Metabolism of Polyunsaturated Fatty Acid in Rat Testis

Expression and Regulation of
Δ^5-Desaturase, Δ^6-Desaturase, Stearoyl-CoA Desaturase 1,
Stearoyl-CoA Desaturase 2 and The Nuclear Receptors
Peroxisome Proliferator-activated Receptor α, δ and γ

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TABLE OF CONTENTS

PREFACE AND ACKNOWLEDGEMENTS

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SUMMARY

In mammalian cells essential polyunsaturated fatty acids (PUFAs) are converted to longer PUFAs by alternating steps of elongation and desaturation. In contrast to other PUFA-rich tissues, the testis is continuously drained of these fatty acids, as spermatozoa are transported to the epididymis. Alteration of the germ cell lipid profile from spermatogonia to condensing spermatids and mature spermatozoa had been described prior to this work, but the male gonadal gene expression of the desaturases, responsible for the PUFA-metabolism, was still not established.

The focus of this study was to characterise the expression and regulation of stearoyl-Coenzyme A (CoA) desaturase 1 (SCD1), stearoyl-CoA desaturase 2 (SCD2), Δ^5- and Δ^6-desaturase in rat testis. Desaturase gene expression was detected in testis, epididymis and fractionated cells from seminiferous tubulus using Northern blot analysis. For the first time SCD1 and SCD2 expression are demonstrated in rat testis and epididymis. Both SCDs are expressed in epididymis, while testis mainly contains SCD2. Examination of the testicular distribution of Δ^5- and Δ^6-desaturase and SCD1 and -2 shows that all four desaturases seem to be localised in the Sertoli cells, with far lower expression in germ cells. In light of earlier published results showing that germ cells are richer in PUFAs than Sertoli cells, this strengthens the hypothesis of a lipid transport from the Sertoli cells to the germ cells.

As opposed to what is shown in liver, Δ^5- and Δ^6-desaturase mRNA levels in Sertoli cells are upregulated by dexamethasone. Furthermore, dexamethasone induces SCD2 mRNA. Insulin also upregulates these three genes in the Sertoli cell, while SCD1 mRNA is downregulated by both insulin and dexamethasone.

Δ^5- and Δ^6-desaturase, SCD1 and SCD2 are all upregulated by follicle-stimulating hormone (FSH). A similar upregulation of the desaturases is observed when treating Sertoli cells with $(Bu)_2$cAMP, indicating that the desaturase upregulation observed with FSH treatment results from elevated levels of cAMP. Testosterone has no influence on the desaturase gene expression, at age 19 days. Thus, FSH seems to be a key regulator of the desaturase expression in the Sertoli cell.

Neither SCD1 or -2, nor Δ^5- and Δ^6-desaturase are upregulated in whole testis tissue in response to two weeks feeding of a fat free diet. This makes a great contrast to what is seen in liver and kidney. Both in liver and kidney SCD1 and Δ^5- and Δ^6-desaturase are upregulated, accompanied by increased levels of fatty acids in the *n*-9 family. In liver the Mead acid (20:3(*n*-9)) to arachidonic acid (20:4(*n*-6)) ratio reached 0,75. Also in testis increased *n*-9

levels are seen; 20:3(*n*-9)/20:4(*n*-6)=0,36. However, this is probably resulting from decreased supply of *n*-3 and *n*-6 substrates. At present we hypothesise that the desaturases in testis are insensitive to essential fatty acid deficiency (EFAD), and furthermore, that the vacant desaturase response is due to differences on the transcription factor level.

The low testicular responsiveness to changes in the PUFA pool is confirmed as Sertoli cells treated with arachidonic or tetradecylthioacetic (TTA) acid only show a weak to non-excising downregulation of the desaturases (less than 1,5-fold). The downregulation of the desaturases in response to PUFAs in hepatocytes has been reported to be more than 4-fold. However, the fact that the net effect of TTA, a strong PPARα agonist, is desaturase-repressive, indicates that some nuclear mediator of desaturase repression must be present in low doses, also in testis.

Peroxisome proliferator-activated receptor (PPAR) α, δ and γ are expressed in rat testis, with PPARα showing a gene expression pattern quite similar to Δ^5- and Δ^6-desaturase, as the Sertoli cell is the cell type with the highest expression. Furthermore, PPARγ expression also dominates in the Sertoli cells. PPARδ expression is for the first time demonstrated in rat germ cells, with high levels in round spermatids. PPARα and -γ are known to control numerous genes related to lipid metabolism including PUFA desaturation. Hence, the co-localisation of the desaturases and e.g. PPARα can not be coincidental. Since PPARα expression in the Sertoli cells has been proven to be stage specific, and regulated by FSH, the induction of the desaturases in response to FSH reported here, could be resulting from a combined primary FSH-cAMP-CREB action and secondary FSH-PPARα-ligand action.

Finally, we show that PPARα is slightly downregulated in testis in response to the fat free diet, while upregulated in liver and kidney. Acknowledging that PPARα induces Δ^5- and Δ^6-desaturase, and that the same genes are upregulated in the EFAD liver and kidney, we speculate if the increased level of PPARα mRNA adds to the SREBP-1c-governed induction of Δ^5- and Δ^6-desaturase under EFAD conditions in these tissues. Furthermore, the small downregulation of PPARα in the EFAD testis could explain how an abolished desaturase repression seen in PUFA-treated Sertoli cells, would disappear in whole testis tissue from fat free diet-fed rats.

The localisation and hormonal regulation of the desaturases are featured in the forthcoming article; *Expression and Regulation of Δ^5-Desaturase, Δ^6-Desaturase, Stearoyl-Coenzyme A (CoA) Desaturase 1, and Stearoyl-CoA Desaturase 2 in Rat Testis*, now in press in Biology of Reproduction [Sæther et al., 2003].

ABBREVIATIONS

ABP	Androgen binding protein
Amp	Ampicillin
apo	Apolipoprotein
AR	Androgen receptor
ACS	Acyl-CoA synthetase
$(Bu)_2$cAMP	N^6,2'-O-dibutyryl cAMP
B-ZIP	Basic region and leucine zipper-region
CoA	Coenzyme A
CRE	cAMP response element
CREB	cAMP response element binding protein
ds	Double-stranded
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
DHA	Docosahexaeonic acid.
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
E.coli	*Eschericia coli*
ECL	Enhanced chemiluminescence
*Eco*RI	*E.coli* restriction enzyme 1
EFAD	Essential fatty acid deficiency
EPA	Eicosapentaenoic acid
ER	Endoplasmatic reticulum
EtBr	Ethidium bromide
FF	(Essential) fatty acid free
FA	Fatty acid
FATP	Fatty acid transport protein
FBS	Foetal bovine serum
FSH	Follicle-stimulating hormone
GA	Gauge
GC	Germ cells
GC-FID	Gas chromatograph - Flame ionising detector
GnRH	Gonadotropin-releasing hormone
GPI	Glycosylphosphatidyl inositol
GTCM	Guanidine isothiocyanate - Mercaptoethanol
HBSS	Hanks' balanced salt solution
Ins	Insulin
IU	International units
LB	Luria broth
LDL	Low density lipoprotein
LH	Luteinising hormone
LRP	LDL receptor-related protein
LXR	Oxysterol liver X receptor
MEM	Minimum essential medium
NAP-column	Sodium phosphate column
NDA	2-Naphthol-3-6-disulfonic acid disodium salt
NMR	Nuclear magnetic resonance
oFSH	Ovine FSH
OLE1	Yeast delta-9-desaturase (origin; oleic acid)
PKA	cAMP-dependent protein kinase
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element
PS	Pachytene spermatocytes
PUFA	Polyunsaturated fatty acid
PVP	Polyvinylpyrrolidone
RBP	Retinol binding protein
rcf	Relative centrifuge force (g)
rpm	revolutions per minute
RST	Round spermatids
RXR	Retinoid X receptor
S	Svedberg units
ss	Single-stranded
SC	Sertoli cells
SCAP	SREBP cleavage-activating protein
SCD	Stearoyl-CoA desaturase
SDS	Sodium dodecyl sulphate
SGP	Sulphated glycoproteins
SHBG	Sex hormone-binding globulin
SLS	Sodium N-laurylsarcosine
SOB	Hanahan's broth
SOC	SOB modified for growing of competent *E.Coli*
SoP	Sodium phosphate
SRE	Sterol regulatory element
SREBP	Sterol regulatory element-binding protein
SSC	Sodium chloride - Sodium citrate buffer
Taq	*Thermus aquaticus*
TBE	Tris base- boric acid - EDTA
TE	Tris base - EDTA.
TES	Tris base - EDTA - SDS
TF	Transcription factor
TTA	Tetradecylthioacetic acid
TTR	Transthyretin
UV	Ultra violet

Single abbreviations are omitted following the guidelines of Biochemical Journal (www.biochemj.org). See *2.9 Chemicals* for explanations to reagents.

1. INTRODUCTION

1.1 POLYUNSATURATED FATTY ACIDS

Fatty acids are chains of carbons with a methyl group in one end and a carboxyl group in the other end. The alkyl chain may be saturated, or it may contain one ore more double bonds as in mono- or polyunsaturated fatty acids. The polyunsaturated fatty acids (PUFAs) comprise a huge variety of functions in the cell, spanning from membrane structures, energy reserves, eicosanoid precursors and protein acetylators to enzyme substrates and transcription factor ligands.

Cell membranes are sheet-like structures consisting of phospholipid bilayers, cholesterol, proteins and carbohydrates. The membranes compartmentalise the cell and form barriers to the flow of polar molecules. At the same time, they function as structural support for membrane proteins involved in e.g. transport, movement, adhesion, signal transduction and cell modelling. A phospholipid is roughly built up by a polar, hydrophilic head and a hydrophilic tail (fig 1.1). The head consists of glycerol, phosphate and a variable molecule, and the tail consists of two fatty acids. Depending on the polar head group and its bonds to the fatty acids, the phospholipids can be divided into several classes and subclasses. Important examples are phosphatidylcholine, phosphatidylethanolamine, phophatidylserine and sphingomyelin.

The degree of unsaturation and the position of the double bonds in the phospholipid fatty acids, together with the cholesterol content, affect the membrane fluidity. The right membrane fluidity is crucial for several cellular events and for the cell as a whole regarding mobility. Two examples given are the oocyte-sperm fusion and the paddling movement of the sperm-tail.

Through β-oxidation the cell can free the chemical energy stored in fatty acids. Both saturated and (poly)unsaturated fatty acids undergo β-oxidation in the peroxisomes. However, degradation of 22:6(*n*-3) and 22:5(*n*-6) requires both NADPH-dependent 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase [Voet & Voet, 1995]. Thus, catabolising (poly)unsaturated fatty acids is not so energetically favourable as saturated fatty acid.

PUFAs with 20 carbons of the *n*-6 and *n*-3 families are important precursors of the eicosanoids, i.e. prostaglandines, prostacyclines, thromboxane, lipoxines, leucotrienes and hydroxy fatty acids [Drevon, 1992; van Dorp et al., 1964; Crawford, 1983]. Eicosanoids are important for several cellular functions like platelet aggregability, chemotaxis and cell growth. Eicosanoids are formed in the cells where they execute their effect, and they are rapidly degraded. The eicosanoids resulting in the strongest effects are the metabolites of arachidonic acid, 20:4(*n*-6).

Polyunsaturated fatty acids can perform transcriptional regulation when binding as ligands to nuclear receptors/transcription factors. Fatty acids are known to bind to the peroxisome proliferator-activated receptor [PPAR; Kersten et al., 2000]. Three PPARs are described in rat; PPARα, PPARδ and PPARγ. The PPARs are known to be promiscuous receptors regarding ligand-binding and bind several different PUFAs and eicosanoids [Kersten et al., 2000; see *1.1.4.1 Peroxisome proliferator-activated receptors*]. The liver X receptor, LXR, and the sterol regulatory element-binding proteins, SREBPs, are also potential candidates in the mediation of PUFA-regulated gene expression [Ou et al., 2001; Yoshikawa et al., 2002].

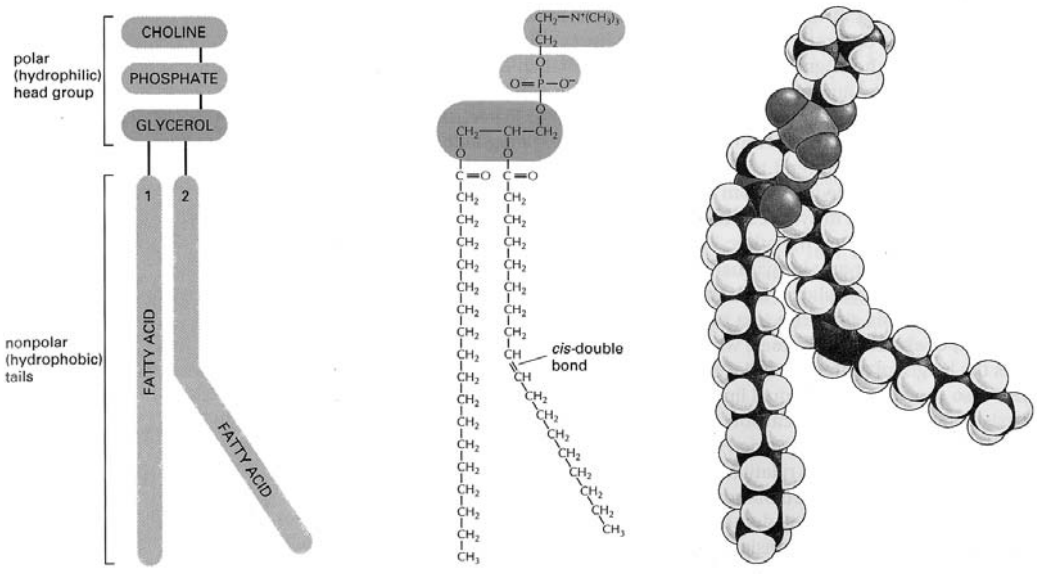

Figure 1.1 The parts of a phospholipid molecule. Phosphatidylcholine represented schematically, in structural formula and as a space-filling model. The kink on the monounsaturated fatty acid is due to the *cis*-double bond. (The Cell, 3rd. Edition, Garland Publ. 1994)

1.1.1 Physiological functions of polyunsaturated fatty acids

Long-chain polyunsaturated fatty acids such as 20:4(*n*-6) and 22:6(*n*-3) play pivotal roles in a number of biological functions including brain development, cognition, reproduction, inflammatory responses and hemostasis [Helland et al., 2003; Birch et al., 1998; Neuringer et al., 1994; Lim et al., 1999; Samuelsson, 1983; Stuhlmeier et al., 1997; Goetzl et al., 1995]. Cell membranes in the retina are rich in very long-chain PUFAs, with DHA, 22:6(*n*-3), as the dominating *n*-3 acid. The light sensitivity of retinal photoreceptors is significantly reduced in new-borns with *n*-3 fatty acid deficiency, while DHA supplementation significantly enhances visual acuity maturation [Uauy, 2001]. Furthermore, children born by mothers, who supplemented their diets with cod liver oil during pregnancy and lactation, scored higher on the Mental Processing Composite of the Kaufman Assessment Battery at age 4 years, as compared to children whose mothers had taken corn oil supplements [Helland et al., 2003].

1.1.2 Metabolism of polyunsaturated fatty acids

In mammalian cells the dietary fatty acids linoleic acid, 18:2(*n*-6), and linolenic acid, 18:3(*n*-3), are converted to the important fatty acids 20:4(*n*-6), 22:5(*n*-6) and 22:6(*n*-3) by alternating steps of elongation and desaturation [Mohammed et al., 1995; Voss et al., 1991]. These modifications include both Δ^5-desaturation and Δ^6-desaturation, where Δ^6-desaturase catalyses the rate-limiting step [Brenner, 1977]. Linoleic acid (18:2(*n*-6)) and linolenic acid (18:3(*n*-3)) can not be synthesised *de novo* in mammals, hence they are essential fatty acids. Rich sources of essential polyunsaturated fatty acids are green leaves, vegetables, linseed, soybean, canola oil, vegetable oils and fish oils.

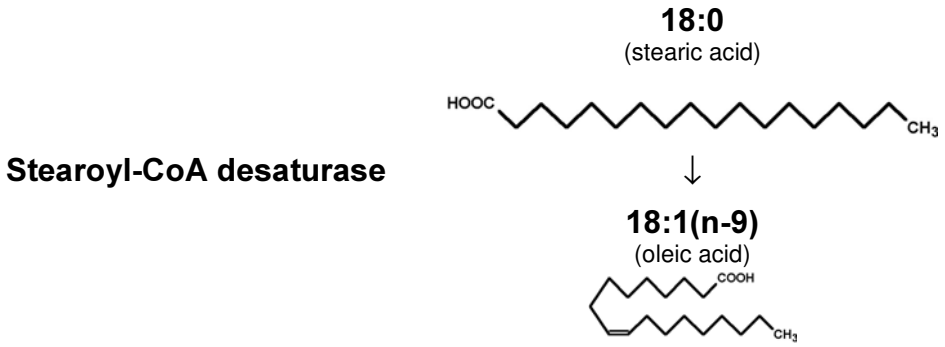

Figure 1.2 The synthesis of the *n-9* polyunsaturated fatty acids. The stearoyl-CoA desaturases introduce a double bond in position 9 in the non-essential stearic acid, 18:0, to form oleic acid, 18:1(*n*-9).

