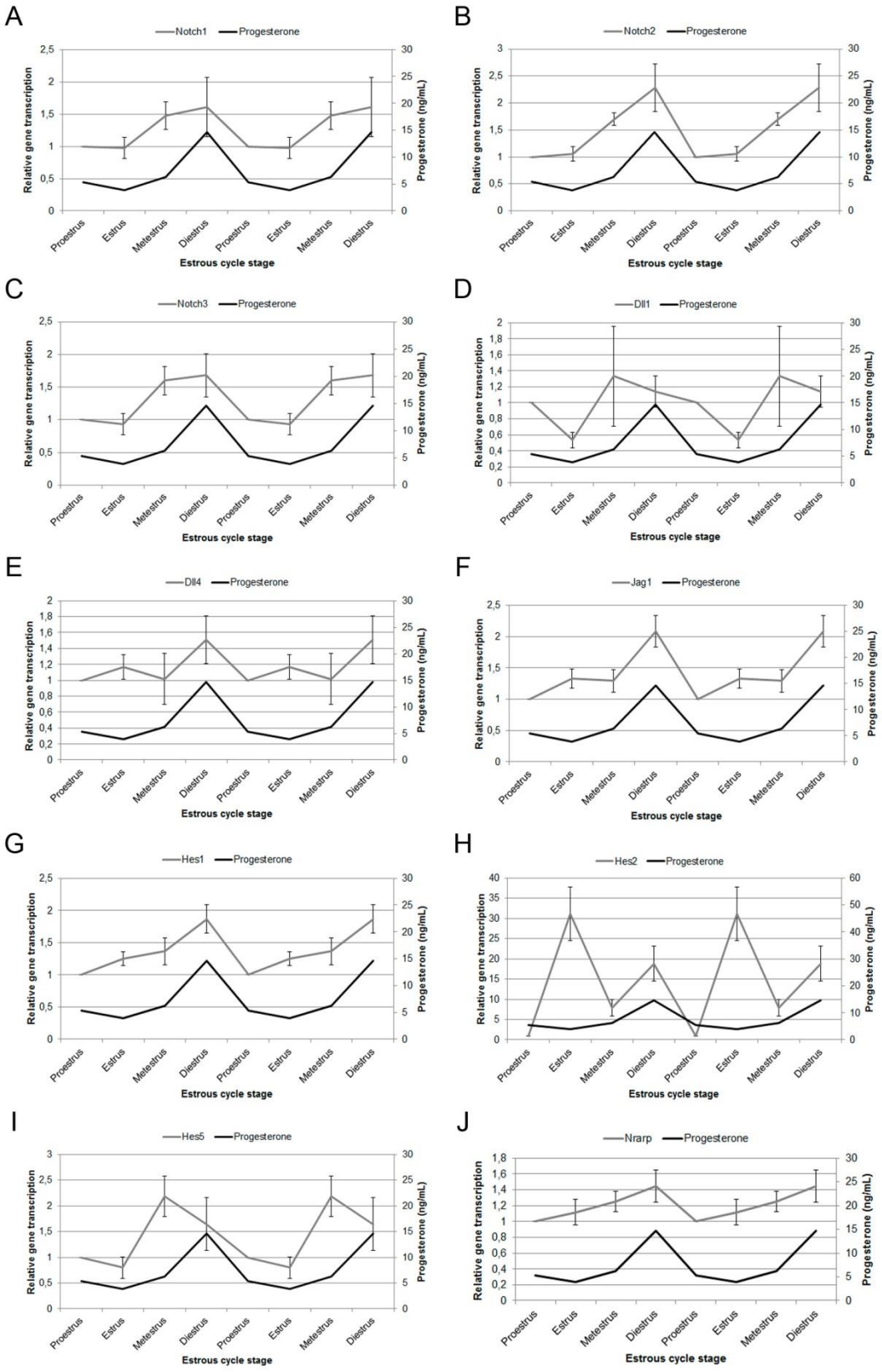
4.1. Notch pathway component and effector genes are differentially transcribed in the oviduct along the estrous cycle

Figure 41 shows the association between oviduct gene transcription and plasma P4 profiles along the EC. To ease interpretation, each graph illustrates a duplication of the EC. As shown, Notch receptors and ligands *Dll4* and *Jagged1* genes (A-C and E-F, respectively) increase transcription along the EC, from the proliferative to the secretory phases of the EC, following the pattern of plasma P4 concentrations. Ligand gene *Dll1* has low transcription levels along the EC, although shows a mild increase in transcription during metestrus (D). Transcription of Notch effector genes *Hes1*, *Hes5* and *Nrarp* (G, I and J, respectively) also increase along the EC, following the pattern of plasma P4 concentrations, although transcription of *Hes5* peak in metestrus.

4.2. Oviduct gene transcription of Notch pathway components and effectors are correlated, and correlate with plasma P4 concentrations along the EC

Table 2 shows results of the Pearson's correlation coefficient analysis between transcription levels of Notch components and effectors, and plasma P4 concentrations. As shown significant correlations are observed between transcription levels of Notch receptors, ligands and effectors, and between those and plasma P4 concentrations and EC stage.

Figure 41 – Association between plasma P4 concentrations and transcription levels of Notch pathway component and effector genes in the oviduct along the estrous cycle.



Transcription of each gene in each EC stage is compared with mean proestrus transcription. *RPS29* gene was used as endogenous control. Error bars represent the standard error of the mean (SEM).

Table 2 – Pearson's correlation coefficient analysis between transcription levels of Notch pathway component and effector genes, and estrous cycle stage and plasma P4 concentrations.

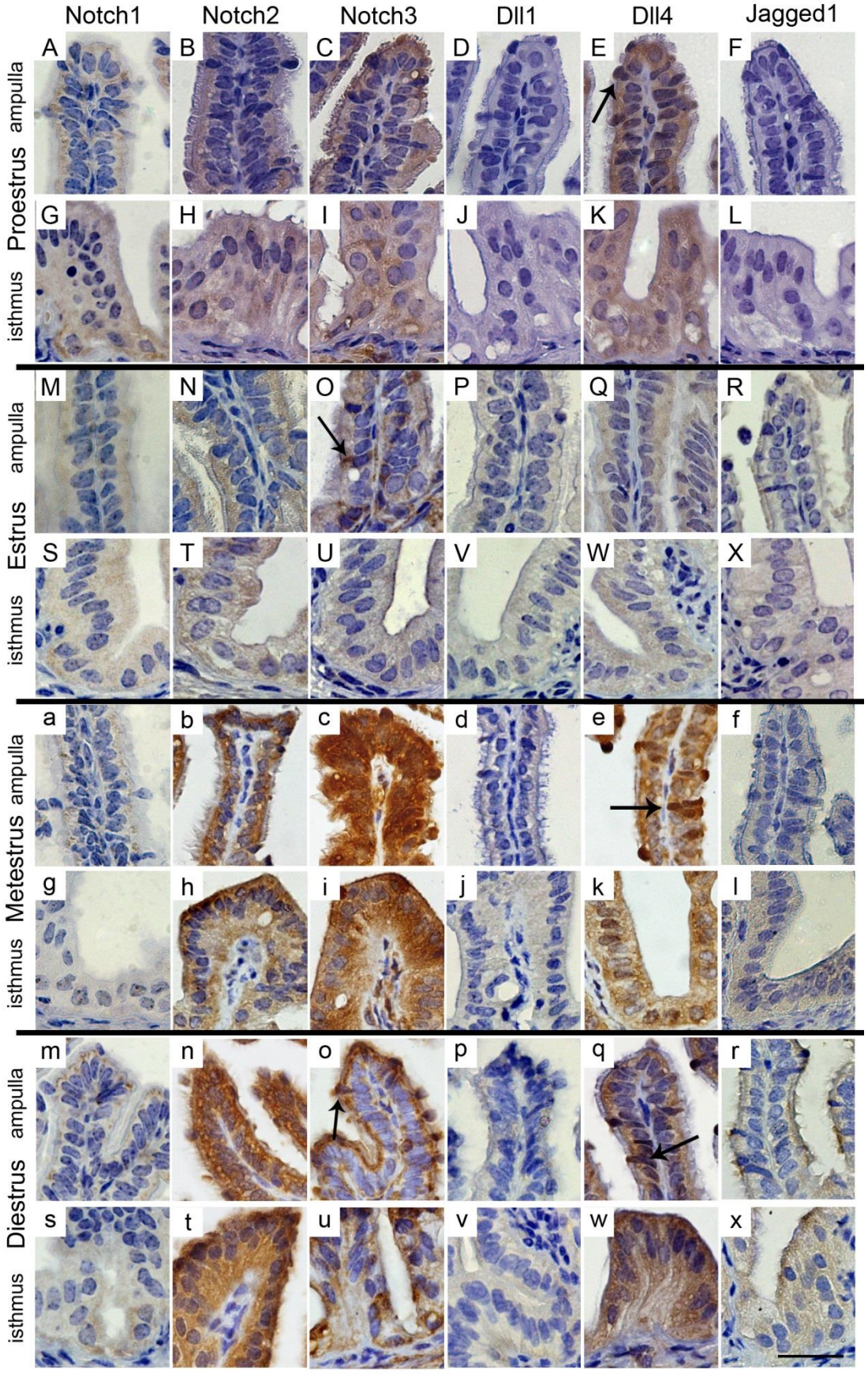
Correlations													
		P4	EC stage	*Notch1*	*Nocth2*	*Notch3*	*Dll1*	*Dll4*	*Jag1*	*Hes1*	*Hes2*	*Hes5*	*Nrarp*
P4	r	1,00	,625*	-0,10	0,23	0,12	0,06	0,31	,457*	0,19	0,06	-0,06	0,13
	p		0,02	0,68	0,36	0,62	0,81	0,20	0,05	0,43	0,82	0,82	0,60
EC stage	r	,625*	1,00	0,38	,669**	0,51*	0,30	0,24	,575*	,552*	-0,38	0,36	0,39
	p	0,02		0,16	0,01	0,05	0,29	0,38	0,02	0,03	0,17	0,19	0,17
Notch1	r	-0,10	0,38	1,00	,749**	,847**	0,18	-0,30	,462*	,815**	0,06	,784**	,690**
	p	0,68	0,16		0,00	0,00	0,46	0,20	0,04	0,00	0,81	0,00	0,00
Notch2	r	0,23	,669**	,749**	1,00	,792**	0,19	-0,04	,788**	,790**	0,21	,568*	,767**
	p	0,36	0,01	0,00		0,00	0,44	0,88	0,00	0,00	0,40	0,01	0,00
Notch3	r	0,12	0,51*	,847**	,792**	1,00	0,25	-0,08	,503*	,837**	0,07	,694**	,887**
	p	0,62	0,05	0,00	0,00		0,30	0,75	0,02	0,00	0,76	0,00	0,00
Dll1	r	0,06	0,30	0,18	0,19	0,25	1,00	-0,09	0,09	0,03	-0,27	,599**	0,22
	p	0,81	0,29	0,46	0,44	0,30		0,70	0,70	0,90	0,26	0,01	0,37
Dll4	r	0,31	0,24	-0,30	-0,04	-0,08	-0,09	1,00	0,34	0,12	0,04	-0,26	0,04
	p	0,20	0,38	0,20	0,88	0,75	0,70		0,14	0,62	0,89	0,28	0,88
Jag1	r	,457*	,575*	,462*	,788**	,503*	0,09	0,34	1,00	,648**	0,29	0,39	,534*
	p	0,05	0,02	0,04	0,00	0,02	0,70	0,14		0,00	0,23	0,09	0,02
Hes1	r	0,19	,552*	,815**	,790**	,837**	0,03	0,12	,648**	1,00	0,29	,501*	,753**
	p	0,43	0,03	0,00	0,00	0,00	0,90	0,62	0,00		0,23	0,02	0,00
Hes2	r	0,06	-0,38	0,06	0,21	0,07	-0,27	0,04	0,29	0,29	1,00	-0,22	0,34
	p	0,82	0,17	0,81	0,40	0,76	0,26	0,89	0,23	0,23		0,36	0,17
Hes5	r	-0,06	0,36	,784**	,568*	,694**	,599**	-0,26	0,39	,501*	-0,22	1,00	,473*
	p	0,82	0,19	0,00	0,01	0,00	0,01	0,28	0,09	0,02	0,36		0,04
Nrarp	r	0,13	0,39	,690**	,767**	,887**	0,22	0,04	,534*	,753**	0,34	,473*	1,00
	p	0,60	0,17	0,00	0,00	0,00	0,37	0,88	0,02	0,00	0,17	0,04	