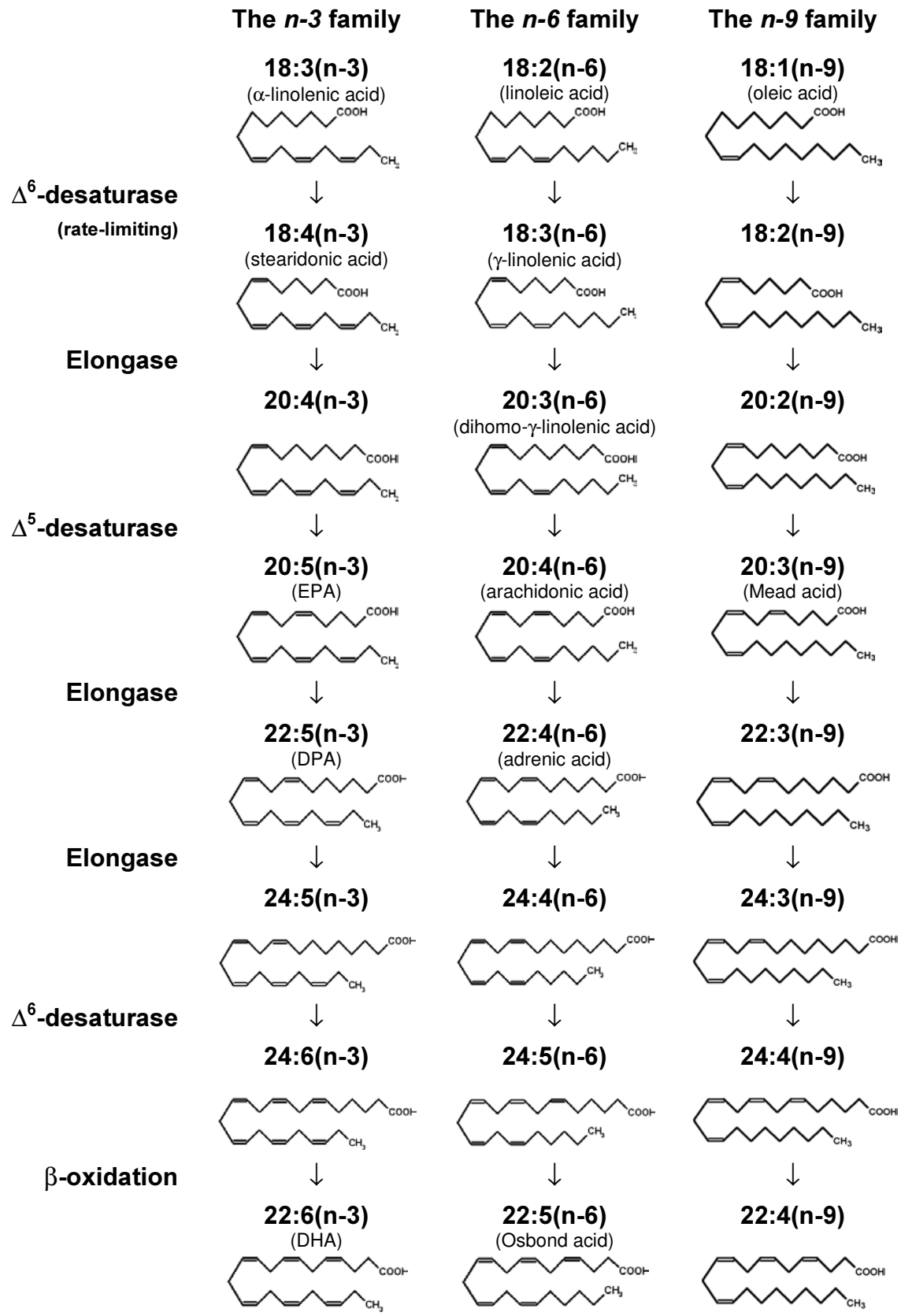

Figure 1.3 The synthesis of the polyunsaturated fatty acids. Dietary and endogenous short-chain PUFAs are converted to long-chain PUFAs by alternating steps of elongation and desaturation. EPA: eicosapentaenoic acid, DPA: docosapentaenoic acid, DHA: docosahexaeonic acid.

The Δ^9-desaturases introduce a double bond in position 9 in the non-essential stearic acid, 18:0, to form oleic acid, 18:1(*n*-9). Oleic acid can go through the same steps of desaturation and elongation as linoleic and linolenic acid, providing the cell with 20:3(*n*-9) and 22:4(*n*-9). In this way the Δ^9-desaturation provides alternative precursors when the cell suffers from essential fatty acid deficiency (EFAD). In rodents two isoforms of Δ^9-desaturase are found: stearoyl-CoA desaturase 1 (SCD1) and stearoyl-CoA desaturase 2 (SCD2). Essential fatty acid deficiency is known to induce the gene expression and activity of SCD1 [Ntambi, 1999], Δ^5-desaturase [Cho et al., 1999a] and Δ^6-desaturase [Cho et al., 1999b] in liver. This induction is actually an abolished PUFA-mediated suppression of these genes, possibly propagated through SREBP-1c (see *1.1.4.2 Sterol regulatory element-binding proteins*).

The rodent SCD1 and SCD2 were already cloned in the eighties [Thiede et al., 1986; Kaestner et al., 1989]. But it was not until 1999, when Cho and Clarke cloned the human Δ^5- and Δ^6-desaturase [Cho et al., 1999a; Cho et al., 1999b], opening up for the cloning of the rat Δ^5- and Δ^6-desaturase [Zolfaghari et al., 2001; Aki et al., 1999], that studies of the expression and regulation of all the four genes were made possible. Earlier reports show that SCD1 is expressed in liver and adipose tissue, while SCD2 is expressed in brain, spleen, heart, lymphocytes and lungs [Tochter et al., 1998]. Δ^5-desaturase and Δ^6-desaturase are expressed in different rodent tissues with the highest level of expression in adrenal gland, liver, brain and testis and lower levels in white adipose tissue and kidney [Matsuzaka et al., 2002]. Several reports have been published on the hormonal regulation of desaturase activity, mainly in liver [Mercuri et al., 1966; Brenner, 1990; Brenner et al., 1968, 2001], while only a few studies have been performed with respect to the regulation of desaturase gene expression. However, Δ^6-desaturase mRNA is reported to increase eightfold in the liver of insulin-treated diabetic rats, as compared to non-treated animals [Rimoldi et al., 2001].

1.1.3 Structural properties of the desaturases

None of the fatty acid desaturases has yet been isolated and characterised with respect to their physical properties, including x-ray crystallography or NMR-analysis. The only analytic tool used so far is therefore a comparison of the predicted primary protein structure between various desaturases and proteins with high homology. Earlier published papers report high

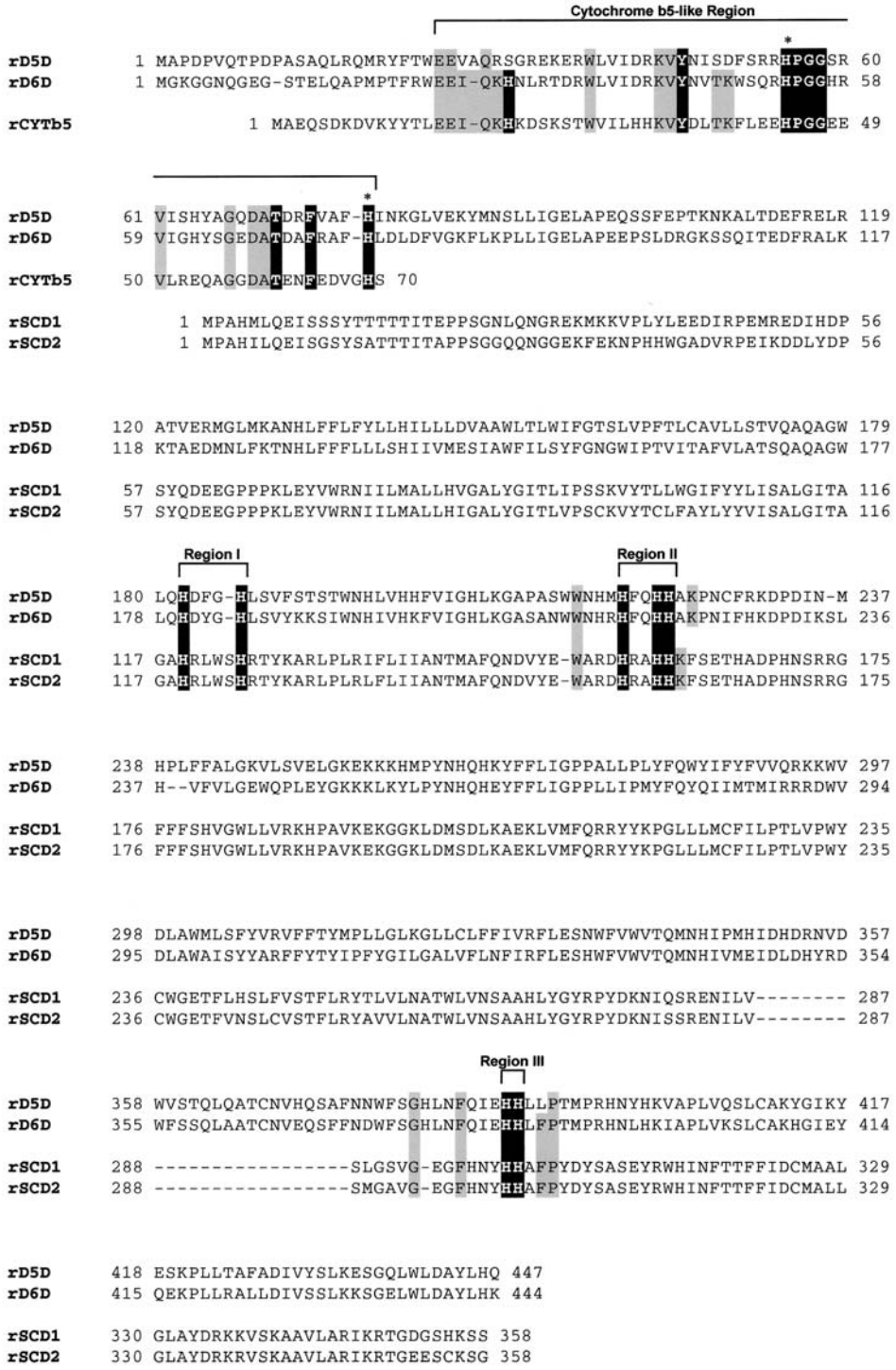

Figure 1.4 Structural properties of the four rat desaturases. D5D and D6D: Δ^5- and Δ^6-desaturase, SCD1 and SCD2: stearoyl-CoA desaturase 1 and 2. The three histidine-rich regions are termed Regions I (HX_3H), II (HX_2HH) and III (HH). The histidines marked with an * within the cytochrome b_5-like region align with the heme-binding histidines in rat cytochrome b_5.

homology between Δ^5- and Δ^6-desaturase [Cho et al., 1999a] and particularly between SCD1 and SCD2 [Kaestner et al., 1989]. All four desaturases also seem to be highly conserved through evolution [Cho et al., 1999b]. A noteworthy feature of the mammalian desaturases is the presence of three histidine-rich regions (fig. 1.4). Regions I (HX_3H) and II (HX_2HH) are located between two transmembrane domains, and region III (HH) is located near the carboxyl terminus of the peptide. These histidine-rich regions are also found in plant membrane desaturases and mammalian stearoyl-CoA desaturase, and reportedly bind non-heme iron required for enzymatic activity [Shanklin et al., 1994]. The enzymatic activity of the desaturases is associated with the microsomal membrane fraction [Sprecher, 1981], which is consistent with the presence of the two membrane-spanning domains that are characteristic of membrane-anchored proteins.

In addition, the amino terminus of the rat Δ^5- and Δ^6-desaturase peptide contains a hydrophilic domain of 54 amino acids that is highly homologous with the heme-binding domain of rat cytochrome b_5 (fig. 1.4). This cytochrome b_5-like domain is conserved among species. [Sayanova et al., 1997; Napier et al., 1998]. The $His^{55/53}$ and $His^{78/76}$ residues located within this domain of the rat Δ^5- and Δ^6-desaturase are exactly aligned with the two heme-binding histidines in cytochrome b_5. Moreover, these two histidines are surrounded by charged amino acids that may contribute to the stabilisation of the heme-histidine complex [Lederer, 1994]. In addition, the sequence $^{55/53}HPGG^{58/56}$ predicts the existence of a β-turn that may render $His^{55/53}$ more accessible to heme iron binding [Lederer, 1994]. This cytochrome b_5-like domain is not a component of the rat SCD1 and SCD2. Early reconstitution studies with mammalian Δ^9-desaturase indicated that the conversion of 18:0(*n*-9) to 18:1(*n*-9) required Δ^9-desaturase, cytochrome b_5 reductase, and cytochrome b_5 itself [Strittmatter et al., 1974].

It has been assumed from these early studies, that all mammalian desaturases require cytochrome b_5 for enzymatic activity [Brenner, 1989; Strittmatter et al., 1974]. However, the cytochrome b_5-like domain of yeast OLE1 was recently reported to replace the requirement for cytochrome b_5; i.e. desaturation occurred in the absence of cytochrome b_5. Removal of the cytochrome b_5-like domain rendered the OLE1 enzyme inactive [Mitchell & Martin, 1995]. This observation raises the possibility that cytochrome b_5 reductase transfers electrons to the catalytic domains of the Δ^5- and Δ^6-desaturase via their cytochrome b_5-like domains, and not via cytochrome b_5.

1.1.4 Transcription factors involved in regulation of the desaturases

1.1.4.1 Peroxisome proliferator-activated receptors

The peroxisome proliferator-activated receptors (PPARs) are members of the steroid/thyroid superfamily of nuclear receptors [Issemann & Green, 1990]. The PPARs heterodimerise with RXR when binding to its DNA recognition site (PPRE; PPAR response element). PPRE have been shown to consist of a direct repeat with one nucleotide spacing; TGACCTNTGACCT. In addition to a dimerisation- and a DNA-binding domain, the PPARs consist of a transactivation- and a ligand-binding domain. The PPARs are promiscuous receptors regarding ligand binding. PPARα and -γ are known to bind several different PUFAs and eicosanoids, e.g. linoleic acid, arachidonic acid, leucotriene B4 and prostaglandine J2 [Kersten et al., 2000].

Three isoforms of PPARs (PPARα, PPARδ and PPARγ) have been identified in mammals. PPARα is primarily expressed in brown adipose tissue and liver, and to a lesser extent in kidney, heart and digestive tract (oesophagus – colon) in the rat [Escher et al., 2001]. The PPARδ is ubiquitously expressed with the highest level in the digestive tract [Escher et al., 2001]. PPARγ exists in two splice variants, PPARγ1 and γ2. They are both highly expressed in adipocytes and to a lesser extent in other organs [Escher et al., 2001]. The overall expression of PPARs in whole testis tissue is small compared to other tissues in the adult rat, but on a cellular level PPARδ is shown to be highly expressed in Sertoli cells and moderately in Leydig cells. In germ cells the expression has been reported to be absent [Braissant et al., 1996]. In the same study Braissant and co-workers found that PPARγ was barely detectable in Sertoli cells, while absent in germ cells and Leydig cells. A later study showed that PPARα protein is present in the Sertoli cell nuclei in a stage-specific manner, with the highest expression in stages II-VI and XIII-I of the seminiferous epithelial cycle [Shultz et al., 1999] (see *1.2.1.2 The spermatogenesis*). This suggests that PPARα expression is controlled by FSH in the testis (see *1.2.1.4 Endocrinology of the testis*).

Studies with PPARα knockout mice have demonstrated that PPARα controls numerous genes related to lipid metabolism in liver. These include genes involved in β-oxidation, acyl-CoA thioesterases [Hunt et al., 1999; Hunt et al., 2000], fatty acid uptake, fatty acid binding protein

and lipoprotein assembly and transport [Motojima et al., 1998; Aoyama et al., 1998; Peters et al., 1997]. The hepatic fatty acid oxidation was dramatically impaired in PPARα knockout mice, resulting in an induction of fatty liver when feeding the mice a high fat diet [Kersten et al., 1999]. The function of PPARδ is not yet fully understood. PPARγ on the other hand regulates the transcription of many genes involved in glucose and lipid homeostasis and genes involved in maintaining normal insulin responsiveness. Furthermore, PPARα and -γ are also known to regulate the expression of fatty acid transport protein (FATP) and acyl-CoA synthetase (ACS) in a tissue specific way [Martin et al., 1997]. FATP and ACS have been suggested to play a crucial role in the transport of fatty acids into the cell.

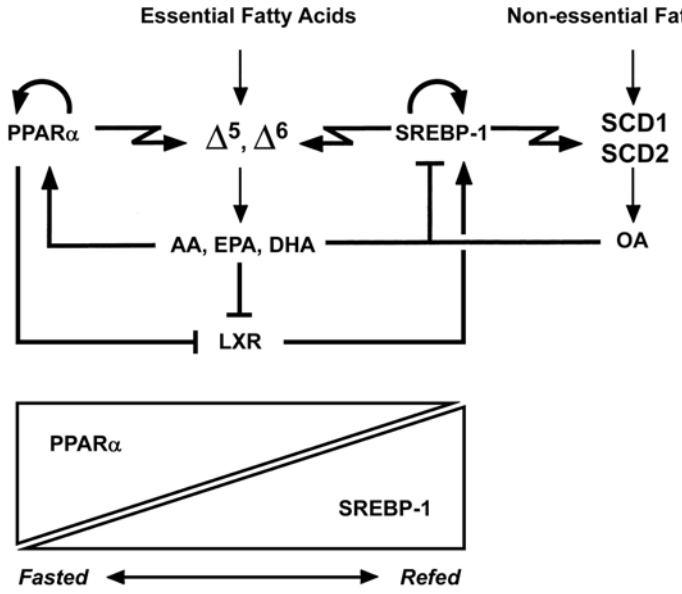

Figure 1.5 Dual regulation of the desaturases by PPARα and SREBP-1c in liver. Depending on the dietary status, the desaturase expression is controlled by PPARα (fasted) or SREBP-1c (refed). The cross-talk between the two is mediated by PUFAs and LXR. For details, see *1.1.4.1 PPARs* and *1.1.4.2 SREBPs*. Δ^5, Δ^6: Δ^5- and Δ^6-desaturase, AA: arachidonic acid, EPA: eicosa-pentaenoic acid, DHA: docosa-hexaenoic acid, OA: oleic acid.

PUFA-rich diets have been shown to inhibit the expression of SCD1, Δ^5-desaturase and Δ^6-desaturase in liver (fig. 1.5) [Ntambi, 1999; Cho et al., 1999a; Cho et al., 1999b]. However, it has become evident that PUFAs when bound to PPARα induce the expression of the same genes [Ntambi, 1999; Matsuzaka et al., 2002]. These contradictory observations can be explained by the dual regulation of the desaturases by PPARα and SREBP-1c (fig 1.5). Depending on the nutritional state, PPARα and SREBP-1c alternate in being the dominating transcription factor controlling the desaturase expression. PPARα is the dominant in fasted animals, while SREBP-1c dominates in the refed or normal state. The cross-talk between

PPARα and SREBP-1c is probably mediated by oxysterol liver X receptor (LXR), since PPARα seems to inhibit LXR expression [Johnson & Ledwith, 2001], while LXR induces SREBP-1c expression [fig. 1.5; Yoshikawa et al., 2002]. Finally, activated PPARα has been shown to induce its own expression, forming an autoloop [Valmaseda et al., 1999].

The PPARs' earlier mentioned promiscuousness regarding ligand binding, has led scientists to search for isoform-specific, highly potent ligands. In this work we make use of a PPARα-specific fatty acid analogue termed tetradecylthioacetic acid (TTA). TTA, which has the chemical structure $CH_3(CH_2)_{13}–S–CH_2–COOH$, was first synthesised at the Institute of Medical Biochemistry, University of Oslo, in 1989 [Spydvold & Bremer, 1989].

1.1.4.2 Sterol regulatory element-binding proteins

The sterol regulatory element-binding proteins are membrane bound transcription factors that belong to the basic helix-loop-helix leucine zipper family [Brown & Goldstein, 1997]. The inactive form of the SREBPs is bound to ER through two transmembrane domains, where the N- and C-terminal domains face the cytosol. A sterol-regulated proteolytic cleavage by SCAP (SREBP cleavage-activating protein) is required for the release of the active N-terminal fragment [Sakai et al., 1996]. SREBP can then enter the nucleus and activate the transcription of target genes, involved in synthesis and receptor-mediated uptake of cholesterol and fatty acids [Sakai et al., 1996]. SREBPs bind to sterol regulatory elements (SREs) which tend to vary. The SREs often contain direct repeats of C/TCAC [Magana & Osborne, 1996; Sakai et al., 1996] and an E-box motif (CANNTG) [Tabor et al., 1998].

Three forms of SREBP have been identified in mammals; SREBP-1a, SREBP-1c (also known as ADD1) and SREBP-2. SREBP-1a and c, which originate from alternative splicing of the same gene, seem to be ubiquitously expressed in mouse and human, but at different ratios [Shimomura et al., 1997]. The 1c:1a ratio is more than 6 in mouse and human liver, while less than 0,1 in spleen. SREBP-1a is a strong activator of cholesterol and fatty acid biosynthesis, whereas SREBP-1c is selective for fatty acid biosynthesis [Horton et al., 1998; Shimano et al., 1997; Pai et al., 1998]. As an exception, whole testis does not express SREBP-1c and only very low levels of SREBP-1a mRNA. SREBP-2 also seems to be ubiquitously expressed both

in man [Hua et al., 1993] and rodents [Wang et al., 2002]. SREBP-2 is relatively selective in inducing genes participating in cholesterol biosynthesis [Horton et al., 1998; Pai et al., 1998]. Recently a germ cell specific SREBP-2 isoform termed SREBP-2gc, was discovered in rodent germ cells. SREBP-2gc protein is expressed in a stage-specific manner, as a constitutively active transcription factor that is not subjected to feedback control by sterols [Wang et al., 2002].

SREBP-1 gene expression has been shown to be directly activated by LXR-RXR through an LXR response element [Schultz et al., 2000; Repa et al., 2000]. Furthermore, promoter analysis has revealed that SREBP-1c, in addition to LXR, is regulated by SREBP-1c itself, forming an autoloop [fig.1.5; Amemiya-Kudo et al., 2000]. As mention above, PPARα induces SCD1, Δ^5-desaturase and Δ^6-desaturase in liver, Interestingly, SREBP-1c does the same [Ntambi, 1999; Matsuzaka et al., 2002]. But while long-chain PUFAs, the desaturase products, activate PPARα, they inhibit SREBP-1c activity as shown in figure 1.5. This inhibition may be mediated through several mechanisms. PUFAs has been shown to inhibit the transcription of SREBP-1 in part by antagonising ligand dependent activation of LXR [Ou et al., 2001; Yoshikawa et al., 2002], and furthermore, PUFAs and oleic acid inhibit the proteolytic maturation of SREBP-1 by SCAP [Worgall et al., 1998; Thewke et al., 1998].

1.2 THE TESTIS AND THE EPIDIDYMIS

1.2.1 The testis

The testes in all mammals are paired encapsulated ovoid organs consisting of seminiferous tubules (*tubuli seminiferi*). Their size varies, depending on the species. In rodents they can represent up to 1% or more of the body weight. The capsule covering each testis is called *tunica albuginea*. This thick capsule cannot distend, and thus, any oedema-producing injury of the testis can result in ischaemic damage to the spermatogenic elements within. Most of the testis is made up by the seminiferous tubules, where the spermatozoa are formed. In most species these tubules are between 200 and 350 μm in diameter. Arising from the tunica albuginea and extending into the substance of the testis are a series of fibrous *septa* which divides the testis into *lobules*. The convoluted seminiferous tubules reside within these

lobules. In most mammal species both ends of the seminiferous tubules are opened into the *rete testis*. The rete testis is a complicated network of intercommunicating channels. Through this network the spermatozoa, and the fluid in which they are surrounded, are transported to the *epididymis*. In the rat, rete testis is a fairly simple sac under tunica albuginea where the *efferent ductules* run to the epididymis.

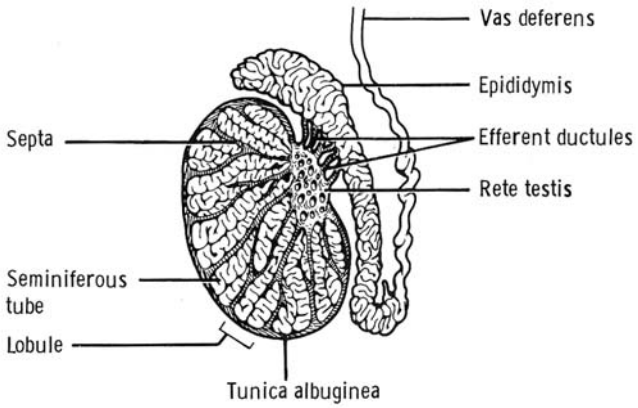

Figure 1.6 A cross-section of the testis, its excurrrent ducts together with the epididymal duct and vas deferens (Semen Analysis – A practical guide, Blackwell Scientific Publ. 1986).

The walls of the seminiferous tubules in the rodent testis are composed of four layers. First a layer of smooth muscle-like or *myoid cells* surrounds an innermost layer of non-cellular material or the *basal lamina* (fig. 1.7). This layer is thought to be responsible for the peristaltic movement of the tubulus seminiferus. Then there is a layer of *collagen fibers*, and finally, on the outside, a layer of *endothelial cells*. The endothelial layer partially lines groups of *Leydig cells* in the *interstitial tissue* (fig. 1.7).

The basal lamina forms a matrix upon which the *germinal stem cells*, the *spermatogonia*, and the *Sertoli cells* attach (see fig. 1.7). The Sertoli cells, the somatic cells in the seminiferous tubules, stretch from the basal lamina and protrude all the way into the lumen of the tubules. Tight junctions between adjacent Sertoli cells near the lamina form the so-called *"blood-testis barrier"*. The blood-testis barrier prevents an undiscriminated import of large molecules from the interstitial tissue and the tubular wall into the adluminal compartment. However, steroids penetrate this barrier with ease, and some proteins pass from the Sertoli cells to the Leydig cells and vice versa. Another important feature of the blood-testis barrier is preventing contact

between the lymphatic system and the tubular cells. Such a connection could cause auto-immuno responses towards e.g. germ cells [Byers et al., 1993]. Sperm antibody production is known from certain groups of patients causing the sperms to aggregate in the seminal fluid lowering the fertilisation potential [Shibahara et al., 2002].

The Sertoli cells support the germ cells as they mature and divide. The germ cells pass through the blood-testis barrier and move to the lumen as they mature. This appears to occur without disruption of the barrier, by progressive breakdown of the tight junctions on the luminal side of the germ cells, with concomitant formation on the basal side.

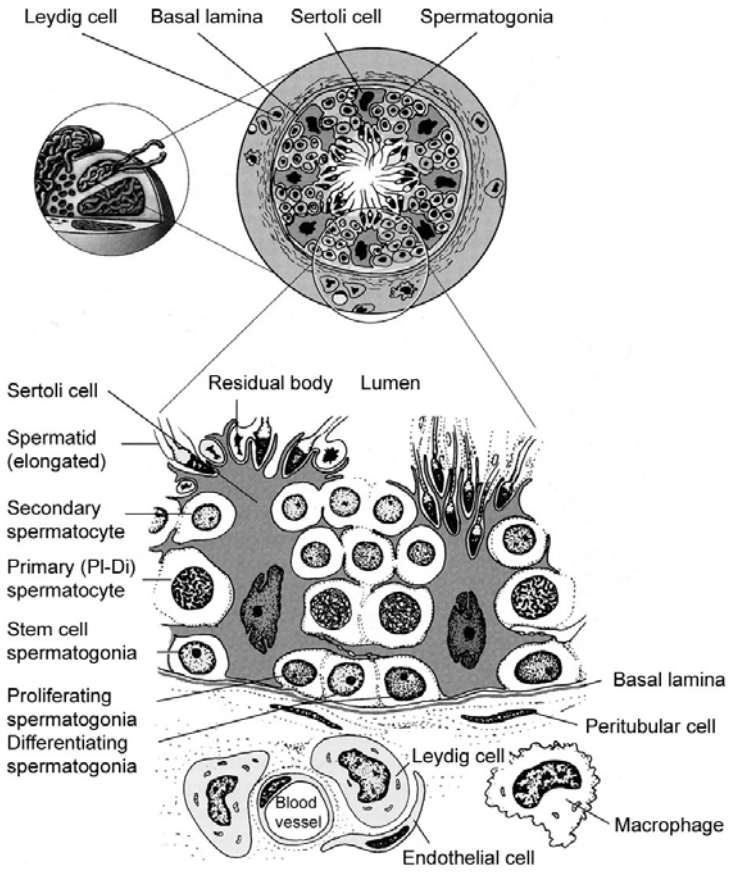

Figure 1.7 The testis. Localisation, ultrastructure and cellular organisation of tubuli seminiferi. The main cell types are described in the text. Pl-Di: preleptotene to diplotene, see tab 1.1. (Andrologi, Liber Utbildning, 1994).

1.2.1.1 The Leydig cell

The Leydig cells are located in the interstitial tissue partly lined by the tubular endothelial layer. The main function of the Leydig cells is production and secretion of testosterone. In the male, the Leydig cell is the main target of luteinising hormone (LH), hence the cell expresses serpentine LH receptors (see *1.2.1.4 Endocrinology of the testis*). LH enhances both production and secretion of testosterone by inducing the expression of genes involved in the steroidogenesis. Testosterone is synthesised from cholesterol, but there is still uncertainty about the relative substrate contribution from circulatory LDL-cholesterol and testis-synthesised cholesterol-esters, respectively.

The secretion of testosterone has been correlated to the amount of smooth endoplasmatic reticulum and Golgi membranes within the Leydig cell [Wing et al., 1984]. In fact, smooth endoplasmatic reticulum is the dominant organelle in the Leydig cell. Another histological feature of these cells is lipid inclusions. These lipid stores are shown to be rapidly depleted in response to trophic LH-stimulation, accompanied by an increased testosterone secretion. The opposite effect is seen when examining Leydig cells after withdrawal of gonadotropins [Aoki & Massa, 1975]. Independent of their origin, these inclusions must be substrate stores for the biosynthesis of testosterone.

1.2.1.2 The spermatogenesis

Most mammalian species form millions of sperm each day in their sexually active period. The process in which the germ stem cells (spermatogonia) form the spermatozoa (sperms) is called the spermatogenesis. Spermatogenesis may be divided into three phases (tab. 1.1):

1. The *proliferative* phase, in which cells undergo rapid successive division.
2. The *meiotic* phase in which genetic material is recombined and segregated.
3. The *differentiation* or *spermiogenic* phase in which the spermatids transform into cells structurally equipped to reach and fertilise the oocyte.

Reproductive strategies in male of all species dictate that cell numbers are increased early in the process of spermatogenesis. The spermatogonial cell population is fulfilling this need.

These relatively immature cells undergo numerous mitoses to build a large population of cells that will subsequently undergo two meiotic divisions and differentiation to form sperms. Despite all the energy consumed in producing spermatozoa, up to 75% of all germ cells fail to complete the spermatogenic cycle and undergo apoptosis [Huckins, 1978]. This selection is a necessity in removing abnormal spermatozoa and reducing the amount of germ cells to a level maintainable for the Sertoli cells.

Cell types	Ploidity	Histological Features & Developmental Checkpoints	Fig. 1.8
Stem cell spermatogonia	2n	Flattened along the basal lamina, rounded surface in contact with the Sertoli cells. Resistant, potential to repopulate tubuli seminiferi after testicular insults.	-
Proliferating spermatogonia	2n	See *Differentiating spermatogonia.* The cells undergo rapid successive division.	-
Differentiating spermatogonia	2n	Shaped like the stem cells. Connected by cytoplasmatic intercellular bridges. This promotes synchronous development.	A_1-A_4, In, B
Preleptotene spermatocyte	4n	Slightly smaller than differentiating spermatogonia. The last cells of the spermatogenic sequence to go through S-phase (synthetic phase).	Pl
Leptotene spermatocyte	4n	The cell and nucleus take on a rounded from. Moves slightly away from the base of the tubuli.	L
Zygotene spermatocyte	4n	Pairing of homologous chromosomes. The cell becomes isolated in the intermediate compartment of the tubulus.	Z
Pachytene spermatocyte	4n	Fully paired chromosomes, genetic recombination (crossing over). Cell and nuclei enlarge greatly. The sex vesicle containing the sex chromatin is visible.	P
Diplotene spermatocytes	4n	Brief phase. Chromosomal pairs separate, except at crossing over regions known as chiasmata. Largest of all germ cell types	Di
Secondary spermatocytes	2n	Completes the first meiotic division (MI), the second meiotic division (MII) follows rapidly. MII cells are considerably smaller than MI cells	II
Round Spermatids	n	Forming of the axoneme (proflagella), a structure containing microtubules (9+2 arrangement) and peri-nuclear Golgi apparatus that later give rise to the proacrosomal vesicle.	1-7
Elongated spermatids	n	Polarisation of the cell with the growing flagella at the luminal pole and the acrosomal vesicle (now contacting the nucleus) at the basal pole. Mitochondria are recruited from the cytoplasm to form a helical pattern around the middle piece of the flagellum. Condensation of the nucleus.	8-18
Spermatozoa	n	Further condensation; separation of a cytoplasmic package (the residual body) formed at sperm release. The residual body contains RNA and packed organelles no longer necessary for sperm survival. This fragment is phagocytosed and digested by the Sertoli cells.	19

Table 1.1 Description of the cell types participating in the spermatogenesis; from the stem cells at the basal lamina to the spermatozoa released in the lumen.

As shown in figure 1.7, the germ cells are not randomly scattered among the Sertoli cells. Embedded in the Sertoli cells and with fixed spermatogenic intervals, the germ cells proliferate, recombine and differentiate as they move from the base of the tubulus towards the lumen. This establishes discrete cross-sections of tubuli seminiferi with certain combination of spermatogonia, spermatocytes and spermatids. In rat it is possible to discriminate between 14 types of cross-sections or "stages" (fig. 1.8). These stages organise the tubulus in a functional way. The stages follow directly after each other; I-II---XIII-XIV-I-II---XIII along the tubulus seminiferus, constituting a seminiferous wave [Perey et al., 1961]. As can be seen in figure 1.8, the germ cell has to go through four and a half cycle before it is released as spermatozoon, a process that spans 52 days in the rat. Consequently, the spermatozoa are released at "the top of the wave" (stage VIII) approximately equally spaced from the leading and lagging crest.

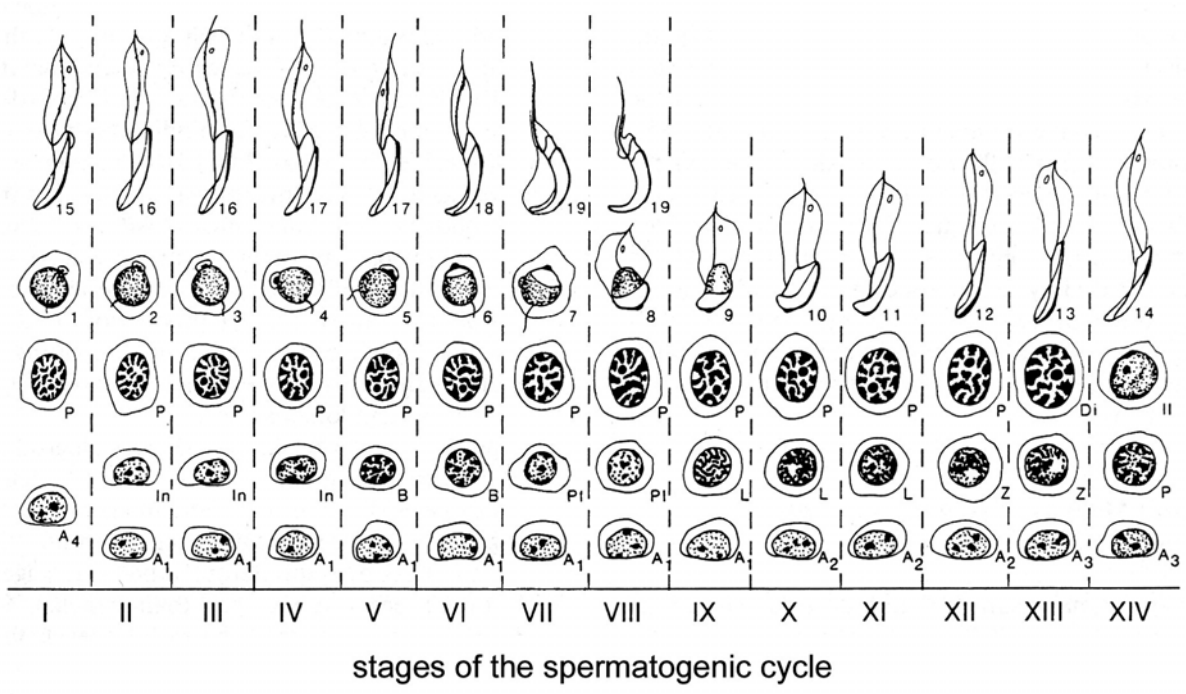

Figure 1.8 Stages of the spermatogenic cycle in rat. One cycle consists of 14 stages. Stages I-VI are FSH dependent, while stages VII-VIII are androgen dependent. The germ cell has to go through four and a half cycle before it is released as a spermatozoon, a process that spans 52 days in the rat. See table 1.1 for abbreviations. (Modified from The Physiology of Reproduction Vol. 1, Raven press, 1994).