Significant associations in grey. Jag1 – Jagged1; r – Pearson's correlation coefficient; EC – estrous cycle; * $p < 0.05$; ** $p < 0.01$;

4.3. Notch pathway component and effector genes are differentially expressed in oviduct epithelial cells along the estrous cycle

Figure 42 shows the expression patterns of Notch components in oviduct (isthmus and ampulla) epithelial cells along the EC. Below follows a description of most notorious features. Notch1 is expressed in the ampulla along the EC (A, M, a, m); in the isthmus is expressed in the epithelium cripts, and is not expressed in metestrus (G, S, g, s). Notch2 and Notch3 are expressed in the ampulla and isthmus along the EC (B, H, N, T, b, h, n, t and C, I, O, U, c, i, o, u, respectively); expression of Notch2 is increased during metestrus and diestrus (b, h, n, t) and Notch3 is mainly expressed in ampulla secretory cells during estrus and diestrus (O, o).

Figure 42 – Notch pathway component genes are differentially expressed in the oviduct epithelium along the estrous cycle.



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(Figure 42 – Legend) Positive staining in brown, counterstaining with haematoxylin (400x magnification). Expression is analyzed in two oviduct segments: ampulla and isthmus. Arrows indicate ampulla secretory cells. Scale bar –50 µm. Notch1 is expressed in the ampulla along the EC (A, M, a, m), and in the isthmus epithelium of the cripts, except at metestrus (G, S, g, s). Notch2 is expressed along the entire oviduct and EC (B, H, N, T, b, h, n, t), although expression is increased during metestrus and diestrus (b, h, n, t). Notch3 follows a similar expression pattern to Notch2 (C, I, O, U, c, I, o, u). Dll1 is not expressed in the oviduct epithelium (D, J, P, V, d, j, p, v). Dll4 is expressed along the EC, although with low expression during estrus (E, K, Q, W, e, k, q, w). Jagged1 is not expressed during proestrus, and expression increases along the EC (F, L, R, X, f, l, r, x)

Dll1 is not expressed in the oviduct (D, J, P, V, d, j, p, v). Dll4 has low expression during estrus, and increases expression, mainly in ampulla secretory cells during metestrus and diestrus (E, K, Q, W, e, k, q, w). Jagged1 is not expressed during proestrus, and increases expression along the EC (F, L, R, X, f, l ,r, x).

Figure 43 shows the detection of Notch effectors in the oviduct along the EC. Hes1 and Hes5 are detected in the oviduct along the EC (B, F, J, N, b, f, j, n, and D, H, L, P, d, h, l, p, respectively), whereas Hes2 is only detected during estrus and diestrus (C, G, K, O, c, g, k, o). Nuclear detection of Notch effectors indicates that Notch signaling is active in the oviduct epithelium.

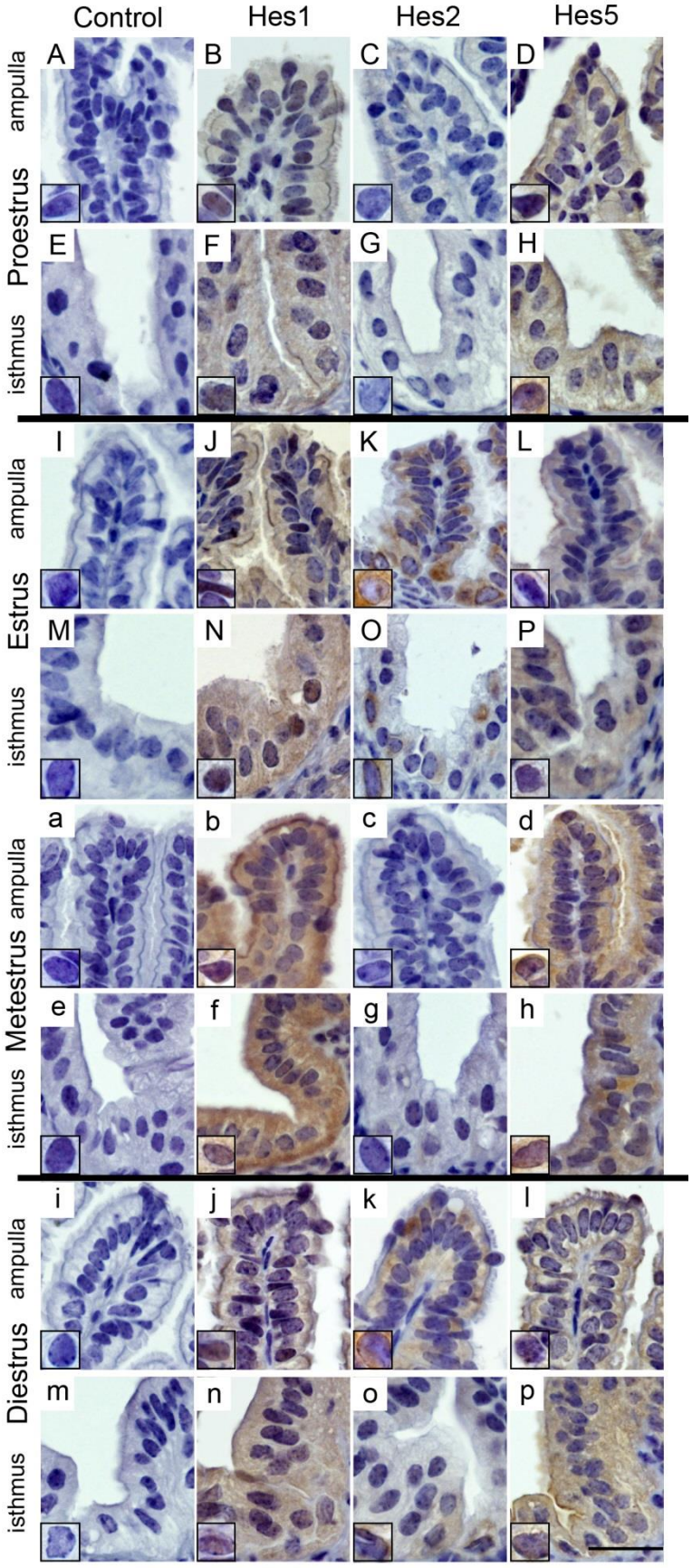
4.4. Notch pathway component and effector genes are differentially expressed in uterus luminal and glandular epithelial cells along the estrous cycle

Figure 44 shows the expression patterns of Notch components in the uterus epithelium (luminal and glandular) along the EC. Below follows a description of most notorious features.