The different stages are hormonally controlled. Stages I-VI are FSH dependent or at least FSH-modulated, while stages VII-VIII are androgen dependent. Following testosterone

withdrawal (e.g. by destroying the Leydig cells with ethane dimethanesulphonate), appearance of degenerating germ cells is first observed in stage VII-tubules [Kerr et al., 1993]. Succeeding this, degenerating germ cells appear in stages VIII-XIV, indicating that these stages are secondary androgen dependent [Kerr et al., 1993].

The FSH modulation of stages I-VI ensures germ cell availability. FSH increases the availability of germ cells at four different steps of development (B-spermatogonia, pachytene spermatocytes, step 6 spermatids and step 18 spermatids) for entry into the androgen-dependent phase (stages VII-VIII). In this way it is clear that FSH and testosterone have synergistic effects on the spermatogenesis. It should be kept in mind, though, that the effect of both hormones is partly (testosterone) or entirely (FSH) mediated through the Sertoli cells described next (see also *1.2.1.4 Endocrinology of the testis*).

1.2.1.3 The Sertoli cell

The Sertoli cell is a tall (75-100 μm) simultaneously columnar and stellate cell with the base solidly attached to the basal lamina and an apex that reaches the lumen of the tubulus seminiferus. Numerous lateral and apical extensions surround and support every neighbouring germ cell (fig 1.7). The Sertoli cells make up a continuing circle along the periphery of the tubule by binding to each other in tight junctions and gap junctions, forming the "blood-testis barrier". This establishes two compartments within the seminiferous epithelium; a *basal* and an *adluminal* compartment. The Sertoli cell is the only cell in the male expressing FSH receptors, and thus, is the only male cell type responding to follicle-stimulating hormone. Furthermore, the Sertoli cell expresses the nuclear androgen receptor, making them responsive even to androgen action.

The supportive cell

The most obvious Sertoli cell function is their supportive role in keeping in place the clones of differentiating germ cells. The basal compartment contains spermatogonia and early spermatocytes, while the adluminal compartment contains meiotic spermatocytes and spermatids at various step of the spermiogenesis (fig 1.7. and 1.8) Following directly from this organisation is the "Sertoli cells lift"-function. As the germ cells mature, early meiotic spermatocytes are "lifted" from the basal to the adluminal compartment, through concomitant

breakdown and formation of tight junctions (see *1.2.1. The testis*). This perfectly timed and synchronised process is highly selective since only spermatocytes are lifted, leaving the spermatogonia behind. Information about the molecular mechanisms involved in this process is now accumulating [Beach & Vogl, 1999].

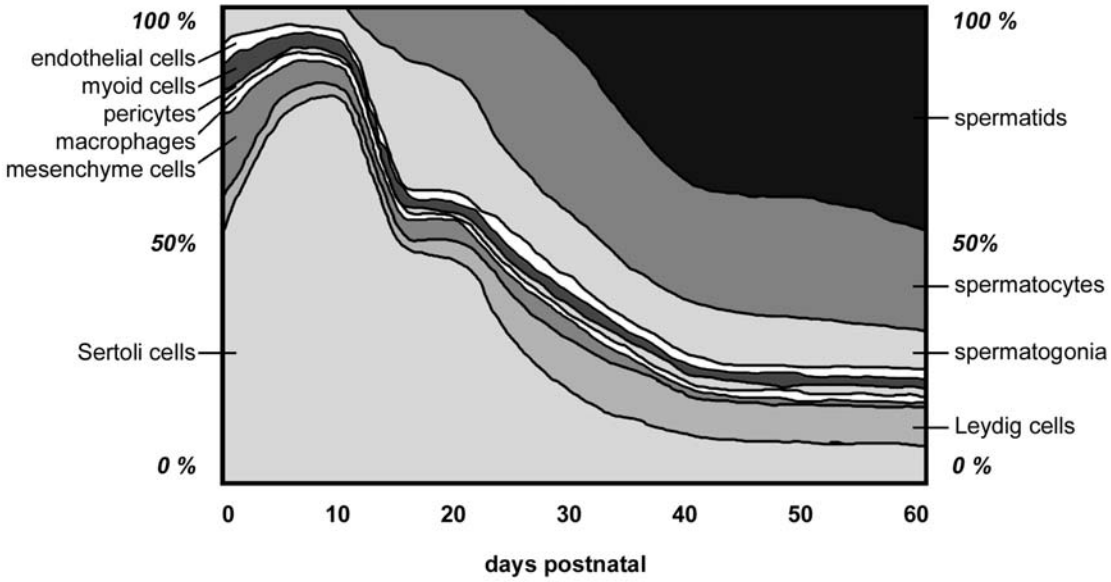

Figure 1.9 The cellular composition of the rat testis as a function of age. Estimated cellular composition of the rat testis (percent of all nuclei) as a function of age in the developing rat testis. The onset of spermatogenesis is observed between day 15 and day 20 in rat. On day 25 the first haploid cells appear, and as they become more abundant, the relative amount of Sertoli cells decrease until levelling off between day 40 and day 50. (Extrapolation of data published by Hardy et al. 1989, Clausen et al. 1979 and Gondos et al. 1993)

The nurturing cell

When acknowledging the close association between the Sertoli cells and the germ cells, and the selectivity of the blood-testis barrier, the nutritive function of the Sertoli cells is generally considered plausible. Sugars, amino acids, lipids and metallic elements are of great importance to the developing germ cell. For the spermatocytes and spermatids in the adluminal compartment, these substances are canalised via the Sertoli cells. Whether or not this flow through the Sertoli cells is facilitated in any way is not fully determined for different substances, e.g. lipids (see *1.3 Polyunsaturated fatty acids in testis and epididymis*). On the other hand, it is stated through several studies that the germ cells lack desired activities necessary to metabolise some of these nutrients, while they are found in Sertoli cells. Two important and descriptive examples can be given:

The Sertoli cells metabolise glucose to lactate, which is utilised by the germ cells. This theory is supported by numerous studies stating that Sertoli cells actively metabolise glucose. The majority of the glucose is converted to lactate (75%) and is not oxidised via the citric acid cycle [Robinson & Fritz, 1981]. FSH and insulin further stimulate this lactate production [Oonk et al., 1989], and finally *in vitro* studies have show that germ cells prefer lactate as an energy source over glucose and [Jutte et al., 1982]. Interestingly, two Japanese studies point in the direction that spermatocytes and spermatids differ with respect to energy metabolism. In pachytene spermatocyte cultures both pyruvate and lactate maintained the endogenous ATP-level, while in cultures of round spermatids only lactate did [Nakamura et al., 1984a; Nakamura et al., 1984b].

The Sertoli cells take up vitamin A from the circulation, and after esterification deliver it as chylomicrons to the germ cells [Shingelton et al., 1989]. This hypothesis was proposed by Shingelton and co-workers in 1989. Retinol has been shown to regulate gene transcription when binding as a ligand to the heterodimeric nuclear receptor RAR-RXR, and in testis vitamin A deficiency leads to germ cell degeneration. The Sertoli cells seem to be resistant to vitamin A deficiency, while the mature germ cells in the adluminal compartment all degenerate. In blood, vitamin A or retinol is transported as a complex, coupled to retinol binding protein (RBP) and transthyretin (TTR). Retinol is taken up by both Sertoli cells and germ cells in the basal compartment, facilitated by postulated retinol-RBP receptors [Sundaram et al., 1998]. The Sertoli cells efficiently metabolise retinol to retinyl ester [Shingelton et al., 1989], before it is transported as chylomicrons to the adluminal germ cells via a tentative LDL receptor-related protein (LRP) [Kowal et al., 1989].

The secreting cell

Another important feature of the Sertoli cell is its secretory function. It is now recognised that the Sertoli cells secrete a wide variety of proteins, as well as water, ions and many other substances, both at their apical and basal poles. Evidence for the secretion of proteins such as sulphated glycoproteins (SGP-1 and -2), androgen binding protein (ABP), metal carrier proteins like transferrin, proteases and inhibitors, growth factors and hormones (e.g. inhibin) has accumulated during the nineties. As the load of information has grown, it has become clear that the Sertoli cells, through their secretory products, are able to regulate the flux of fuels, hormones and factors, both locally in the testis and globally in the organism via the

circulatory system. However, most of this information has been derived from *in vitro* investigations on isolated Sertoli cells from immature animals. The importance of some of these data, *in vivo* in adult animals, remains to be clarified. Once again two examples can be given:

SGP-2 or clusterin is the dominant secreted protein by primary rat Sertoli cell cultures [Wilson & Griswold, 1979], and it is secreted exclusively at the apical side of the Sertoli cell [Danahey et al, 1986]. Studies have shown that SGP-2 is a heterodimeric protein which is localised in the cytoplasm of the Sertoli cell and on the head and tail plasma membrane of late spermatids and released spermatozoa [Sylvester et al., 1991]. However, SGP-2 mRNA is only found in Sertoli cells in the testis [Morales et al., 1987; Collard & Griswold, 1987]. Many hypotheses have been set forth to explain the role of SGP-2 in testis; one is linked to lipid transport. In 1990 de Silva and co-workers described a human high-density lipoprotein-associated protein which they designated apoJ [de Silva et al., 1990]. ApoJ was found to occur in complex with apolipoprotein A1 (apoA1) that harboured cholesterol ester transferase activity in human plasma. Later, amino acid and cDNA sequencing led to the discovery that apoJ was the human homologue of rat SGP-2. Studies done by Law and Griswold revealed that SGP-2 in plasma, seminiferous tubule fluid and epididymal fluid formed complexes with other proteins and lipids [Law & Griswold, 1994]. However, nor they or others have yet shown any specific SGP-2 association with the apolipoprotein-like particles containing apoA1.

APB was the first Sertoli cell secretion product to be discovered, mainly because of its androgen binding activity [French & Ritzén, 1973]. ABP or androgen binding protein is a homodimeric protein, produced and secreted by the Sertoli cells in rat [Hagenas et al., 1974], and in liver and testis, probably in the Sertoli cells, in man [Wang et al., 1989]. FSH has been shown to induce ABP [Hansson et al., 1974], and in this way FSH and testosterone action are linked. Joseph and co-workers have identified 3 functional domains, which includes the steroid-binding domain that binds both testosterone and the reduced form dihydrotestosterone [Joseph, 1994]. Furthermore, ABP harbours a dimerisation domain and a plasma membrane receptor-interacting domain. The ABP secretion is regulated by FSH, insulin [Mather et al., 1983], testosterone and glucocorticoids [Lim et al., 1996]. Studies have shown that 80% of the total ABP secreted are released on the apical side, entering the tubuli seminiferi lumen. The remaining 20% are secreted on the basal side, ending up in the circulatory system

[Gunsalus et al., 1980], where for historical reasons ABP is termed sex hormone-binding globulin (SHBG). The ABP released into the lumen is transported to the epididymis where ABP-testosterone/dihydrotestosterone is internalised via receptor-mediated endocytosis in androgen-regulated epididymal endothelial cells [Gerard et al., 1998]. In body fluids the ABP-androgen complex exist in equilibrium with free androgens. At the target cell, the free hydrophobic androgens pass the cell membrane, bind to the androgen receptor, enter the nucleus and perform downstream action at transcriptional level. In this way androgen-ABP executes local or primary action in the testicular tissue and global or secondary action in the organism as such.

The phagocytosing cell

The last of the four main Sertoli cell functions is the active involvement in the release of spermatids from the seminiferous epithelium called *spermiation*. This phenomenon, which has been well illustrated by several electron microscopic studies [Russell, 1984], includes three distinct phases. During the first phase the late spermatids are expulsed from the Sertoli cell crypt, observed as a migration of the spermatids from the depth of the seminiferous epithelium towards the lumen. Next follows a retention of the spermatids along the surface of the epithelium, each spermatid head individually encapsulated by large Sertoli cell extensions. In the last phase the spermatid head separate from the Sertoli cell embrace. Concomitantly, the spermatid's cytoplasmic surplus or residual body (see *1.2.1.2 The spermatogenesis*) is selectively phagocytosed by the Sertoli cell. From this point the spermatid is termed spermatozoon. The molecular basis for this complex event is still a matter of speculation.

In a lipid perspective, one special remark must be done. A histological hallmark for functional Sertoli cells is the presence of lipid droplets in the cytoplasm. These droplets can easily be observed through a light microscope (fig. 2.5 and 2.7B). In rodent Sertoli cells, as well as in Sertoli cells from other species, the amount of droplets tends to vary in a cyclic fashion, with the highest density in the spermatogenic stages following spermiation [Niemi & Kormano, 1965]. This observation has led to the hypothesis that the accumulated lipid droplets are a result of the breakdown of phagocytosed residual bodies [Lacy, 1962]. The disappearance of lipid droplets in the following stages has further led investigators to hypothesise a lipid transport from the Sertoli cells to the germ cells. In sum, this suggests that the residual body lipids is re-utilised by the germ cells in a cyclic fashion [Chen et al., 1977]. Once again, the

lack of molecular proofs is evident. However, results that will nurture these theories, are presented in this thesis.

1.2.1.4 Endocrinology of the testis

With the main cell types in the testis described and some of its endocrine factors mentioned, a brief review of the testis endocrinology is needed. It has already been stated that Leydig cells are responsive to luteinising hormone (LH) resulting in the production of testosterone. Sertoli cells have been shown to be responsive to both testosterone and follicle-stimulating hormone (FSH). Furthermore, Sertoli cells produce and secrete inhibin. These and other factors are interconnected in what is often referred to as the pituitary-gonadal axis (fig. 1.11).

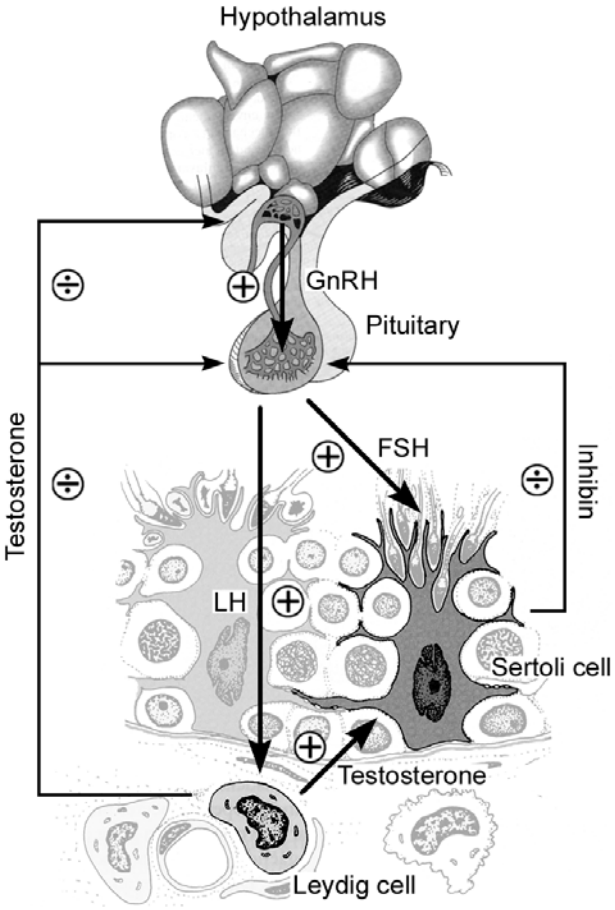

Figure 1.11 Endocrinology of the testis. Simplified sketch of the factors and cell types involved in the hormonal control of testicular function. +: increased activity, ÷: decreased activity. See text for details. (Modified from Andrologi, Liber Utbildning, 1994 & Review of Medical Physiology, 19th. Edition, Appleton and Lange, 1999).

The *hypothalamus*, which is also included in this axis, secretes the gonadotropin-releasing hormone (GnRH) in a pulsatile fashion. This secretion originates from neuroendocrine cells in an area called the *median eminence*. GnRH is transported via the portal hypophysial vessel to the anterior pituitary lobe. Here GnRH stimulates the release of the gonadotropins, FSH and LH. The gonadotropins follow the circulatory system ending up at their target cells in the gonads; LH acting on the Leydig cells inducing the production of testosterone and FSH acting on the Sertoli cells inducing the production and secretion of among others ABP and inhibin.

The first cross over in this axis has already been mentioned, namely the action of testosterone on the Sertoli cells. To control the levels of the gonadotropins, there exist feedback mechanisms. Serum testosterone acts on the hypothalamus, inhibiting the secretion of GnRH, and on the pituitary, inhibiting LH secretion. Serum inhibin, originating from the Sertoli cells, also acts on the pituitary, but this hormone inhibits FSH secretion. Activin, a member of the inhibin family, still controversial with respect to relevance and site of production, stimulates FSH secretion. In the healthy adult male this endocrine interplay regulates the levels of the factors mentioned globally. This results in optimal local concentration, e.g. in testis, ensuring a functional spermatogenesis.

In prepubertal mammals no gonadotropins are secreted, even though GnRH is produced by the hypothalamus. The onset of puberty, hence spermatogenesis in males, coincides with the start of pulsatile secretion of GnRH (see fig 1.9). A further discussion of the molecular basis for the endocrinological events both during puberty, adulthood, and senescence (the andropause) is beyond the scope of this thesis.

FSH – mechanism of action

FSH is a peptide hormone and thus can not pass the plasma membrane. The external FSH signals are therefore translated to internal messages through a transduction mechanism localised within the Sertoli cell membrane: Arriving FSH molecules are recognised by the Sertoli cells via the membrane-spanning FSH receptors that undergo a conformational change and thereby transmit information through the plasma membrane and into the cell. This is a G protein-coupled process. Like other peptide hormone receptors, the FSH receptor in the Sertoli cell interacts with the G_S protein [Casey & Gilman, 1988]. G proteins are “transducer molecules” belonging to the guanine nucleotide-binding protein family. The G-proteins

activate or inhibit effector systems, e.g. adenylyl cyclase, phospolipase C, phospolipase A_2, Ca^{2+}-channels or cyclic GMP phosphodiesterases. The two major signalling pathways; the cAMP signal transduction pathway and the phosphatidylinositol pathway are present in Sertoli cells [Scott, 1991; Nishizuka, 1986]. However, only the cAMP signal transduction pathway, which employs the second messenger cyclic adenosine monophosphate (cAMP,), is activated by FSH via the transducer G_S. A simplified presentation of this can be seen in figure 1.10.

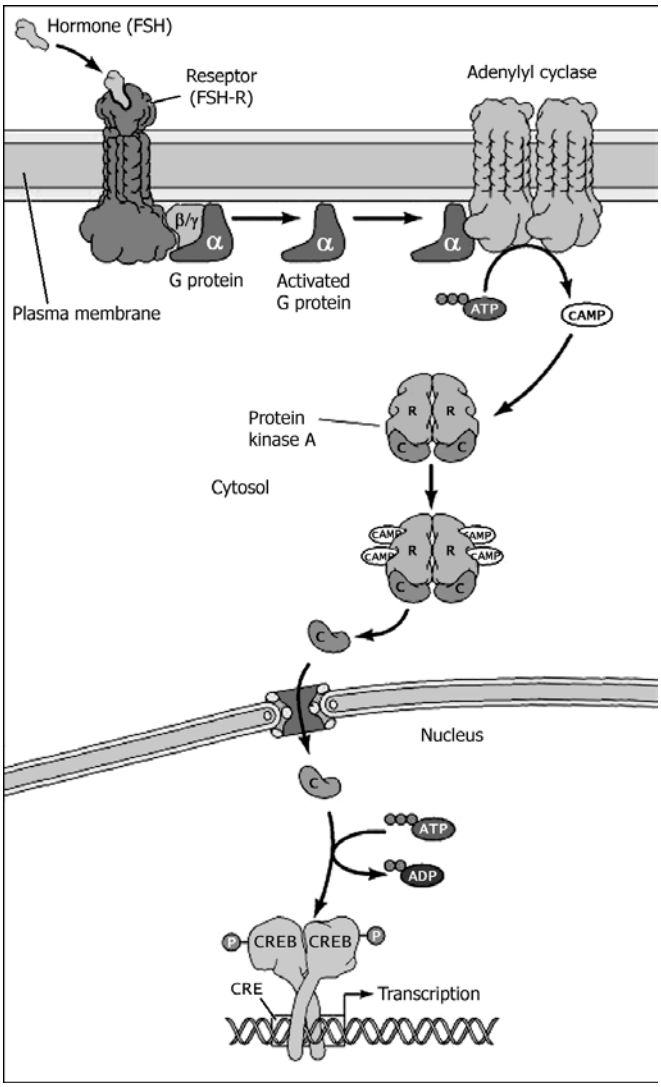

Figure 1.10 FSH – mechanism of action. FSH bound to the FSH receptor starts a signalling cascade from receptor-bound G_S protein via adenylyl cyclase, cAMP, PKA, catalytic subunit C, to phosphorylated nuclear CREB. P-CREB dimerises, binds to cAMP response element (CRE) and performs downstream action. See text for details. (Modified from The Cell - A Molecular Approach, 2nd Edition, Sinauer Associates Inc, 2000)

When FSH binds to the its receptor, the conformational changes that follows facilitate binding of the heterotrimeric G_S protein (G_S; α, β, γ) to the cytolsolic domain of FSH-R. This leads to the release of GDP from, and concomitant binding of GTP to, the α-subunit of the G protein. GTP-$G_S\alpha$ modulates the activity of distinct effector systems, i.e. adenylyl cyclase. Activated adenylyl cyclase then catalyses the formation of cAMP from ATP. cAMP can now bind to cAMP-dependent protein kinases (PKAs) in the cytosol. PKA is a tetrameric protein consisting of two regulatory and two catalytic subunits (C_2R_2), which dissociate upon binding of four cAMPs to the regulatory subunits.

The catalytic subunits then pass the nuclear membrane and activate transcription factors within the nucleus by phosphorylation. One example given is the cAMP response element binding protein or CREB, which belongs to B-ZIP family of transcription factors. The B-ZIP family is named so because of their basic region and leucine zipper-region within the DNA-binding domain. Dimerisation through the Lucien zipper-region is required for DNA-binding and transactivation. CREB binds to upstream cAMP response element (CRE; 5'-TGACGTCA-3') and activates gene transcription.

Due to the large number of PKAs and PKA-activated transcription factors, FSH-action is far more complex than described here. Cross-talk between different signalling pathways may increase the complexity even more.

In this thesis, FSH-induced desaturase expression was observed (see *3.1.2 Hormonal regulation of the desaturases*). Furthermore, dibutyryl cAMP, $(Bu)_2$cAMP, was employed to confirm the FSH effects. $(Bu)_2$cAMP is a cAMP analogue, capable of crossing the cell membrane. In the cytosol dibutyryl cAMP is cleaved into butyrate and monobutyryl cAMP, the latter with the capability of activating PKA.

1.2.2 The epididymis

The epididymal duct is a single, highly convoluted duct, closely applied to the surface of the testis, extending from the anterior to the posterior pole of the organ. The epididymis is held more or less firmly, depending on the species, to the tunica albuginea by connective tissue.

Except from the initial segment where the efferent ductules empty, the epididymis is loosely subdivided into three parts, termed *caput*, *corpus* and *cauda epididymis*. The initial segment, caput and corpus epididymis are primarily concerned with sperm maturation, whereas cauda epididymis represents the region where mature sperm are stored before ejaculation via *vas deferens* into the urinary tract.

The epididymal sperm maturation process, leading to the acquisition of motility and fertilising capacity, involves different membrane remodelling steps. These include the uptake of secreted epididymal glycoproteins, removal or utilisation of specific phospholipids from the inner leaflet of the sperm lipid bilayer, processing of acquired or existing glycoproteins by endoproteolysis and the re-positioning of both proteins and lipids to different membrane domains. When passing through the epididymis, the spermatozoon has little capacity to alter its membrane through *de novo* synthesis. Thus, the physical and functional changes in the sperm membrane are mediated through modification of the cell surface governed by the surrounding environment and through reorganisation of endogenous components. The sperm maturation process is therefore dependent on functional epididymal cells.

The main sperm lipid changes occurring in the epididymis are treated in chapter *1.3 Polyunsaturated fatty acids in testis and epididymis*, but some examples of the changes at membrane protein level can be given. About 50% of the glycoproteins found on the sperm membrane in an ejaculate, originate from the epididymis [Jones, 1998]. Most of these proteins are uniquely produced and secreted in the epididymal epithelial cells. They attach to the spermatozoa either by ionic interactions, like HIS50 [Rifkin and Oslon, 1985], by GPI anchors, like CD52 [Yeung et al., 1997], or by covalent binding to lipids or membrane proteins [Rooney et al., 1993; Olson et al., 1997]. One quite rare mechanism is the “protein-off-protein-on” exchange: Testicular SGP-2 or clusterin (see *The secreting cell in 1.2.1.3 The Sertoli cell*) is selectively lost at the initial segment of the epididymis, and thereafter, replaced with a low molecular weight-form, secreted by the caput epididymis [Sylvester et al., 1991].

CE9, PH20 and fertilin are examples of rat sperm membrane glycoproteins that undergo processing and rearrangement during maturation in the epididymis. All three are site-specifically endoproteolytically cleaved [Petruszak et al., 1991; Lum & Blobel, 1997; Jones et al., 1996] and repositioned. CE9 changes its position from all over the tail of caput spermatozoa to the midpiece region of caudal spermatozoa [Cesario & Bartles, 1994]. PH20

and fertilin, which originate from all over the sperm head, move to the postacrosomal region. The rearrangement of PH20 and fertilin may be coupled directly to the endoproteolysis [Phelps et al., 1990].

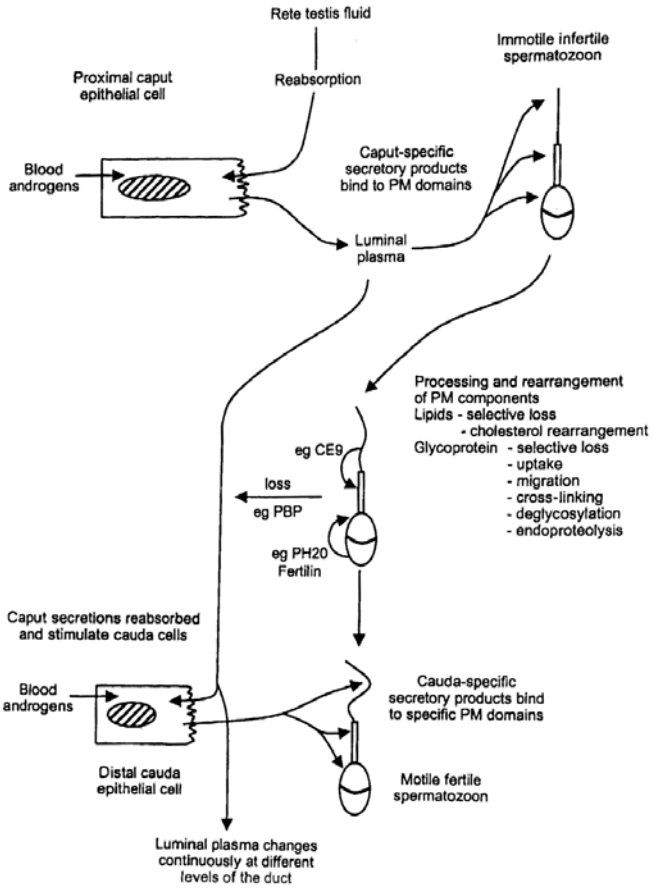

Figure 1.12 Summary of processes involved in plasma membrane remodelling during maturation of spermatozoa in the mammalian epididymis. See text for details. PM: plasma membrane, PBP: phospholipid-bound proteins. (Picture taken from Jones, 1998).

1.3 POLYUNSATURATED FATTY ACIDS IN TESTIS AND EPIDIDYMIS

The testis is an extraordinary organ regarding the metabolism of polyunsaturated fatty acids (PUFAs). In contrast to other PUFA-rich tissues, like the brain and retina, the testis is continuously drained of these fatty acids, as the spermatozoa are transported to the epididymis. Testicular cells are rich in PUFAs, with different fatty acids dominating depending on the species. The rat testis has a high content of 22:5(*n*-6) [Chanmugam et al., 1991], whereas 22:6(*n*-3) is dominating in human and monkey testis [Nissen et al., 1978;

Connor et al., 1997]. It has earlier been described that germ cells are especially rich in PUFAs, more than the Sertoli cells [Beckman & Coniglio, 1979], while the Sertoli cells seem to be more active in converting the dietary essential fatty acids to 22:5(*n*-6) and 22:6(*n*-3) than germ cells are [Retterstøl et al., 2001]. A PUFA transport from the Sertoli cells to the germ cells has therefore been postulated [Beckman & Coniglio, 1979].

Fatty acid transport protein (FATP; see *1.1.4.1 Peroxisome proliferator-activated receptors*) is highly expressed in the rodent brain, adipose tissue, testis and epididymis [Martin et al., 1997]. Not surprisingly, these are tissues rich in PUFAs and with a high desaturase expression. An exception here is the liver; though active in lipid metabolism, it does not express FATP. If modified PUFAs are transported from the Sertoli cells to the germ cells, FATP would be a likely candidate in mediating this transfer. In the absence of spermatogenesis, whatever the cause, lipids accumulate in the Sertoli cells [Lynch & Scott, 1951]. When impairing the spermatogenesis by destroying the Leydig cells with ethane dimethanesulphonate, abnormal accumulation of lipid droplets can be seen after 8 days in Sertoli cells [Kerr et al., 1993]. FATP is reported to be regulated by PPARα and PPARγ in rodents, since induced by fibrate and thiazolidinedione BRL 49653 [Martin et al., 1997]. Bearing this in mind, an earlier paper reporting abnormal spermatogenesis in RXRβ mutant mice, should be given new attention. Here the researchers found a progressive accumulation of lipids within the Sertoli cells, which were histochemically characterised as unsaturated triglycerides [Kastner et al., 1996].

In the progression of germ cell differentiation from spermatogonia to condensing spermatids in rodents, the total amount of 22:5(*n*-6) increases from 2 to 20% of total fatty acids, but is lowered in mature sperm [Beckman et al., 1978; Grogan et al., 1981]. During sperm maturation in the epididymis, the acrosome membrane undergoes a well-defined series of chemical, physical, and functional changes. These changes include an enrichment of highly unsaturated phosphatidylcholine. Studies on ram sperm indicate an increase in total 22:6(*n*-3) content from 14% to 25% [Nolan & Hammerstedt, 1997], which leads to a decrease in general membrane stability. At the same time, addition of cholesterol and an actively maintained asymmetric transmembrane phospholipid distribution modulate this effect, stabilising the membrane of the mature sperm for storage [Nolan & Hammerstedt, 1997]. Furthermore, the tail of the monkey spermatozoa has a higher content of 22:6(*n*-3) than the head. It has therefore been speculated if this distribution of PUFAs is important for the membrane fluidity

needed for proper motility [Connor et al., 1998]. These PUFA-asymmetries, both the acrosome transmembrane and the head-to-tail asymmetry, are necessary to produce a fertile gamete.

Nutritional studies have shown that rats fed diets deficient in essential fatty acids show testicular atrophy, which can not be prevented by the inclusion of linolenic acid [Leat et al., 1983]. Moreover, male rats were infertile when raised on a linoleic acid deficient diet [Leat et al., 1983]. It has also been reported that there is a significant lower level of 22:6(*n*-3) in the fatty acid composition of phospholipids of spermatozoa collected from patients with impaired sperm motility and patients with low sperm concentration, compared to normal patients [Zalata et al., 1998]. The reason for this difference is not known.

1.4 AIM OF THE STUDY

Even though the testis has been proven to be one of the most PUFA-rich organs in mammals together with liver, brain and retina, little work had been done in disclosing the localisation and regulation of the PUFA-metabolism in this organ. With the publication of the desaturase sequences and the establishment of collaboration between the Institute of Clinical Biochemistry at Rikshospitalet and the Andrology laboratory, we had the possibility to "add some pieces to the puzzle". Through several years the techniques necessary to isolate highly purified cell fractions of both germ cells and Sertoli cells, have been available at the Andrology laboratory. Furthermore, protocols for different rat feeding regimes and the extraction of lipids from biological materials have been running at the Institute of Clinical Biochemistry.

In this study we wanted to localise the primary site(s) of Δ^5-and Δ^6-desaturase, SCD1 and SCD2 expression, hence PUFA-metabolism, in the testis. Furthermore, we wanted to study the effects of essential fatty acid deficiency in the sexually immature rat testis, both on lipid and desaturase gene expression level. Nutritional studies have shown that the activity of the desaturases is upregulated in liver, when feeding rats a fat free diet. Resulting from the localisation studies, we further wanted to investigate the hormonal and nutritional regulation of the desaturase expression in primary cultures of Sertoli cells. Finally, with the PPARα, -β and -γ probes, provided by the Institute for Nutrition Research, we were tempted to try to

correlate the expression and regulation of the desaturases with PPAR expression, still incompletely described in testis.

As referred in the introduction, phospholipids of spermatozoa collected from patients with impaired sperm motility and patients with low sperm concentration have been reported to contain a significant lower level of 22:6(*n*-3), compared to normal patients [Zalata et al., 1998]. The reason for this difference is not known. The question of male fertility has during the past few years come into focus, due to alarming reports indicating a time-related decline in male reproductive function [Carlsen et al., 1992]. Although this question is still a matter of discussion, it has become evident that environmental, genetical and life-style related factors may have a significant impact on sperm parameters, and thereby on the fertility of the male. Therefore, in a wider perspective, understanding the mechanisms controlling male reproductive function is crucial for diagnosis and treatment of male infertility.

2. MATERIALS AND METHODS

2.1 ANIMALS – LOCALISATION STUDIES

Weaning Sprague-Dawley rats were obtained from M&B, Ry, Denmark. After separation from their mother, the rats were given a standard pellet diet from Grimstone Aldbrough, Hull, UK. Whole testis, epididymis, liver and kidney were dissected from CO_2-asphyxiated rats, frozen directly over liquid nitrogen and kept at –80°C for later RNA extraction.

2.2 ANIMALS – DIETARY STUDIES

Weaning Wistar rats were obtained from M&B. At the age of 21 days the rats, 6 littermates, were separated from their mother. The littermates were split into two groups of three. One group was fed a standard pellet diet from Grimstone Aldbrough, whereas the other group was fed a fat free diet consisting of 0,4% methionine, 1% cellulose, 2% vitamin mix, 5% salt mix, 20% casein, 20% sucrose and 52% maize starch [Tran et al., 2001]. The vitamin mix, salt mix and maize starch were a gift from Prof. Erling N. Christiansen at the Institute for Nutrition Research, University of Oslo. The feeding regimes lasted for 14 days before the rats were killed by CO_2-asphyxiation. Whole testis, liver and kidney were dissected from the rats and frozen directly over liquid nitrogen. The tissue was kept at –80°C for later RNA and lipid extraction.