In the luminal epithelium, Notch1 is expressed at proestrus and diestrus (A, G, M, S), Notch2 and Notch3 are expressed throughout the EC (B, H, N, T and C, I, O, U, respectively), Dll1 is expressed during proestrus and diestrus (D, J, P, V), Dll4 is expressed throughout the EC (E, K, Q, W), and Jagged1 is not expressed during estrus (F, L, R, X).

In the glandular epithelium, Notch1 has a low expression during proestrus and estrus and is not expressed during metestrus and diestrus (a, g, m, s); Notch2 is expressed along the EC (b, h, n, t), whereas Notch3 is not expressed during estrus (c, i, o, u); Dll1 is only expressed during proestrus (d, j, p, v), Dll4 is expressed throughout the EC (e, k, q, w), and Jagged1 is not expressed during estrus (f, l, r, x).

Figure 43 – Detection of Notch pathway effectors in the oviduct epithelium along the estrous cycle.



(Legend in the next page)

(Figure 43 – Legend) Positive staining in brown, counterstaining with haematoxylin (400x magnification; bottom-left windows show nuclear detection at 1,000x magnification). Only nuclear detection of Notch effectors is indicative of active Notch signaling. Expression is analyzed in two oviduct segments: ampulla and isthmus. Arrows indicate ampulla secretory cells. Scale bar – 50 µm. Negative controls were performed with the polyclonal rabbit IgG (A, E, I, M, a, e, i, m). Hes1 is detected along the EC, showing increased detection during metestrus (B, F, J, N, b, f, j, n). Hes2 is only detected during estrus and diestrus, in the ampulla and in the isthmus cripts (C, G, K, O, c, g, k, o). Hes5 is detected along the EC but decrease expression during estrus (D, H, L, P, d, h, l, p).

Figure 45 shows the detection of Notch effectors in uterus luminal and glandular epithelia along the EC. In the luminal epithelium, Hes1 and Hes5 are detected throughout the EC; however, during proestrus and diestrus Hes1 has a low detection (B, F, J, N), and Hes5 is only detected in the nucleus of epithelial cells during proestrus (D, H, L, P); Hes2 is not detected in the uterus luminal epithelium during the EC (C, G, K, O).

In the glandular epithelium, Hes1 and Hes5 are detected along the EC. As in the luminal epithelium, Hes1 has a low detection during diestrus (b, f, j, n), and Hes5 is only unequivocally detected in the nucleus of epithelial cells during proestrus, (d, h, l, p); Hes2 is only detected during diestrus (c, g, k, o).

As observed in the oviduct, nuclear detection of Notch effector genes indicates that Notch signaling is also active in the uterus luminal and glandular epithelia.

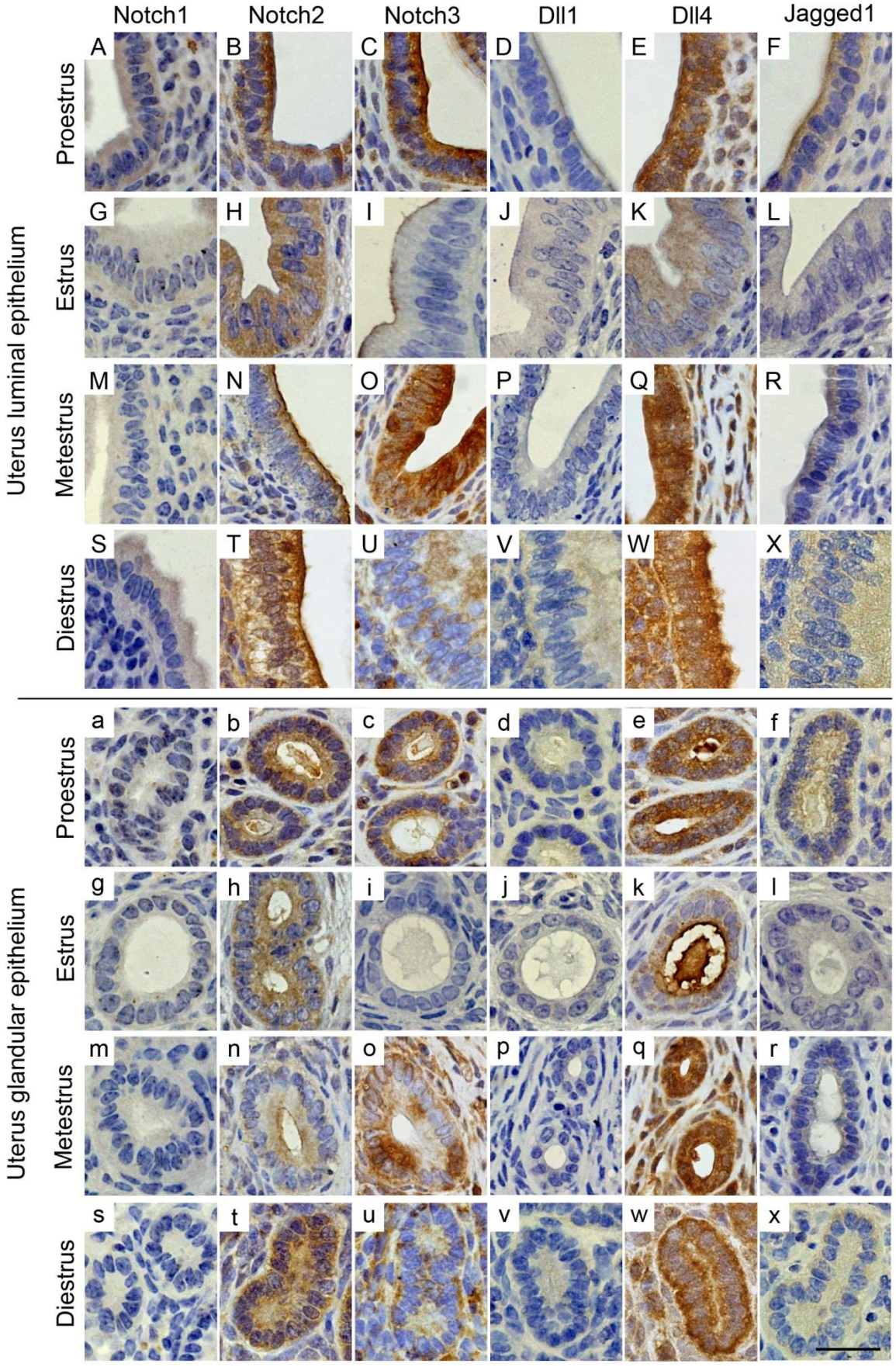
4.5. Uterine luminal and glandular epithelia show unique combinations of Notch receptor/ligand/effector expression along the estrous cycle

This information is summarized in Table 3. As can be depicted, uterus luminal and glandular epithelial Notch receptor/ligand/effector expression combinations are specific of each stage of the EC.

4.6. Notch pathway component and effector genes are expressed in uterus stroma cells along the estrous cycle, and Notch2 and Notch3 are expressed in endometrial neutrophils at metestrus

Figure 46 shows the expression patterns of Notch pathway component and effector genes in uterus stroma cells along the EC. As shown, all Notch components are expressed in stroma cells along the EC (B-G). Notch 2 and Notch3 are specifically expressed in endometrium neutrophils during metestrus (C and D). Notch effectors Hes1 and Hes5 are detected in the stroma cells along the EC (H and J), whereas Hes2 is not detected in the stroma (I). Again, nuclear detection of Notch effector genes indicates that Notch signaling is active in uterus stroma cells.

Figure 44 – Notch pathway component genes are differentially expressed in uterus luminal and glandular epithelia along the estrous cycle.



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(Figure 44 – Legend) Positive staining in brown, counterstaining with haematoxylin (400x magnification). Expression is evaluated in luminal (A-X) and glandular (a-x) epithelia. Scale bar – 50 µm. Luminal epithelium: Notch1 is expressed during proestrus (A) and diestrus (S), but not during estrus (G) and metestrus (M). Notch2 is expressed along the EC (B, H, N, T). Notch3 is expressed along the EC (C, I, O, U), but with weak expression during estrus (I). Dll1 is not expressed during estrus (J) and metestrus (P), and has a weak expression during proestrus (D) and diestrus (V). Dll4 is expressed along the EC (E, K, Q, W). Jagged1 is expressed during proestrus (F), metestrus (R), and diestrus (X), but not during estrus (L).
Glandular epithelium: Notch1 shows low expression during proestrus (a) and estrus (g) and is not expressed during metestrus (m) and diestrus (s). Notch2 is expressed along the EC (d, h, n, t). Notch3 is expressed during proestrus (c), metestrus (o), and diestrus (u), but not during estrus (i). Dll1 shows a very weak expression during proestrus (d) and is not expressed during the remaining EC stages (j, p, v). Dll4 is expressed along the EC (e, k, q, w). Jagged1 is expressed during proestrus (f), metestrus (r) and diestrus (x) but not during estrus (l).