2.3 GERM CELL PREPARATION

Enriched germ cell suspensions from juvenile Sprague-Dawley rats (32-, 44- and 58-day-old) were obtained by consecutive treatment with collagenase, trypsin and DNase [Bellve et al., 1975; Jutte et al., 1981]. The cells were further fractionated by centrifugal elutriation, followed by separation in density gradients of Percoll™, into pachytene spermatocytes (PS) and round spermatids (RST) [Meistrich et al., 1981]. For each germ cell preparation (PS and RST) testes from 5 animals were used. Two separate preparations representing all three ages were analysed. The protocol presented is designed for 5 animals resulting in approximately $2{,}5\times10^7$ PS and $6{,}0\times10^7$ RST:

2.3.1 Disintegration of tissue

1. Decapsulate testes from 5 rats, and transfer them to a 50 mL Falcon tube with 20 mL tissue medium.
2. Shake, remove the tissue medium, and add the collagenase solution.
3. Incubate the tissue for 20 min submerged in a shaking water bath; 34°C, 120 rpm.
4. Let the suspension sediment, and remove the supernatant.
5. Fill the Falcon tube with washing medium, and stir with a sterile wooden stick to remove blood veins.
6. Let the suspension sediment for 3 min, and remove the supernatant.
7. Add the trypsin/DNase solution, and incubate the suspension for 15 min submerged in a shaking water bath; 34°C, 205 rpm.
8. Add a spatula pinch of trypsin inhibitor to stop the reaction, and incubate at room temperature for 2-3 min.
9. Filter the suspension through a sterile 70 µM nylon membrane, and wash the membrane with elutriation medium.
10. Dilute the cell suspension to 100 mL, and determine the cell concentration.

Primary medium:
330 mg pyruvate
340 µL DL-lactic acid
1,5 L Hanks' balanced salt solution with Ca/Mg

Remove 180 mL → Washing medium.

3,1 g NDA
6,6 g BSA

Elutriation medium:
1320 mL primary medium
Rest of the DNase solution (~2 mL)
Adjust pH to 7,4 with 1M NaOH (appr. 2 mL).

Tissue medium:
10 mg BSA
10 mL washing medium

Collagenase:
10 mg collagenase
10 mg BSA
10 mL washing medium

Washing medium:
180 mL (see **Primary medium**)

DNase:
6 mg DNase
2 mL washing medium

Trypsin/DNase:
5 mg trypsin
50 µL DNase
10 mL washing medium

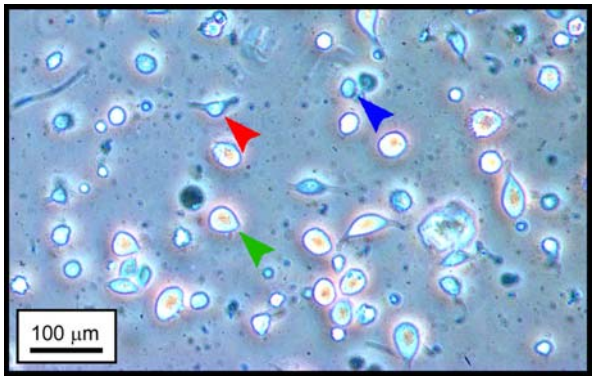

Figure 2.1 Disintegration of the testis tissue yields a crude germ cell extract (10^7-10^9 cells), which contains mononucleated PS, polynucleated PS, RST and elongated cells (elongating spermatids or endothelial cells). Pachytene spermatocytes can be seen (green arrows) together with round spermatids (blue arrows). An example of an elongated cell is marked with a red arrow. This cell could be an early, elongating spermatid, or more likely an endothelial cell. The picture is taken with a Spot Insight Colour digital camera connected to a Nikon Eclipse E400 microscope.

2.3.2 Centrifugal elutriation

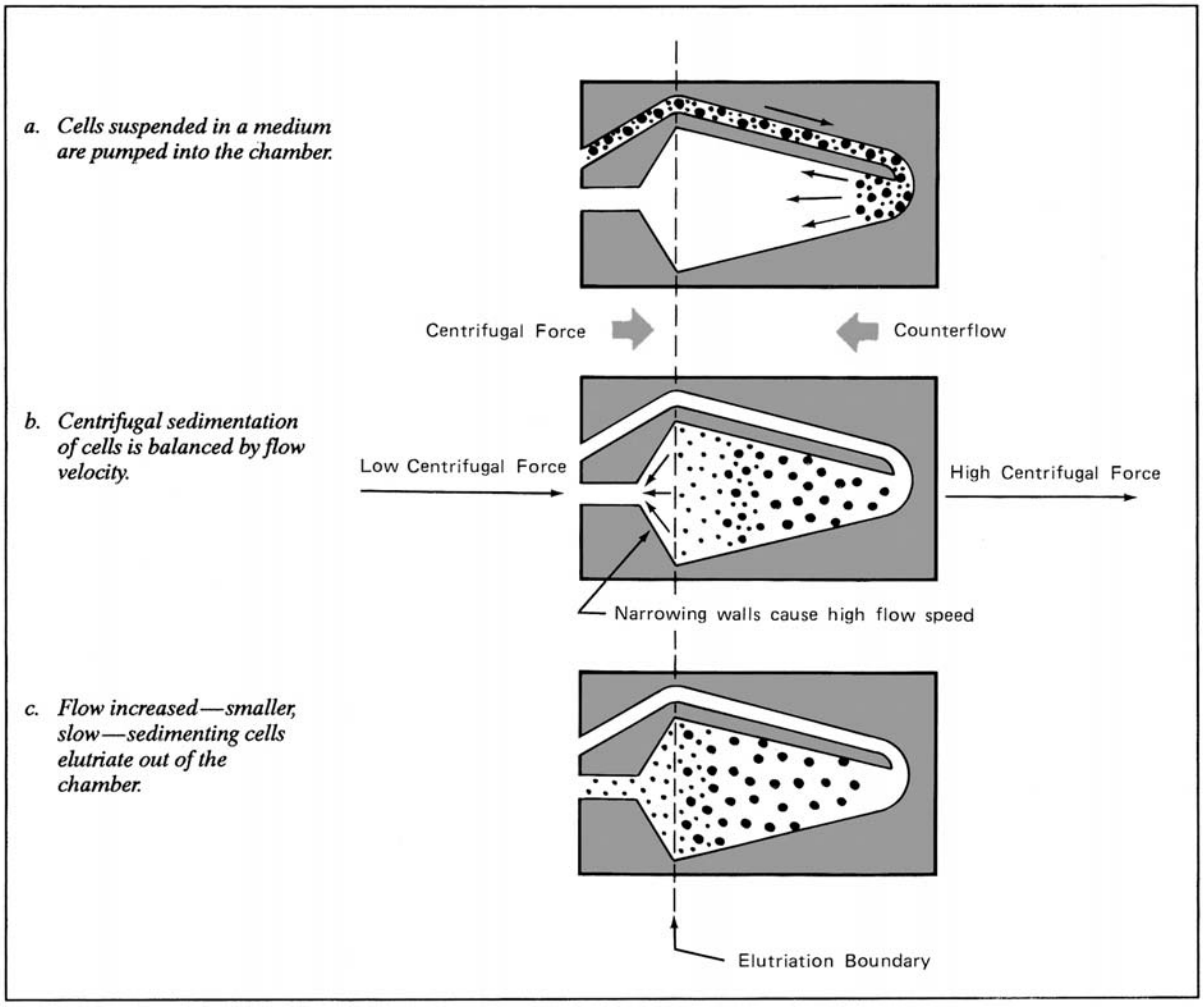

Figure 2.2 The elutriation process principals. (The JE-5.0 Elutriation System, Spinco, 1993).

The protocol is designed for centrifugal elutriation of 10^7-10^9 germ cells:

1. Load the cell preparation into the elutriation system through the tygon inlet line, and start collecting fractions right away. When the cell extract is loaded, place the inlet line in elutriation medium.
2. Adjust the speed of the centrifuge and peristaltic pump following table 2.1, and collect the fractions on ice.
3. Sediment fractions 2A and 2B (RST) and 5 (PS) by centrifugation; 600 rcf, 4°C, 5 min.
4. Pour off the supernatant directly after centrifugation, and place the tubes upside-down on a paper towel to remove as much of the medium as possible.
5. Optional: validate the cells by resuspending the cell pellets in 10 mL cold 1×PBS.
6. Resuspend the cell pellets in 4,15 mL 18% or 20 % Percoll™ for RST and PS, respectively.

Fraction	rcf	flow rate (mL/min)	volume (mL)
0	1750	15.5	300
1	=	23.0	150
2A (RST)	=	**31.0**	**75**
2B (RST)	=	**40.0**	**75**
3	750	23.0	150
4	=	29.0	150
5 (PS)	=	**40.0**	**150**

Table 2.1 Settings for centrifuge and peristaltic pump during centrifugal elutriation.

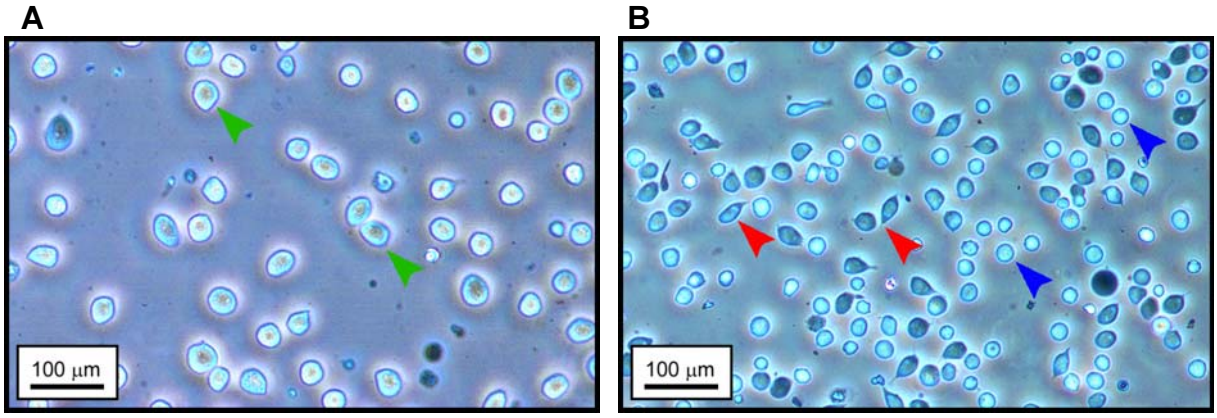

Figure 2.3 After the centrifugal elutriation the PS and RST fractions still contain some contaminating cells. In the PS fraction (A) the main contaminant is polynucleated pachytene spermatocytes. PS are marked with green arrows. In the RST fraction (B) endothelial cells/elongating spermatids (red arrows) is the main contaminant. RST are marked blue arrows. The pictures are taken with a Spot Insight Colour digital camera connected to a Nikon Eclipse E400 microscope.

2.3.3 Separation in density gradients of Percoll™

The protocol is designed for separation of crude RST and PS fractions (10^7-10^9 cells) in density gradients of Percoll™:

1. Prepare the Percoll™ solutions following tables 2.2 and 2.3.
2. Prepare the 23-33 % and 25-37% Percoll™ gradients following tables 2.4 and 2.5. The solutions (1-11) are placed carefully on top of each other with a Pasteur pipette in a centrifuge tube on ice.
3. Carefully load the RST dissolved in 18% on top of the 23-33 % Percoll™ gradient, and the PS dissolved in 20% Percoll™ on top of the 25-37% Percoll™ gradient.
4. Centrifuge the gradients; 11.000 rcf, 4°C, 10 min. Let gravity slow down the rotor head, use no breaks.
5. RST: collect the battery of band 2,0-3,5 cm from the tube bottom. PS: collect the broad band 1,0-3,0 cm from tube bottom. Employing Pasteur pipettes, transfer the cells into two 50 mL tubes on ice.
6. Adjust the volume to 50 mL with cold 1×PBS, and resuspend the cells.
7. Sediment the cells by centrifugation; 600 rcf, 4°C, 5 min.
8. Remove the supernatant, resuspend the cell pellets in 10 mL cold 1×PBS, and determine the cell concentration.

Reagents	18% (µL)	20% (µL)	sum (µL)
Percoll™	900	1000	1900
10×PBS	500	500	1000
4 mM Pyruvate	110	110	220
6 mM Lactate	110	110	220
dH_2O	3360	3260	6620
1 mg/mL DNase **(Fresh)**	20	20	40
Total Volume (µL)	5000	5000	

Table 2.2 18 and 20% Percoll™ solutions

Reagents	23% (mL)	25% (mL)	33% (mL)	37% (mL)	sum (mL)
Percoll™	3,8	4,2	5,5	6,2	19,7
10×PBS	1,7	1,7	1,7	1,7	6,8
50 mM $MgCl_2$	0,33	0,33	0,33	0,33	1,32
90 mM Pyruvate	0,37	0,37	0,37	0,37	1,48
Lactate	8,7 µL	8,7 µL	8,7 µL	8,7 µL	34,8 µL
dH_2O	10,2	9,8	8,5	7,8	36,3
5 mg/mL DNase **(Fresh)**	0,33	0,33	0,33	0,33	1,32
Total Volume (mL)	16,73	16,73	16,73	16,73	

Table 2.3 23, 25, 33 and 37% Percoll™ solutions.

Percoll™	1	2	3	4	5	6	7	8	9	10	11	Sum
23% (mL)	0,00	0,25	0,50	0,75	1,00	1,25	1,50	1,75	2,00	2,25	2,50	13,75
33% (mL)	2,50	2,25	2,00	1,75	1,50	1,25	1,00	0,75	0,50	0,25	0,00	13,75
% (mix)	33	32	31	30	29	28	27	26	25	24	23	

Table 2.4 23-33 % Percoll™ gradient.

Percoll	1	2	3	4	5	6	7	8	9	10	11	Sum
25% (mL)	0,00	0,25	0,50	0,75	1,00	1,25	1,50	1,75	2,00	2,25	2,50	13,75
37% (mL)	2,50	2,25	2,00	1,75	1,50	1,25	1,00	0,75	0,50	0,25	0,00	13,75
% (mix)	37,0	35,8	34,6	33,4	32,2	31,0	29,8	28,6	27,4	26,2	25,0	

Table 2.5 25-37 % Percoll™ gradient.

10×PBS: 1 tablet of PBS
20 mL dH_2O

50 mM $MgCl_2$: 0,5 mL 1 M $MgCl_2$
9,5 mL dH_2O.

90 mM Pyruvate: 50 mg pyruvate
5 mL dH_2O

4 mM Pyruvate: 220 µL 90 mM pyruvate
4,78 mL dH_2O

Percoll™: Store at 4°C.

Lactate: DL-lactic acid
Store at 4°C.

6 mM Lactate: 5,1 µL DL-lactic acid
10 mL dH_2O

5 mg/mL DNase: 25 mg DNase
5 mL dH_2O

1 mg/mL DNase: 200 µL 5 mg/mL DNase
800 µL dH_2O

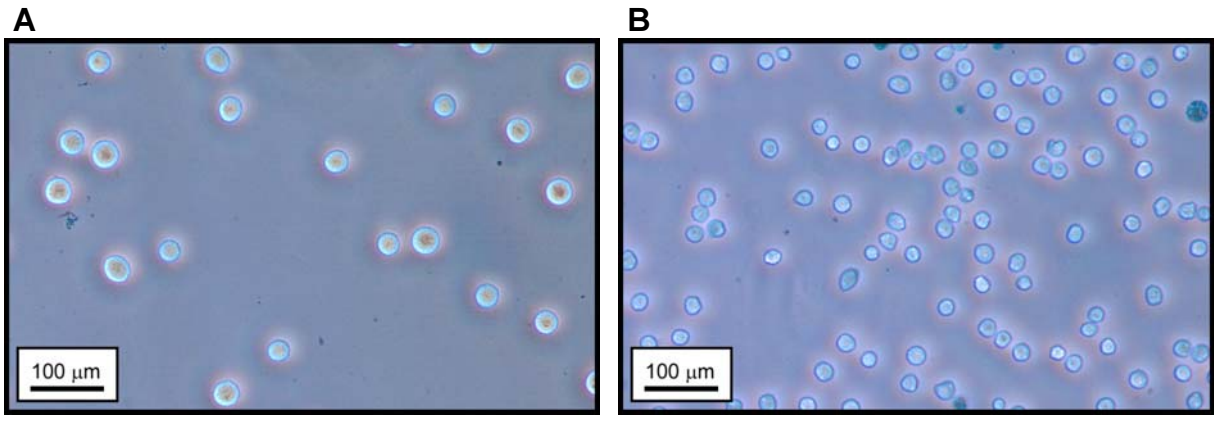

Figure 2.4 After Separation in density gradients of Percoll™ the fractions are relatively pure. This can be seen in figure 2.4A (PS), and figure 2.4B (RST). After validating and counting the cells, the PS and RST fractions were harvested by adding GTCM (3,1 mL GTCM per 10^7 cells). Earlier flow cytometry data show the strength of this protocol, with PS fractions purity higher than 85% and RST fractions purity higher than 90% [Retterstøl et al., 2001]. The pictures are taken with a Spot Insight Colour digital camera connected to a Nikon Eclipse E400 microscope.

2.4 PREPARATION OF SERTOLI CELLS

Primary cultures of Sertoli cells were obtained from testes from infantile (19-day-old) and juvenile (35-day-old) Sprague-Dawley rats [Dorrington et al., 1975]. These time points were chosen in order to obtain highly purified Sertoli cell preparations (19-day-old rats), and to study more mature Sertoli cells (35-day-old rats). Sertoli cells were plated on 10 cm Nunc dishes (Nunc, Copenhagen, Denmark) to a final concentration of ~ 10^6/mL in 12,5 mL Eagles' minimum essential medium (MEM; Gibco BRL, 21090-022, Grand Island, NY; non-essential amino acids included). The MEM was supplemented with L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (0,10 mg/mL), Fungizone™ (2,5 mg/mL) and 10% foetal bovine serum (FBS).

Cultures were kept at 34°C in a humidified atmosphere of 5% CO_2 in air. On day 3 after plating the medium was changed to MEM without FBS, and germ cells remnants (<4,0·10^5/mL) were removed mechanically (19-day-old rats) in combination with hypotonic shock treatment (10% MEM in water, 35-day-old rats). This was repeated on day 4 and 5. On day 5 the cells were harvested for RNA extraction (19-day-old rats: SC>98%, 35-day-old rats: SC>93%). These preparations were used for the localisation studies. For the stimulation experiments, cultured Sertoli cells from 19-day-old rats were used.