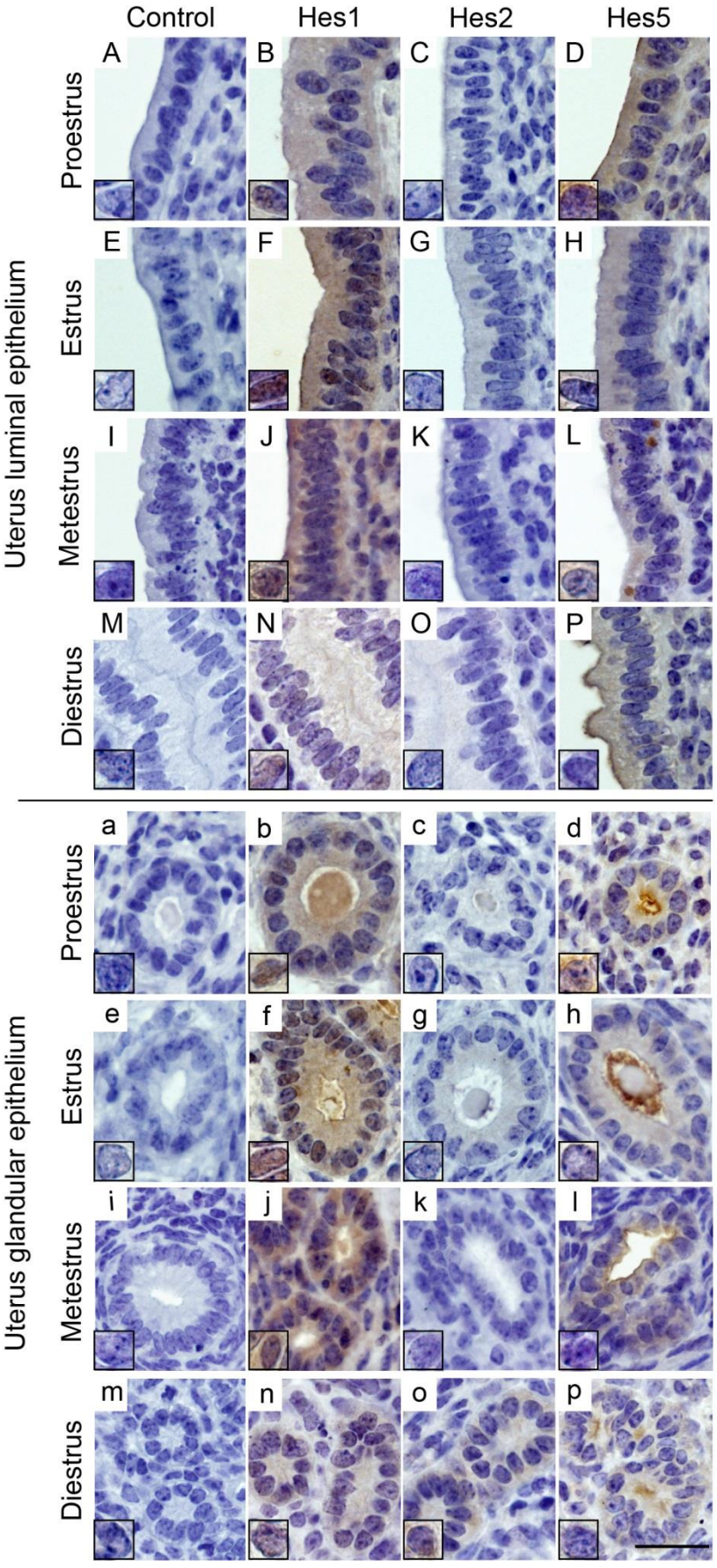
5. Discussion

Here is described for the first time the transcription and dynamic expression patterns of Notch pathway component and effector genes in oviduct and uterus epithelia, and uterus stroma, along the mouse EC. Although some Notch components were identified in the human (Cobellis et al., 2008; Mazella et al., 2008; Mikhailik et al., 2009; Afshar, et al., 2012b) and mouse (Afshar, et al., 2012a; Degaki et al., 2012) uterus, this study is the first to provide an integrated spatial and temporal characterization of epithelial Notch signaling in the oviduct and uterus.

Oviduct luminal ciliated and secretory epithelial cells undergo cyclic changes during the EC (Myoungkun & Bridges, 2011). These epithelial changes require a finely tuned cell signaling and a well-orchestrated gene expression. Notch cell signaling pathway is a major regulator of cell fate decisions in several tissues (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009). Notch pathway component and effector genes are differentially transcribed and expressed in the oviduct along the EC, and nuclear detection of Notch effectors indicates that Notch signaling is active in the oviduct epithelium. This prompts for a regulatory role of Notch signaling in oviduct epithelial function.

As expected EC stage is significantly correlated with plasma P4 concentrations. Notch component and effector genes (except *Hes2*) increase oviduct transcription levels from the proliferative (proestrus and estrus) to the secretory (metestrus and diestrus) stage of the EC, following the profile of plasma P4 concentrations. Significant positive correlations are observed between EC stage and *Notch2*, *Notch3*, *Jagged1* and *Hes1* oviduct transcription levels, and *Jagged1* oviduct transcription levels are significantly correlated with plasma P4 concentrations. These observations point to a P4 regulation of Notch signaling in the oviduct.

Figure 45 – Detection of Notch effectors in uterus luminal and glandular epithelia along the estrous cycle.



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(Figure 45 – Legend) Positive staining in brown, counterstaining with haematoxylin (400x magnification; bottom-left windows show nuclear detection at 1,000x magnification). Only nuclear detection of Notch effectors is indicative of active Notch signaling. Expression is evaluated in luminal and glandular epithelia. Negative controls were performed with the polyclonal rabbit IgG (A, E, I, M, a, e, i, m). Scale bar – 50 µm.

Luminal epithelium: Hes1 is detected during proestrus, estrus and metestrus (B, F, J), and has low detection during diestrus (N). Hes2 is not detected during the whole EC (C, G, K, O). Hes5 is only detected during proestrus (D, H, L, P).

Glandular epithelium: Hes1 is detected along the EC (b, f, j, n), but has a low detection during diestrus (n). Hes2 is only detected during diestrus (c, g, k, o). Hes5 is detected during proestrus, metestrus and diestrus, although nuclear detection only occurs during proestrus (d, h, l, p).

Table 3 – Estrous cycle stage specific expression combinations of Notch pathway component and effector genes in uterus luminal and glandular epithelia.

	Proteins	Proestrus	Estrus	Metestrus	Diestrus
Uterus luminal epithelium	Receptors	N1/N2/N3	N2/N3	N2/N3	N1/N2/N3
	Ligands	D1/D4/J1	D4	D4/J1	D1/D4/J1
	Effectors	H1/H5	H1	H1	H1
Uterine glandular epithelium	Receptors	N1/N2/N3	N1/N2	N2/N3	N2/N3
	Ligands	D1/D4/J1	D4	D4/J1	D4/J1
	Effectors	H1/H5	H1	H1	H1/H2

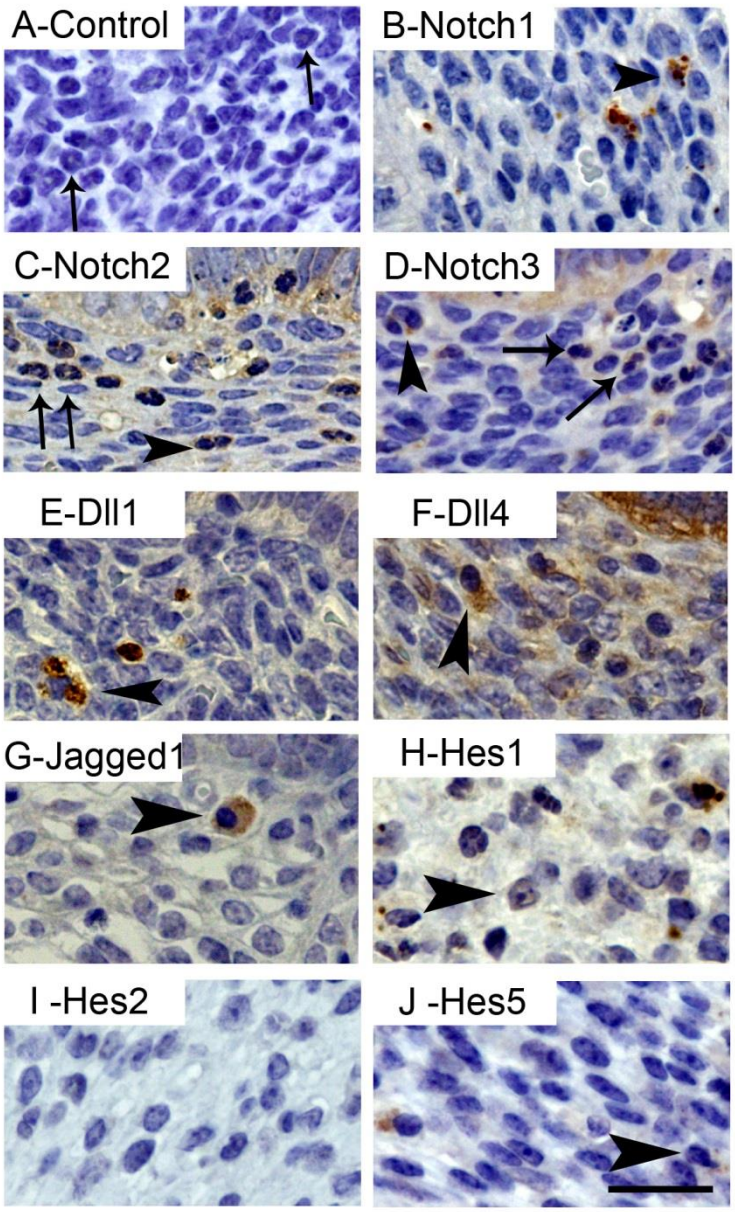
N1 – Notch1, N2 – Notch2, N3 – Notch3, D1 - Dll1, D4 – Dll4, J1 – Jagged1, H1 – Hes1, H2 – Hes2, H5 – Hes5.