In addition, freshly isolated preparations, enriched in Sertoli cells from testes of both 19- and 35-day-old rats, were analysed. For these preparations the cells were harvested for RNA extraction directly after isolation, without being plated [SC>65%; Dorrington et al., 1975]. For both age groups (19- and 35-day-old rats) and isolation steps (cultured and freshly isolated cells) two separate preparations representing 10 animals each, were analysed. The protocol presented is designed for 22 animals, resulting in approximately 2,5×10^8 Sertoli cells or 20 culture dishes.

2.4.1 Sertoli cell isolation

1. Decapsulate testes from 22 rats, and transfer them to 20 mL HBSS-w/o.
2. Wash the testes in 20 mL fresh HBSS-w/o, and pour the tissue and medium in a Peteri dish.
3. Cut the testes in small pieces (~1mm^3) with a pair of scalpels.
4. Transfer the suspension to an Erlenmeyer, and remove the medium by suction.
5. Add the trypsin solution and approximately 10 mL of the DNase solution.
6. Incubate the suspension for minimum 5 min submerged in a shaking water bath; 34°C, 235 rpm.
7. Place the Erlenmeyer at angel 45°, let the suspension sediment, and remove the medium.
8. Add the trypsin inhibitor solution and 50 mL HBSS-w.
9. Shake the suspension, let it sediment, and remove the medium.
10. Wash the cells twice with HBSS-w, and transfer the cells to a 50 mL Falcon tube
11. Add the collagenase solution, and incubate the suspension for 15 min submerged in a shaking water bath; 34°C, 235 rpm.
12. Sediment the cells by centrifugation; 15 rcf, 20°C, 2 min, and remove the medium.
13. Add a small volume of the BSA solution, resuspend the cell pellet by careful pipetting, and add the rest of the BSA solution.
14. Sediment the cells by centrifugation; 50 rcf, 20°C, 2 min, and remove the medium.
15. Add 25 mL HBSS-w, and resuspend the pellet.
16. Filter the suspension through a sterile 100 µM nylon membrane, and wash the membrane with additional 25 mL HBSS-w.
17. Sediment the cells by centrifugation; 50 rcf, 20°C, 3 min, and remove the medium.
18. Resuspend the cells in 10 mL MEM+, and determine the cell concentration. The cells can now be harvested in GTCM (3,1 mL/10^7 cells) for total RNA extraction, or continue being cultured.

Antibiotics: 0,5 mL penicillin (10^5 IU/mL)
0,25 mL streptomycin (0,20 g/mL)
0,1 mL Fungizone™ (12,5 mg/mL)

Trypsin: 250 mg trypsin
10 mL HBSS-w/o
Sterile filter
Adjust to 100 mL with HBSS-w/o

HBSS-w: 500 mL Hanks' balanced salt solution with Ca/Mg
Antibiotics

HBSS-w/o: 500 mL Hanks' balanced salt solution without Ca/Mg
Antibiotics

DNase: 30 mg DNase
5 mL HBSS-w/o
Sterile filter
Adjust to 15 mL with HBSS-w/o

BSA: 500 mg Bovine serum albumin, essential fatty acid free
40 mL HBSS-w
Adjust pH to 7,4 with 1M NaOH (3 drops).
Sterile filter

Collagenase: 50 mg collagenase
10 mL HBSS-w
Sterile filter
Adjust to 50 mL with HBSS-w

Trypsin inhibitor: 4 mg trypsin inhibitor
5,0 mL HBSS-w/o
Sterile filter
Adjust to 10 mL with HBSS-w/o

MEM +: 450 mL Eagles' minimum essential medium
50 mL foetal bovine serum
5 mL L-glutamine
Antibiotics

MEM -: 500 mL Eagles' minimum essential medium
5 mL L-glutamine
Antibiotics

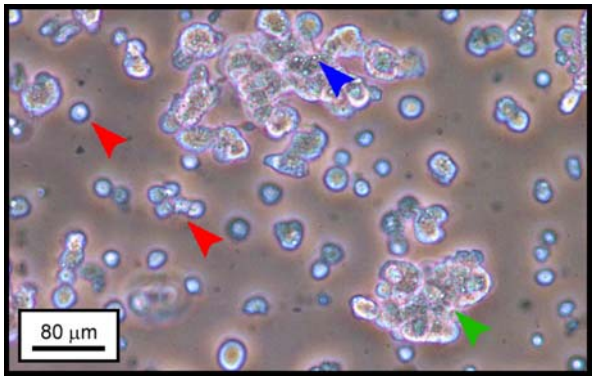

Figure 2.5 The Sertoli cell isolation yields a crude cell extract, which contains a considerable amount of germ cells. Here aggregates of Sertoli cells can be seen (green arrows) together with germ cells (red arrows). Most of the Sertoli cells contain one or more characteristic lipid droplet (blue arrows). The picture is taken with a Spot Insight Colour digital camera connected to a Nikon Eclipse E400 microscope.

2.4.2 Culturing of Sertoli cells

1. Suspend the cells to a final concentration of ~ 10^6/mL with the proper volume of MEM +.
2. Plate 12,5 mL of the cell suspension per Nunc dish.
3. Keep the cell cultures at 34°C in a humidified atmosphere of 5% CO_2 in air.
4. On day 3, remove germ cells by vigorously shaking the dish (19-day-old animals), combined with hypotonic shock treatment (10% MEM in water, 35-day-old animals).
5. Discard the medium, and wash the cells twice with 5 mL MEM–.
6. Add 12,5 MEM–, and continue the incubation. Repeat the washing procedure on day 4 and 5.
7. On day 5 the cells can be harvested in GTCM (3,1 mL/10^7 cells) for total RNA extraction, or start being treated with different stimuli.

MEM +: See *2.4.1 Sertoli cell isolation*

MEM -: See *2.4.1 Sertoli cell isolation*

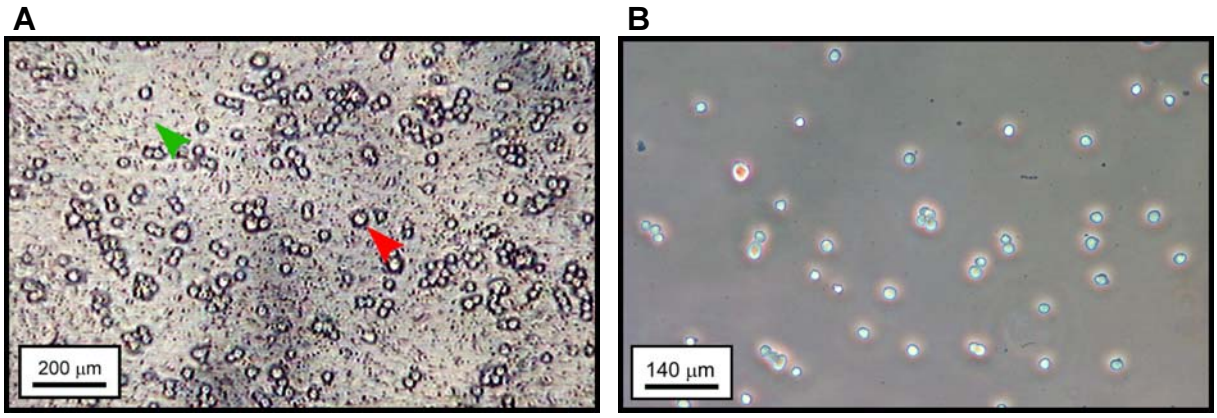

Figure 2.6 After being incubated for 3 days at 34°C in a humidified atmosphere of 5 % CO_2 in air, the Sertoli cells have adhered to the dish as a monolayer. At this moment the Sertoli cells can be viewed as a marble floor (green arrows) upon which the germ cells dance (red arrows) (A). On day 3, 4 and 5 germ cells remnants (total $<4{,}0 \cdot 10^5$/mL) are washed off by handshaking of the dish (B). Picture A is taken with a Sony DXC-107P video camera connected to a Leitz Labovert FS microscope. Picture B is taken with a Spot Insight Colour digital camera connected to a Nikon Eclipse E400 microscope.

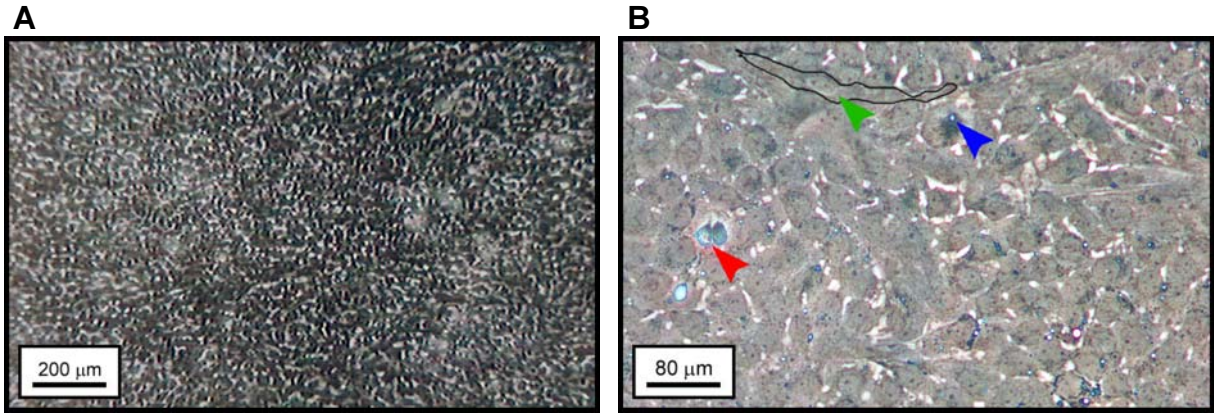

Figure 2.7 After having washed the dishes on day 5, almost all the germ cells are removed (A). When taking a closer look at the «marble floor» at this point, an endless variety of shape and size is revealed (B). E.g. the black outline in fig 2.7B marks the border of one single Sertoli cell. When adhered to the dish, the Sertoli cell stretch and crawls along the dish floor until reaching the border of another Sertoli cell (green arrows). The lipid droplets (blue arrows) are positive controls on functionally active Sertoli cells. Picture A is taken with a Sony DXC-107P video camera connected to a Leitz Labovert FS microscope. Picture B is taken with a Spot Insight Colour digital camera connected to a Nikon Eclipse E400 microscope.

2.5 HORMONAL TREATMENT OF SERTOLI CELLS

Stimulation experiments were started on day 5 after plating. Sertoli cells were treated with hormones, metabolites and analogues; dexamethasone, insulin, testosterone and ovine follicle-

stimulating hormone (FSH), N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate ($(Bu)_2$cAMP), arachidonic acid (20:4(*n*-6)) and tetradecylthioacetic acid (TTA; gift from Professor emeritus Jon Bremer, Department of Medical Biochemistry, University of Oslo) for 3 or 12 hours. The hormones and $(Bu)_2$cAMP were dissolved in PBS, except for testosterone, which was dissolved in ethanol to a final concentration of 0,001%. In half of the controls ethanol was added to a final concentration of 0,001%. Arachidonic acid and TTA were dissolved in essential fatty acid free BSA solution to a final concentration of 0,072%. BSA was therefor added in some controls to a final concentration of 0,072%. An additional experiment varying the usage of FBS during isolation and stimulation of the Sertoli cells was also conducted. Before harvested, the cell cultures were washed twice with 5 mL MEM– to remove hormones, metabolites and analogues, respectively. The concentrations and the duration of the different stimulations are described in the figure legends in chapter 3. RESULTS in this thesis. For each Sertoli cell stimulation experiment testes from 22 animals were used, resulting in approximately 20 culture dishes. The duplicates shown in the figures in 3. RESULTS represent RNA extracted from two separate cultures.

2.6 RNA EXTRACTION AND NORTHERN ANALYSIS

Total RNA was isolated by homogenisation of tissue or cells in guanidine isothiocyanate - mercaptoethanol, followed by centrifugation through a CsCl cushion and phenol-chloroform extraction. RNA denatured in 50% formamide and 6% formaldehyde was size-fractionated (20 µg/lane) through a 1,5% agarose gel containing 6,7% formaldehyde with circulating sodium phosphate buffer running buffer (20 mM). The RNA was transferred to a Magna nylon transfer membrane (MSI, Westborough, MA, USA) by capillary blotting. Ethidium bromide staining of the gel was used to verify equal loading. The 18S rRNA signal for each blot is shown in the figures in chapter 3. RESULTS in this thesis. All pipetting of RNA samples was performed with a positive displacement pipette.

2.6.1 Phenol-chloroform extraction of total RNA

1. Lyse and homogenise tissue or cells in guanidine isothiocyanate - mercaptoethanol (GTCM). Use approximately 3,1 mL GTCM per 0,15 g tissue, 10^7 cells, or culture dish.
2. When homogenising tissue, use an ultra knife to cut the tissue submerged in GTCM.
3. Make sure that all cells are lysed by breaking down aggregates with a syringe (21 GA needle).
4. Carefully load the cell lysate on top of a 1,8 mL CsCl cushion in an ultra centrifuge tube.
5. Centrifugation; 140.000 rcf, 20°C, 18 hours (>2×10^6 rcf×h).

6. After this, the RNA is smeared on the tube bottom. Remove the GTCM – CsCl mix with a Pasteur pipette. Gently remove the cell debris by sucking up the viscose band placed in the middle of the tube.
7. Turn tubes upside-down on a paper tissue to get rid of all the fluid.
8. Add 300 µL TES-buffer to resuspend the RNA, and incubate for 10 min.
9. Transfer the crude RNA extract to a fresh Eppendorf tube.
10. Add 300 µL TE-phenol, and vortex the samples for 30 sec.
11. Separate the phases by centrifugation; 13.000 rcf, 20°C, 4 min.
12. Collect the hydrophilic phase (upper phase), and transfer it to a fresh tube
13. Add 150µL TE-phenol and 150 µL 24:1 chloroform:amylalcohol, and vortex the samples for 30 sec.
14. Separate the phases by centrifugation; 13.000 rcf, 20°C, 4 min.
15. Collect the hydrophilic phase (upper phase), and transfer it to a fresh tube
16. Precipitate the RNA in 70% ethanol with 0,3 M sodium acetate (750 µL 100% ethanol, 30 µL 3 M sodium acetate) at -20°C over night.
17. Sediment the precipitated RNA by centrifugation; 13.000 rcf, 4°C, 45 min.
18. Remove the alcohol by pipetting, and add directly 1000 µL of ice cold 70% ethanol.
19. Wash the RNA pellet by vortexing, and sediment it at -80°C for 15 min.
20. Continue sedimenting the pellet by centrifugation; 13.000 rcf, 4°C, 30 min.
21. Remove as much of the ethanol as possible using an RNase free pipette tip.
22. Let the pellet dry under a light bulb for 15 min., and evaluate the pellet size.
23. Resuspend the RNA in suitable volume of DEPC-H_2O.
24. Analyse the RNA extract with respect to concentration, by diluting aliquots of the sample 1:500 with dH_2O, and reading the A_{260} in an UV spectrophotometer. If necessary, dilute the sample with DEPC-H_2O until reaching a final RNA concentration of 4,5-6,5 µg/µL (standardising the RNA concentration in the linear area of the A_{260} makes e.g. the loading of gels easier to equalise).
25. Store the RNA samples at -80°C.

$$[RNA] = \frac{A_{260} \text{ X } \mu L \text{ RNA}}{25 \text{ (abs. coeff. RNA)}}$$

Figure 2.8 Calculation of RNA concentrations

10% SLS
10 g N-laurylsarcosine (SLS)
dH_2O to 100 mL

1M Sodium citrate
29,41 g tri-sodium citrate dihydrate
75 mL dH_2O
Adjust pH to 7,0 with NaOH/HCl.
dH_2O to 100 mL
Autoclave

GTCM:
29,55 g guanidine isothiocyanate
1,25 mL 1 M sodium citrate pH 7,0
2,5 mL 10% SLS
20 mL dH_2O
Dissolve in water bath, 65°C.
dH_2O to 50 mL, store dark.
Before usage add 7µl/ml 14,3 M mercaptoethanol.

CsCl:
48,00 g CsCl
1,86 g EDTA
dH_2O to 50 mL
Will dissolve at room temperature in one day.

Chloroform/ Amylalcohol:
Mix chloroform:amylalcohol 24:1.
Store dark.

TES-buffer:
100 µL 1 M Tris/HCl pH 7,4
100 µL 0,5 M EDTA
1,0 mL 10% SDS
8,8 mL dH_2O

TE-buffer:
0,61 g Trizma® base
0,186 g EDTA· $2H_2O$
450 mL dH_2O
Adjust pH to 8,0 with NaOH/HCl.
dH_2O to 500 mL
Autoclave

H_2O-Phenol:
Melt the phenol in a water bath, 65°C, 1 hour.
1:1 phenol:dH_2O, shake.
Centrifuge; 1700 rcf, 20°C, 5 min.
Remove the hydrophilic phase.
Add dH_2O 1:1.
Store dark and cold.

TE-Phenol:
Remove hydrophilic phase from H_2O-Phenol.
Add TE-buffer 1:1, shake.
Centrifuge; 1700 rcf, 20°C, 5 min.
Remove the hydrophilic phase, and repeat.
Add TE-buffer 1:2, shake.
Centrifuge; 1700 rcf, 20°C, 5 min.
Remove 50% of the hydrophilic phase.
Store dark and cold.