Transcription levels of the three receptors (Notch1-3) and of three effectors (*Hes1*, *Hes5* and *Nrarp*) are significantly correlated with those of *Jagged1*, which implies *Jagged1* as the main Notch pathway ligand in the oviduct. Additionally, significant correlations between transcription levels of Notch components (both receptors and ligands) and *Hes* genes, indicates that *Hes* transcription is a direct result of Notch pathway activation.

These transcription results are in accordance with expression patterns evidenced by IHC analysis. Notch2, Notch3 and Dll4 show increased expression after ovulation. Differential transcription and expression of Notch components and effectors is not only temporal, but also spatial. Notch3, Dll4, Jagged1, Hes1 and Hes5 are specifically expressed in oviduct ampulla secretory cells, although in different combinations along the EC stages. This result leads to the suggestion that Notch signaling is regulating oviduct secretory function along

the EC. Notch1 and Hes2 are specifically expressed in oviduct isthmus crypts, mainly during estrus and diestrus, which may be related with the regulation of cellular turnover along the EC.

Figure 46 – Notch pathway components and effectors are present in uterus stroma cells.



Positive staining in brown, counterstaining with haematoxylin (400x magnification). Only nuclear detection of Notch effectors is indicative of active Notch signaling. Negative controls were performed with the polyclonal rabbit IgG, metestrus image (A). Arrow heads indicate stroma cells. Arrows indicate neutrophils. Scale bar –50 µm.

Notch1 is expressed during diestrus (B). Notch2 and Notch3 are expressed in stroma cells and neutrophils during metestrus (C and D, respectively). Dll1 and Dll4 are expressed during diestrus (E and F, respectively). Jagged1 is expressed during estrus (G). Hes1 and Hes5 are detected during estrus (H and J, respectively). Hes2 is not detected, estrus image (I).

Endometrium undergoes extensive cellular remodeling along the EC, in response to circulating concentrations of E2 and P4. In the mouse, changes that define transition from one stage to the other occur over a 24 hour period, which denotes a rapid cellular response to hormonal fluctuations (Hewitt et al., 2003). The Notch pathway component and effector genes are differentially expressed in uterus luminal and glandular epithelia along the EC. Unique expression combinations of receptor/ligand/effector genes are observed in the luminal and glandular epithelia at each EC stage. It is plausible that these specific expression combinations of Notch receptor/ligand/effector genes are related to definition of cellular events occurring at each EC stage, as Notch signaling results from the specific activation of Notch receptors (Shimizu et al., 2002; Yuan et al., 2012).

Uterine stroma cellular dynamics along the EC is modulated by ovarian steroids (Wood et al., 2007). Notch pathway component and effector genes (except Hes2), are expressed in stroma cells along the EC. As suggested for uterine luminal and glandular epithelial cells, Notch signaling may be involved in the regulation of cellular changes, through signaling between epithelial and stroma cells. Metestrus migration of neutrophils into the luminal epithelium is well documented (Corbeil et al., 1985). Metestrus uterine stroma neutrophils express Notch2 and Notch3 receptors. Notch signaling has recently been implicated in immune response (Degaki et al., 2012; Ito, Connett, Kunkel, & Matsukawa, 2012; Radtke, Macdonald, & Tacchini-Cottier, 2013). Results here described also associate Notch signaling to immunity cells and potentially to endometrial immunity function.

In conclusion, Notch pathway component and effector genes are differentially transcribed and expressed in the mouse oviduct and uterus along the EC. In the oviduct, Notch component and effector genes are transcribed following the pattern of plasma P4 concentrations along the EC, indicating a P4 regulation of Notch signaling. Notch oviduct and uterus dynamic expression patterns are spatially and temporally specific, resulting in unique expression combinations of Notch receptor/ligand/effector genes in oviduct luminal epithelium, uterus luminal and glandular epithelia and endometrium stroma cells, at each EC stage. Nuclear detection of Notch effectors indicates that Notch signaling is active. Altogether, these results prompt for a regulatory role of Notch signaling in oviduct and uterus cellular remodeling and epithelial function along the EC.

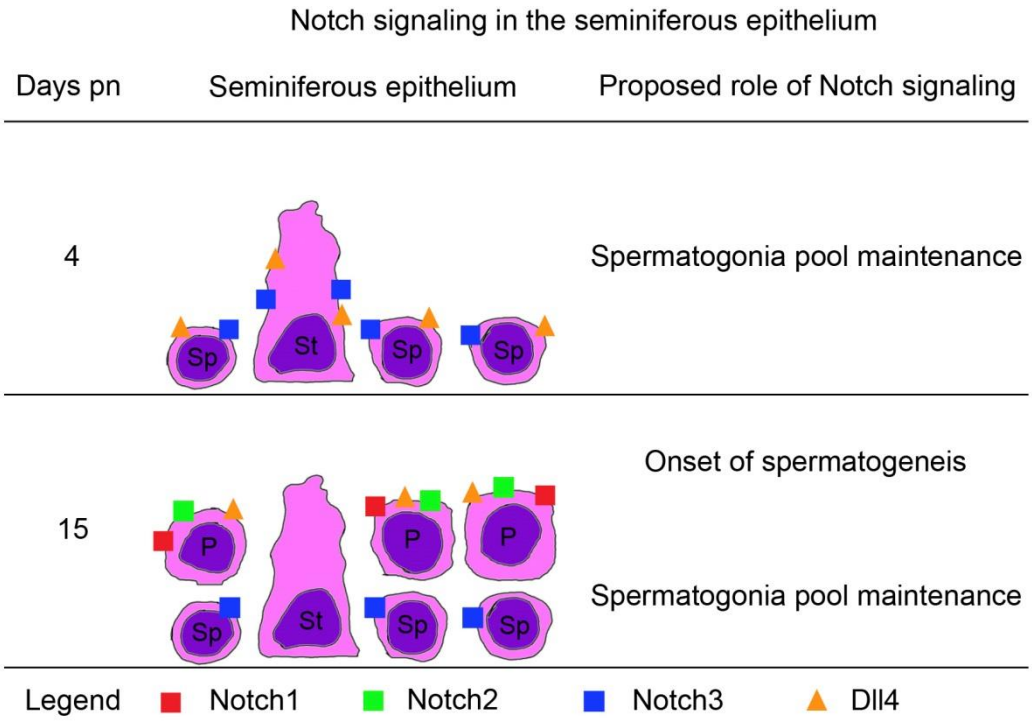
GENERAL DISCUSSION AND CONCLUSIONS

The objective of the experimental work included in this thesis was the evaluation of Notch pathway involvement in male and female reproductive function. Chapters I and II considered the evaluation of the transcription and expression patterns of Notch pathway components and effectors in male reproductive tract (testis and epididymis), and their relationship with key cellular remodeling events occurring along pn testis development and along the adult spermatogenic cycle and epididymal transit of spermatozoa. Chapter III evaluated *in vivo* blockade of Notch signaling in male reproductive function. Chapters IV and V considered the evaluation of the transcription and expression patterns of Notch pathway components and effectors in female reproductive tract (ovary, oviduct and uterus), and their relationship with key cellular remodeling events occurring along the EC.

Results here presented in the first two chapters indicate that Notch pathway is associated with major cellular remodeling events occurring in the testis during spermatogenesis and in the epididymis during transit of spermatozoa. Results presented in the third chapter indicate that Notch blockade induces abnormal spermatogenesis and epididymis function. Overall, these results prompt for a major regulatory role of Notch in testis pn development, spermatogenesis and spermatozoa maturation.

Figure 47 summarizes the Notch pathway components detection and the proposed role of Notch signaling during testis pn development. As shown, we suggest that Notch is regulating spermatogonia quiescence and spermatogenesis onset, through an epithelial canonical pathway (Artavanis-Tsakonas et al., 1995; Borggrefe & Oswald, 2009). Notch pathway components were previously detected during testis pn development (Dirami et al., 2001; Hasegawa et al., 2011). However, results presented in Chapter I constitute the first integrated evaluation of the spatial and temporal presence of Notch components and effectors during testis pn development and along the adult spermatogenesis. Our proposed role of Notch signaling in the regulation of early pn spermatogonia quiescence is in accordance with recent data published by others (Garcia et al., 2013; Garcia & Hofmann, 2013). A role for Notch signaling at the onset of meiosis was previously reported in *C. elegans* (Lambie & Kimble, 1991).

Figure 47 – Proposed role of Notch signaling during testis post-natal development and onset of spermatogenesis.



The main Notch components participating in each role are represented in shapes and colors according to legend. pn – post-natal; St – Sertoli cell; Sp – spermatogonia; P – preleptotene spermatocyte.

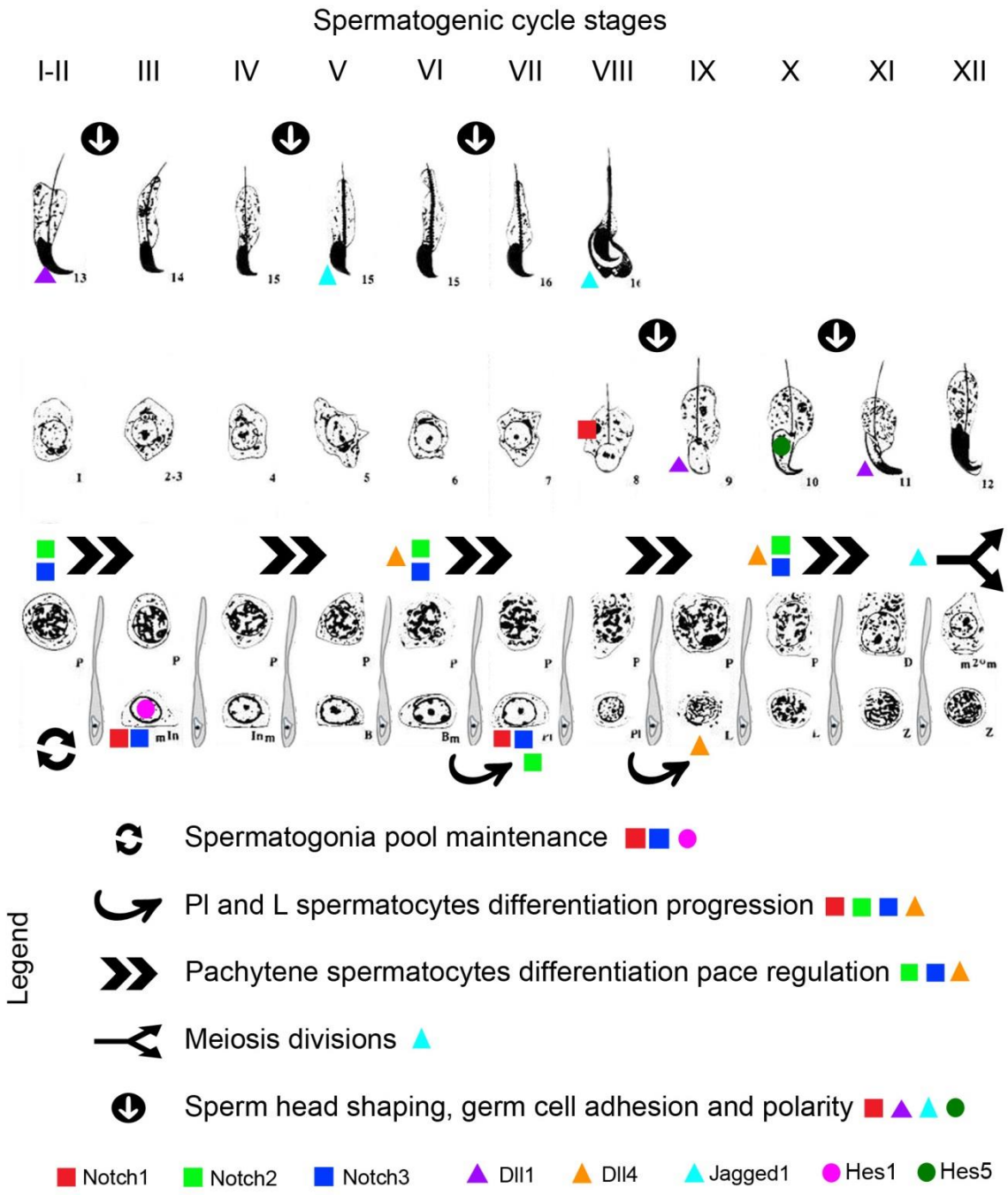
Notch pathway component and effector genes show specific expression patterns during the spermatogenic cycle in adult spermatogenesis. Although the presence of Notch pathway components in male germ cells was previously reported, the so far available information is very fragmentary and controversial (Hayashi et al., 2001; Mori, et al., 2003; Hayashi, et al., 2004b; von Schönfeldt, et al., 2004; Sahin et al., 2005; Hahn et al., 2009; Hasegawa et al., 2011; Garcia et al., 2013). Results described in Chapter I indicate that Notch signaling is active during spermatogenesis, and lead to the suggestion that Notch may regulate germ cells differentiation. This hypothesis is supported by studies associating Notch signaling with male infertility (Hayashi et al., 2001; Hayashi, et al., 2004b; Sahin et al., 2005; Lupien et al., 2006; Garcia et al., 2013; Huang et al., 2013). However, the role of Notch signaling during spermatogenesis is controversial, as recent studies using genetically modified mice (Hasegawa et al., 2011; Batista et al., 2012) reported that Notch signaling was not necessary for spermatogenesis. Although, Notch1 loss of function in germ cells had no phenotype in spermatogenesis and fertility (Batista et al., 2012; Huang et al., 2013), Notch1 gain of function in germ cells induced spermatogenesis failure (Huang et al., 2013). This controversial results may be explained taking in consideration that Notch activity is dosage dependent (Trindade et al., 2012), and redundancy in Notch receptors may be crucial to

normal signaling, since paralogues exert redundant or additive functions in maintaining the balance (Zeng et al., 1998; Kitamoto & Hayasaka, 2005).

Based on results presented in Chapters I and III, Figure 48 summarizes our proposed role of Notch signaling in the regulation of the spermatogenic cycle. Similarly to what happens in somitogenesis (Morimoto et al., 2005; Niwa et al., 2007), Notch pathway components may regulate the pace of spermatogenesis at several key points, contributing to the coordination and orchestration of the complex cellular remodeling events that take place along the spermatogenic cycle. Specific combinations of receptor/ligand expression are not only associated with key events occurring during the spermatogenic cycle, but also these combinations may be determinant for germ cells identity. Notch signaling results from the specific activation of Notch receptors (Shimizu et al., 2002; Yuan et al., 2012). The presence of different receptors within a cell-type may be relevant for cell identity and function. This assumption gain support from results described in Chapter III. Following DAPT treatment, the expression patterns of some Notch components were changed, including ectopic expression in other germ cell types. These changes in expression patterns, probably compensatory following the decrease in Notch activation, induced formation of aberrant germ cells, loss of germ cell identity and increased apoptosis of specific cell types.

Analysis of specific effects on germ cell identity and morphology following DAPT treatment may indicate specific regulatory roles during the spermatogenic cycle. These are the cases of germ cell anchorage to Sertoli cells, polarity (sperm head shaping) and meiosis. The ectoplasmic specialization (the elongated spermatid anchoring system) maintains adherence between Sertoli and germ cells, and also confers cell orientation and polarity within the seminiferous epithelium. This structure is associated with Par complex proteins (Wong et al., 2008), and Notch signaling was associated to cell polarity decisions in tissues involving Par complex proteins (Afonso & Henrique, 2006; Bultje et al., 2009). Results presented in Chapter III allow the association between germ cell defects observed following DAPT treatment and changes in Notch expression in those cellular types. Cellular defects originated during meiosis are also related with changes in expression patterns of Notch components following Notch blockade, and in accordance with recent studies associating Notch signaling with cell division (Bultje et al., 2009; Das & Storey, 2012).

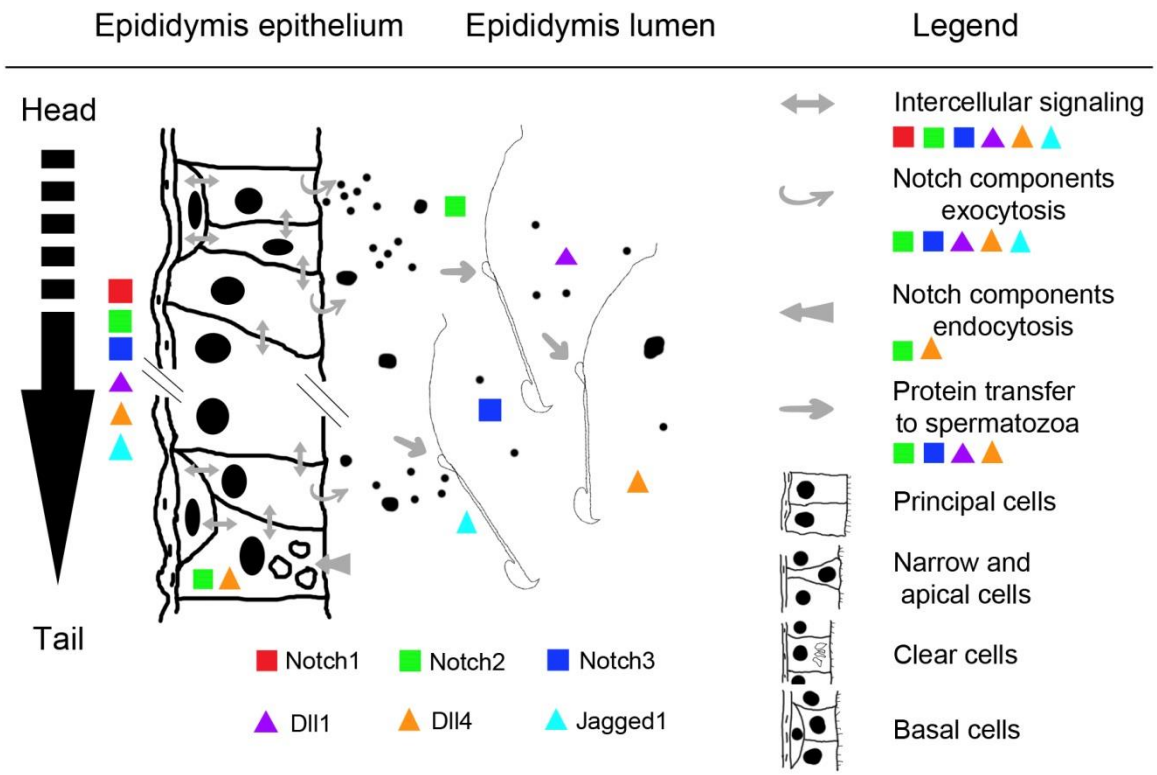
Figure 48 – Proposed roles of Notch signaling in the regulation of the spermatogenic cycle.



Draw-scheme representing stages (I-XII) of the mouse spermatogenic cycle. Spermatogonia (In, B); spermatocytes (Pl- preleptotene, L- leptotene, Z- zygotene, P- pachytene, D- diakinesis, Mi- meiotic division); round spermatids (1-8); elongated spermatids (9-16). Different arrows represent the main roles proposed for Notch signaling. The main Notch components and effectors participating in each role are represented in shapes and colors according to legend. Drawing adapted from Hess & Franca (2008).

Results from Chapter II and III prompt for a role of Notch signaling in spermatozoa maturation along the epididymis. Each epididymis segment shows a specific combination of expression patterns of Notch components and effectors in each cell type. Additionally, some Notch components were detected in spermatozoa and in the epididymis lumen, in a dynamic specific pattern along the epididymis. Although Notch involvement in epididymis development was already reported (Hayashi, et al., 2004c; Lupien et al., 2006; Hahn et al., 2009), Notch gene transcription and expression in the adult epididymis and the enrollment of Notch signaling in spermatozoa maturation is novel. In Chapter II we suggest that Notch components are transferred at distance, between epididymis epithelial cells and spermatozoa. Notch signaling at distance was recently reported in other tissues (Sheldon et al., 2010; Lu et al., 2013), and transfer of Notch components between different germ cell types was also evidenced in work described in Chapter I. Protein transfer through epididymosomes is a key step in spermatozoa maturation (Saez et al., 2003; Caballero et al., 2013). We suggest that transfer of Notch components, within epididymosomes, to spermatozoa and the epididymis lumen may play a role in spermatozoa maturation, or later on, in spermatozoa interactions with oocytes and the oviduct. This proposed mechanism is schematically illustrated in Figure 49

Figure 49 – Schematic illustration of a proposed role of Notch in epididymis function.

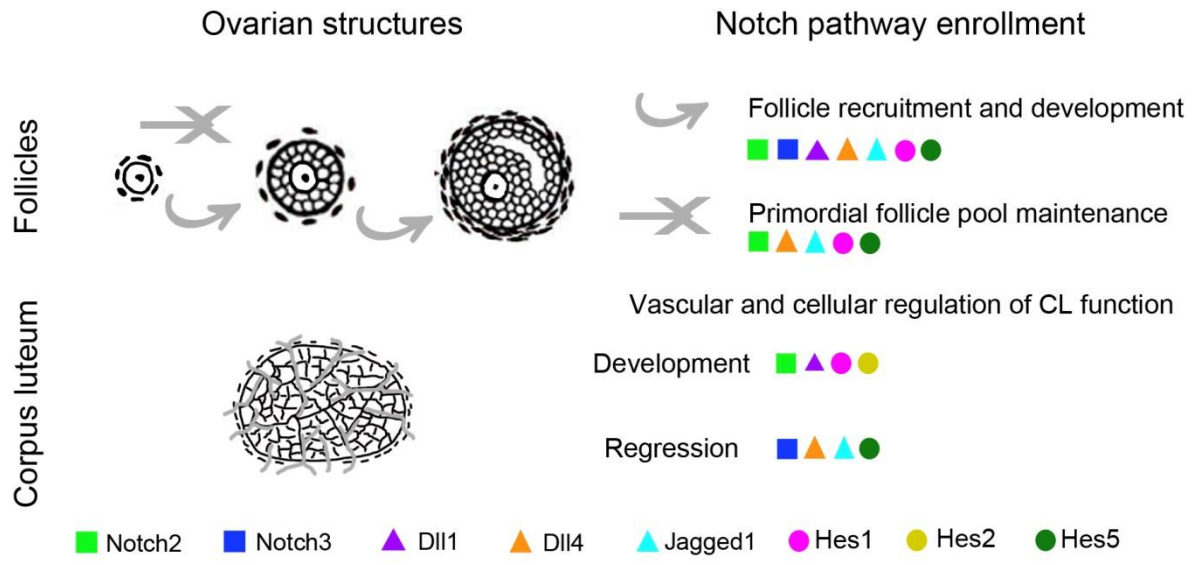


Draw-scheme representing the epididymis epithelium and lumen. Arrows represent Notch components' dynamics along the epididymis. The Notch components dynamics are represented in shapes and colors according to legend.

Notch components and effectors were detected in Sertoli cells, and their expression patterns were changed following DAPT treatment. The role of Notch signaling in Sertoli cells is controversial. Active Notch signaling in Sertoli cells was previously reported, although it was considered unessential to spermatogenesis (Hasegawa et al., 2011). This was not corroborated by results recently reported by (Garcia et al., 2013; Garcia & Hofmann, 2013). Results described in this thesis are in alignment with these latter studies. Notch signaling was also detected in Leydig cells, and Notch expression patterns were changed following DAPT treatment. However, this had no effect on adult plasma testosterone concentrations. The presence of Notch signaling in Leydig cells was also reported by others (Sahin et al., 2005), and the potential role of Notch in the regulation of Leydig cell population during embryonic and post-natal development suggested (Tang et al., 2008; DeFalco et al., 2013). Still, the Notch involvement in adult Leydig cells function is unknown.

Results of Chapter IV established the Notch component and effector gene expression patterns in the ovary, and related them with cellular remodeling events associated with follicle and CL development. Results prompt for a role of Notch signaling in follicle pool maintenance, follicle recruitment and development. Figure 50 schematically illustrates the proposed role of Notch signaling in the above ovarian cellular events.

Figure 50 – Proposed role of Notch signaling in the regulation of follicle and corpus luteum development.



Draw-scheme representing the ovarian follicles and corpus luteum. The main Notch components and effectors participating in each role are represented in shapes and colors according to legend.

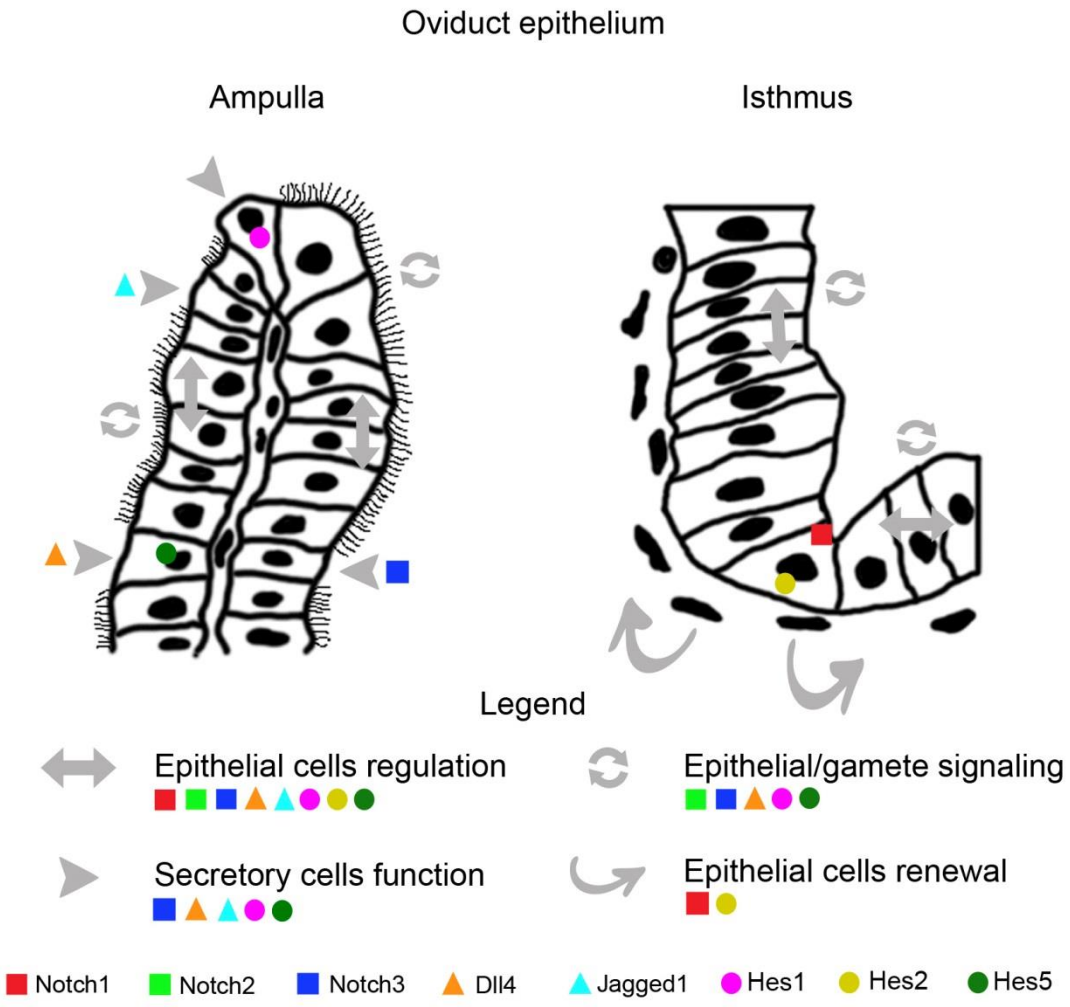
Notch components and effectors had already been identified in follicles and the CL (Johnson et al., 2001; Trombly et al., 2009; Zhang et al., 2011; Jovanovic et al., 2013). However, results described in this thesis are the first to provide an integrated (spatial and temporal)

assessment of Notch components and effectors along the EC, and the first to identify and characterize Dll1 and Dll4 expression in follicle development. The association between Notch signaling and follicle growth was also recently reported by others (Trombly et al., 2009; Zhang et al., 2011; Xu & Gridley, 2013).

Notch components and effectors were identified in luteal, theca and capillary cells within the CL. Expression patterns change along with CL development and regression. Notch signaling was already associated to CL function through the regulation of neo-angiogenesis within the CL (Fraser et al., 2012; García-Pascual et al., 2013). Results here presented also prompt for a direct regulation of luteal cells function, as also suggested by others (Hernandez et al., 2011).

Notch component and effector genes are differentially transcribed and expressed along the oviduct segments. The presence of Notch signaling in the oviduct epithelium is novel. The patterns of expression here described lead us to propose a role for Notch signaling in oviduct epithelial cells along the EC, which is schematically illustrated in Figure 51.

Figure 51 – Proposed role for Notch signaling in the oviduct epithelium.

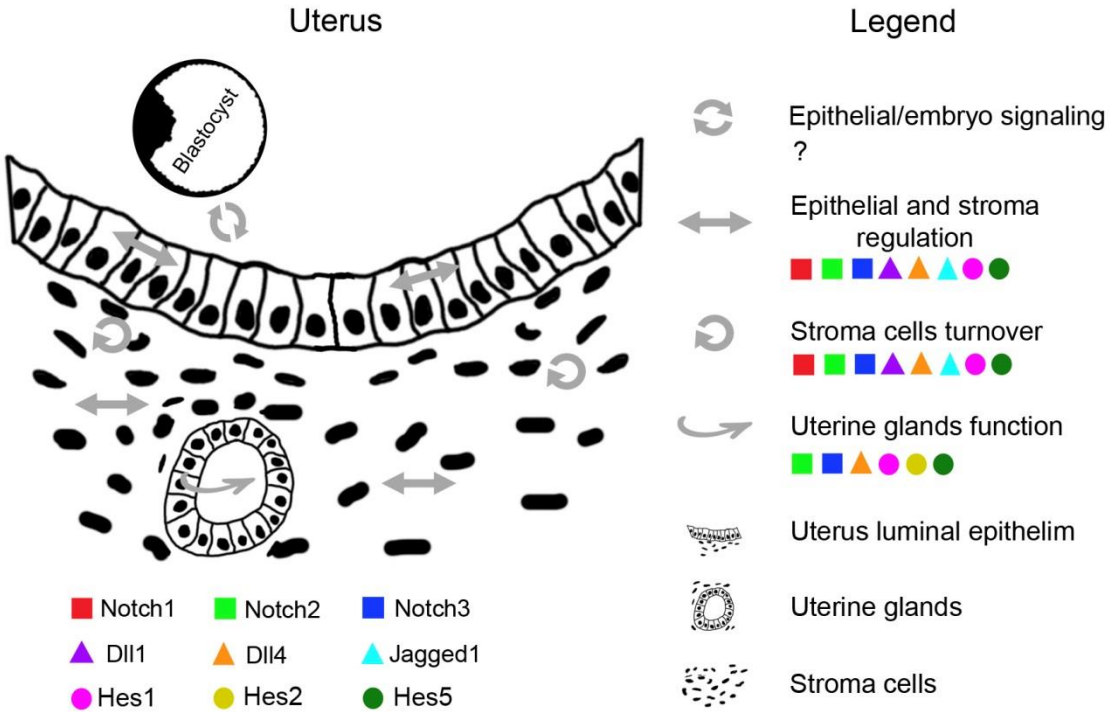


(Legend in the next page)

(Figure 51 – Legend) Draw-scheme representing two portions (ampulla and isthmus) of the oviduct epithelium. The main Notch components and effectors participating in each role are represented in shapes and colors according to legend.

Data regarding Notch presence in the uterus are scarce and incomplete (Cobellis et al., 2008; Mazella et al., 2008; Afshar, et al., 2012a; Afshar, et al., 2012b). Results described in Chapter V, show that Notch component and effector genes are dynamically and specifically expressed in the uterus luminal and glandular epithelium along the EC. This prompts for a major role of Notch signaling in the regulation of uterus epithelial cells identity, fate and function. This regulation is associated to cellular remodeling events occurring along the EC, to prepare the uterus for embryo implantation. Notch enrolment in embryo implantation was already reported (Afshar, et al., 2012a; Afshar, et al., 2012b). As in this thesis, expression of Notch components have been described in uterus stroma cells (Cobellis et al., 2008; Mazella et al., 2008; Afshar, et al., 2012a; Afshar, et al., 2012b). The proposed role of Notch signaling in the regulation of uterus luminal and glandular epithelia and stroma is schematically summarized in Figure 52.

Figure 52 – Proposed role for Notch signaling in the uterus luminal and glandular epithelia, and stroma.



Draw-scheme representing the uterus. The main Notch components and effectors participating in each role are represented in shapes and colors according to legend.

CONCLUSION

The experimental work presented in this thesis contributed to the integrated (spatial and temporal) evaluation of the role of Notch signaling in male and female reproductive function. This area of research has gained increasing interest in the very last few years, due to an increasing evidence of the major regulatory role of this cell signaling pathway in key cellular events occurring in male and female reproductive biology. Progress on the comprehension of the role of Notch in mammalian reproduction has dramatically increased, however several controversial issues remain. Results described in this thesis prompt for a major regulatory role of Notch signaling in the following reproductive events:

- Testis post-natal development (spermatogonia quiescence, onset of spermatogenesis);
- Spermatogenesis (pace of the spermatogenic cycle, germ cell identity and fate);
- Epididymis function (signaling between epithelium and spermatozoa);
- Follicle development;
- Corpus luteum development and regression;
- Oviduct and uterus epithelia function.

All the above events involve extensive cellular remodeling and intercellular communication. Notch signaling may play a pivotal role in the orchestration of these cellular events, and therefore in the autocrine, paracrine and juxtacrine regulation of reproduction.

FUTURE DIRECTIONS

Most published studies involving genetically modified mice are targeted to Notch1 function. Results presented in this thesis indicate that the specific combination of Notch component and effector genes is critical for germ cell identity and fate. Therefore, functional evaluation of other Notch components through the phenotypic analysis of mutant mice is necessary. Mutant mice have now been produced in the host laboratory during the development of this work. Studies with these models, focusing mainly on ligands Dll4 and Jagged1, have already been started and will hopefully add novel and relevant information regarding the role of Notch signaling in mammalian reproductive function. Considering all available information, Notch pathway is a promising therapeutic target. It is expectable that additional advances in this field will lead to the development of new therapeutic strategies of fertility control in the male and female, namely in infertility management and contraception.

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ANNEX I - Primer pair sequences list

	Primer	Sequence	Gene
Receptors	Notch1_F	5’ ACAGTAACCCCTGCATCCAC 3’	*Notch1*
	Notch1_R	5’ GGTTGGACTCACACTCGTTG 3’	
	Notch2-F	5’ GACTGCACAGAAGACGTGGA 3’	*Notch2*
	Notch2-R	5’ GCGTAGCCCTTCAGACACTC 3’	
	Notch3_F	5’ GTGTCAATGGTGGTGTCTGC 3’	*Notch3*
	Notch3_R	5’ GCACACTCATCCACATCCAG 3’	
Ligands	Dll1_F	5’ GTTGTCTCCATGGCACCTG 3’	*Dll1*
	Dll1_R	5’ TGCACGGCTTATGGTGAGTA 3’	
	Dll4 – F	5’ GGAACCTTCTCACTCAACATCC 3’	*Dll4*
	Dll4 – R	5’ CTCGTCTGTTCGCCAAATCT 3’	
	Jag1_F	5’ CCAGCCAGTGAAGACCAAGT 3’	*Jagged1*
	Jag1_R	5’ CAATTCGCTGCAAATGTGTT 3’	
Effectors	Hes1_F	5’ GCGAAGGGCAAGAATAAATG 3’	*Hes1*
	Hes1_R	5’ TGTCTGCCTTCTCTAGCTTGG 3’	
	Hes2_F	5’ CGGATCAACGAGAGCCTAAG 3’	*Hes2*
	Hes2_R	5’ GTCTGCCTTCTCCAACTTCG 3’	
	Hes5_F	5’ GCACCAGCCCAACTCCAA 3’	*Hes5*
	Hes5_R	5’ GGCGAAGGCTTTGCTGTGT 3’	
	Nrarp_F	5’ AGTCGCTGCTGCAGAACAT 3’	*Nrarp*
	Nrarp_R	5’ AACAGCTTCACCAGCTCCAG 3’	
Endogenous controls	B2mg_F	5’ CGGTGACCCTGGTCTTTCTG 3’	β*2mg*
	B2mg_R	5’ TCAGTATGTTCGGCTTCCCATT 3’	
	HPRT1_F	5’ GTCGTGATTAGCGATGATGAACC 3’	*HPRT1*
	HPRT1_R	5’ GCAAGTCTTTCAGTCCTGTCCATAA 3’	
	RPS29_F	5’ CACGGTCTGATCCGCAAATAC 3’	*RPS29*
	RPS29_R	5’ ACTAGCATGATCGGTTCCACTTG 3’	