10% SDS: 50 g sodium dodecyl sulphate (SDS)
450 mL dH_2O
Dissolve by heating to 68°C.
dH_2O to 500 mL

0,5M EDTA: 46,53 g EDTA· $2H_2O$
200 mL dH_2O
5 g NaOH
Adjust pH to 8,0 with 1 M NaOH.
Autoclave

1M Tris/HCl: 30,28 g Trizma® base
200 mL dH_2O
Adjust pH to 7,4 with conc. HCl.
dH_2O to 250 mL
Autoclave

3M Sodium acetate: 102,05 g sodium acetate · $3H_2O$
100 mL dH_2O
Adjust pH to 4,8 with conc. Acetic acid.
Autoclave

DEPC-H_2O: 125 µL diethyl pyrocarbonate
500 mL dH_2O
Shake thoroughly.
Incubate at 37°C for 24 h with open lid.
Autoclave

2.6.2 Size-fractionation of RNA through denaturing agarose gel

1. Dissolve 4,5 g agarose in 30 mL 0,2 M SoP buffer and 216 mL dH_2O by boiling up the solution.
2. Cool to 65°C. This takes 20 min in room temperature.
3. Filter 54 mL formaldehyde through a paper filter, and add 25 µg ethidium bromide (40 µL - 0,625 µg/µL).
4. When cooled, add the formaldehyde-ethidium bromide to the agarose solution.
5. Cast the gel directly in the electrophoresis chamber, and allow it to polymerise for at least 45 min.
6. Pour the 0,02 M SoP electrophoresis buffer in the chamber, and remove the combs. The buffer should cover the gel with at least 0,5 cm.
7. Thaw the RNA samples on ice, mix and spin down.
8. Calculate the volume of the samples in question to be added (20 µg RNA/well).
9. Mix the RNA samples with denaturing buffer (5× µL RNA), and spin down.
10. Incubate in water bath; 15 min, 50°C, and cool on ice for 2 min.
11. Add loading buffer (1,5× µL RNA), mix, and spin down. Thereafter, load the samples onto the gel.
12. Run the gel at 100V for 15 min. This allows the RNA to migrate into the gel.
13. Reduce the voltage to 40V, and run the gel for approximately 17 hours (this allows the smallest transcripts to migrate at least 13,5 cm, as indicated by the blue loading buffer front).

Formamide (deionized): 100 mL formamide
10 g bed resin
Stir 30 min, filter
Store at -20°C

0.2M SoP buffer: 80,4 g Na_2HPO_4 · $7H_2O$ in 1,5 L dH_2O
31,2 g NaH_2PO_4 · $2H_2O$ in 1,0 L dH_2O
Adjust pH to 7,0 in Na_2HPO_4 with NaH_2PO4.
Autoclave

Denaturing buffer (stock solution): 2,38 g hepes
0,34 g sodium acetate
0,19 g EDTA· $2H_2O$
40 mL dH_2O
Adjust pH to 7,0 with 1M NaOH.
Sterile filter
Store at 4°C

Denaturing buffer: 450 µL deionized formamide
146 µL filtered formaldehyde
90 µL denaturing buffer (stock solution)
64 µL dH_2O

Loading buffer: 1,0 mL 0,2 M SoP buffer
1,0 g ficoll-400
0,025 g bromophenol blue
dH_2O to 40 mL
Store at 4°C

0,02 M SoP buffer (electrophoresis): 250 ml 0,2 M SoP buffer
2250 ml dH_2O

2.6.3 Capillary blotting

1. Stop the electrophoresis, and examine the gel on an UV light table (312 nm). Take a photo of the gel.
2. Cut off all empty wells, and cut the gel south of the blue loading buffer band and north of the well border. Make the gel unsymmetrical (e.g. cut of the corner south of well 1).
3. Make the sandwich illustrated in figure 2.9. From bottom:
4. Use a clean electrophoresis chamber as support/reservoir.
5. Cut two layers of 3MM Whatman paper fitting the chamber, so that when soaked with transfer buffer (20×SSC), they will tip down in the reservoir and function as a wick. Remove all air bubbles.
6. Place the gel upside-down (the RNA-side up) on top of the Whatman wick.
7. Frame the gel with Parafilm™ (this prevents buffer from passing outside the gel, rather than through it).
8. Fill up the reservoirs with 20×SSC to approximately 1 cm from the gel stage.
9. Soak the Nylon transfer membrane (slightly larger than the gel) in 20×SSC, and place it on top of the gel. Remove all air bubbles.
10. Soak 2 layers of 3MM Whatman paper (slightly larger than the Transfer membrane) in 20×SSC, and place them on top of the Nylon transfer membrane. Remove all air bubbles.
11. Place a 3,5 cm bundle of paper tissues (cellestoff), and another 3,5 cm of paper towels, on top of the Whatman paper.
12. Finally, place a glass plate on top of the sandwich, and add a weight of 1 kg.
13. Let the capillary blotting run over night.

20×SSC: 350,6 g NaCl
201,0 g tri-sodium citrate dihydrate
1600 ml dH_2O
Adjust pH to 7,0 with 1 M HCl.
dH_2O to 2000 ml
Autoclave

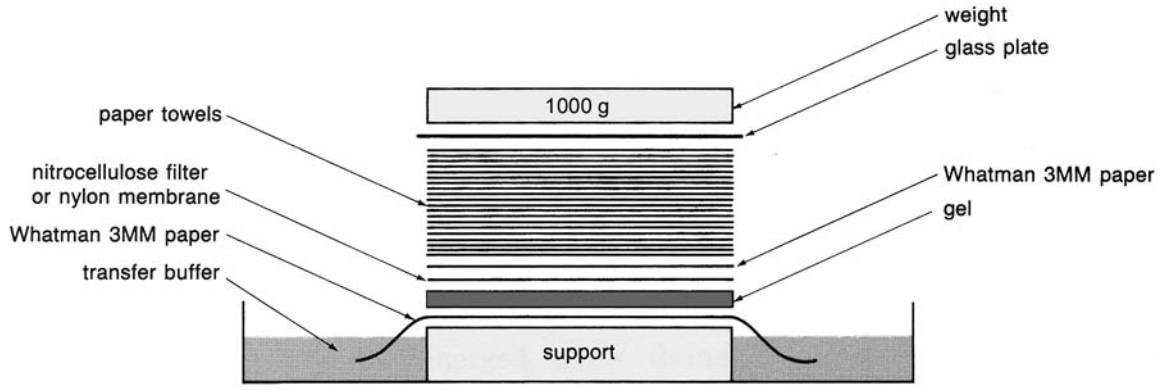

Figure 2.9 The capillary blotting sandwich.

2.6.4 Cross-linking of filter

1. Disassemble the sandwich, and examine the filter (transfer membrane) on an UV light table (312 nm). Mark the 18 S and 28 S rRNA bands, and take a photo of the filter.
2. Turn the filter so that the RNA side faces the light table, and cross-link the filter by exposing it to 312 nm UV light for 1 min.
3. Bake the filter in a dry oven at 80°C for 1 hour (this increase the RNA-membrane cross-linking).
4. Store the filter, wrapped in Whatman paper, dry and dark at room temperature.

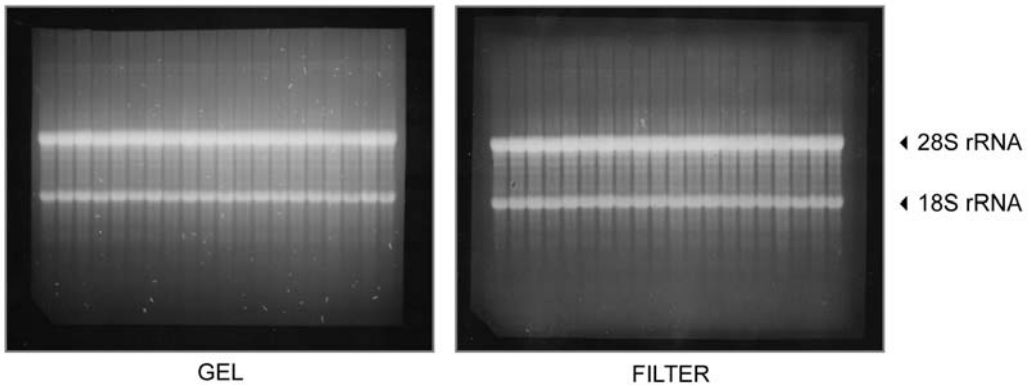

Figure 2.10 Ethidium bromide staining of the gel was used to verify equal loading and a successful transfer to the filer. Here both 18S and 28S rRNA signals can be seen. The 18S rRNA signal for each blot is shown in the figures in chapter 3. RESULTS in this thesis.

2.6.5 Hybridisation

Radio-labelled single stranded and double stranded probes generated by PCR and random priming, respectively, were hybridised to the filters in 50% formamide, 5×Denhart's, 5×SSC, 0,1% SDS, 0,25 mg/mL non-hom. salmon DNA, 50 mM sodium phosphate (pH 6,5) for 12 to 16 hours at 42°C. The single stranded probes had a final activity of approximately $12{,}5\times10^6$ cpm/mL, while the double stranded probes had a final activity of approximately $4{,}5\times10^6$ cpm/mL, in the hybridisation buffer. The filters were washed in 2×SSC/0,1%SDS at room temperature for 4×10 minutes and 0,1×SSC/0,1%SDS at 50°C for 2×30 minutes. Autoradiography was carried out with Hyperfilm™ ECL (Amersham Biosciences, Buckinghamshire, UK).

1. Wet the filter(s) in 2×SSC/0,1%SDS, and make a sandwich of nylon membranes and filter(s); membrane-filter-membrane-filter…membrane (maximum 3 filters per tube).
2. Roll out the sandwich in a hybridisation tube, and remove air bubbles.
3. Boil 10 mL prehybridisation buffer for 10 min, and cool it down to 42°C in a water bath. Pour the buffer in the hybridisation tube.
4. Prehybridise the filters minimum 4 hours by incubating the tubes in an oven with rotation at 42°C.
5. Radiolabel the probe(s), see *2.6.6 Probes and primers*.
6. Make the hybridisation buffer by adding your radio-labelled probe to 10 mL prehybridisation buffer.
7. Boil the hybridisation buffer for 10 min, cool it down quickly on ice (10 min), and exchange the prehybridisation buffer with the hybridisation buffer.
8. Hybridise the filters 12-16 hours by incubating the tubes in an oven with rotation at 42°C.
9. Pour off the hybridisation buffer, and wash the filters 4×10 min at room temperature with 150 mL 2×SSC/0,1%SDS per tube.
10. Wash the filters 2×30 min at 50°C with 150 mL 0,1×SSC/0,1%SDS per tube.
11. Remove the washing solution, dismantle the sandwich, and seal the filter in a plastic bag.

12. Mount the filter on a 3 MM Whatman paper, and mark the 18S and 28S rRNA with fluorescent tape.
13. Place the filter in a film cassette with x-ray film, and expose it at -80°C for the desired time.
14. Develop the film, and re-expose it if necessary.
15. The filter can be rehybridised or "stripped":
    Make a sandwich as described above, incubate it in 100 mL rehybridisation buffer in an oven with rotation at 65°C, and wash the filter in 150 mL 2×SSC/0,1%SDS at room temperature for 15 min. Finally, seal the filter in a plastic bag. Used filters should be kept in sealed plastic bags at 4°C.

Prehybridisation buffer:
10mL 100% deionized formamide
5mL 20×SSC
2mL 50×Denhart's
1mL 1 M SoP buffer, pH 6,5
1mL non-hom. salmon DNA (5 mg/ml)
0,8mL dH_20
0,2mL 10% SDS

Hybridisation buffer:
Prehybridisation buffer
Ss or ds radio-labelled probe.

Rehybridisation buffer:
1,0 mL 1M SoP buffer, pH 6,5
50 mL deionized formamide
dH_2O to 100 mL

Formamide (deionized):
See 2.6.2 *Size-fractionation of RNA through denaturing agarose gel*

50×Denhardt´s:
1,0 g ficoll-400
1,0 g polyvinylpyrrolidone (PVP)
1,0 g BSA
dH_2O to 100 mL
Filter through Whatman paper.
Store at-20°C.

1M SoP buffer:
40,2 g $Na_2HPO_4 \cdot 7H_2O$ in 150 mL dH_2O
15,6 g $NaH_2PO_4 \cdot 2H_2O$ in 100 mL dH_2O
Adjust pH to 6,5 in Na_2HPO_4 with NaH_2PO4.
Autoclave

20×SSC:
See *2.6.3 Capillary blotting*

10% SDS
See *2.6.1 Phenol-chloroform extraction of total RNA*

2.6.6 Probes and primers

Single stranded probes were synthesised and labelled with [α-^{32}P]dCTP (Amersham Biosciences) by PCR, using a short synthetic DNA template [Condon, 1999]. For the Δ^5-desaturase we used a 20-mer anti-sense primer; 5'-GGGCACCAAGGAAGTTCCAA-3', providing a 108-mer probe covering the area between basepair 321and 428 (ORF) of the rat Δ^5-desaturase cDNA (PubMed accession number AF320509). For the Δ^6-desaturase we used a 20-mer anti-sense primer; 5'-GGGAGGTAGCAAGGACAAAG-3', providing a 100-mer probe covering the area between basepair 415 and 514 (ORF) of the rat Δ^6-desaturase cDNA (PubMed accession number AB021980). For the SCD-1 probe we used a 24-mer anti-sense primer; 5'-TATGCATTTATCATGTATGCTTAG-3', providing a 106-mer probe covering the area between basepair 1202 and 1307 (ORF) of the rat SCD-1 cDNA (PubMed accession number J02585). Finally, for the SCD-2 probe we used an 18-mer anti-sense primer; 5'-CACAGCCC GAGTGTTGAA-3', providing a 106-mer probe covering the area between basepair 1828 and 1933 (ORF) of the rat SCD-2 cDNA (PubMed accession number NM_031841). All primers and templates were obtained from MedProbe, Oslo, Norway.

Double stranded cDNA probes were synthesised and labelled with [α-^{32}P]dCTP (Amersham Biosciences) using 0,8-1,6 kb cDNA templates and the Megaprime DNA labelling system kit (Amersham Biosciences). For the PPARα probe we used a 1,6 kb cDNA corresponding to rat PPARα (PubMed accession number NM_013196). For the PPARδ probe we used a 1,0 kb cDNA corresponding to rat PPARδ (PubMed accession number NM_013141). Finally, for the PPARγ probe we used a 0,8 kb cDNA corresponding to mouse PPARγ$_2$ (PubMed accession number NM_011146) that hybridised to both rat PPARγ$_1$ and -γ$_2$ (two splice variants of the PPARγ gene differing in only 3 amino acids, impossible to discriminate with Northern blots).

2.6.6.1 Generating single stranded probes by PCR

1. Make a template/primer/nucleotide-mix (can be stored one week at -20°C):

X µL	template	⇒ *300 ng / 20 µL*
2,0 µL	50 µM primer	⇒ *5,0 µM*
1,3 µL	10 mM dATP	⇒ *0,65 mM*
1,3 µL	10 mM dTTP	⇒ *0,65 mM*
1,3 µL	10 mM dGTP	⇒ *0,65 mM*
DEPC-H_2O to 20 µL		

2. Make the PCR-mix (mix the solutions directly in a PCR tube):

2,0 µL	template/primer/nucleotide-mix:	
	template	⇒ *30 ng / 25 µL*
	primer	⇒ *400 nM*
	dNTP	⇒ *50 µM*
2,5 µL	10×PCR buffer	⇒ *1×*
1,5 µL	25 mM $MgCl_2$	⇒ *1,5 mM*
3,75 µL	DEPC-H_2O	
15,0 µL	3000Ci/mmol [α-^{32}P] dCTP	⇒ *2 µM*
0,25 µL	5 IU/µL *Taq* polymerase	⇒ *1,25 IU*

3. Start the PCR reaction (table 2.6):

Step	Event	Temperature	Duration	Cycles
1	Hot start:	95°C	30 sec	
2	Denaturation:	94°C	30 sec	30
3	Annealing:	55°C	60 sec	
4	Elongation:	72°C	60 sec	
5	Final elongation:	72°C	300 sec	
6	Stop:	4°C	∞	

Table 2.6 PCR programme for Δ^5-desaturase, Δ^6-desaturase, SCD1 and SCD2 used in our experiments. The same programme was used for all four probes.

4. When the reaction is finished, purify the probe on a NAP-5 column (Sepadex™ G-25; this desalts the probe and removes the majority free primers and nucleotides).
5. Start with equilibrating the column with 10 mM SoP buffer, pH 6,8 (3×column volume).
6. Add the PCR product carefully on the column surface, and let the solution soak in.
7. Add 475 µL 10 mM SoP buffer, and discard the eluate.
8. Add another 1000 µL 10 mM SoP buffer, and collect the eluate. This fraction contains your radio-labelled ss anti-sense probe.
9. Estimate the activity of the probe by counting an aliquot of the 1000 µL eluate in a liquid scintillation counter (Charenkov-counting).

10 mM SoP buffer: 0,780 g $NaH_2PO_4 \cdot 2H_2O$ in 500 mL dH_2O
1,341 g $Na_2HPO_4 \cdot 7H_2O$ in 500 mL dH_2O
Adjust pH to 6,8 in $NaH_2PO4$with Na_2HPO_4.
Autoclave

PCR reagents: Nucleotides, PCR buffer, $MgCl_2$ and *Taq* polymerase bought from ABgene.

2.6.6.2 Generating double stranded probes by random priming

1. Mix minimum 25 ng cDNA template with 5 µL primer solution, and adjust to 33 µL with DEPC-H_2O.
2. Denature by boiling for 5 min, cool on ice, and spin down.
3. Add 10 µL labelling buffer, 5 µL [α-^{32}P]dCTP and 2 µL enzyme (Klenow, 1 IU/µL).
4. Mix thoroughly by pipetting, and incubate at 37°C for 15 min.
5. Stop the reaction by adding 2 µL 0,5M EDTA.
6. Purify the probe on a NICK column (Sepadex™ G-50; this desalts the probe and removes the majority free primers and nucleotides).
7. Start with equilibrating the column with TE-buffer, pH 8,0 (3×column volume).
8. Add 40 µL TE-buffer, pH 8,0 to the random priming product.
9. Mark 15 Eppendorf tubes to collect the eluates in.
10. Carefully load the probe on the column surface, let it soak in, and start collecting the eluate.
11. Fill up the column with TE-buffer, pH 8,0, and collect; 5 drops in tube 1-6 and 3 drops in tube 7-15.
12. Estimate the activity by counting aliquots of the fractions in a liquid scintillation counter (Charenkov-counting).

Random priming: chemicals Primer solution, labelling buffer and enzyme (Klenow, 1 IU/µL) all found in Megaprime DNA labelling system kit (Amersham Biosciences)

0,5M EDTA: See *2.6.1 Phenol-chloroform extraction of total RNA*

TE-buffer: See *2.6.1 Phenol-chloroform extraction of total RNA*

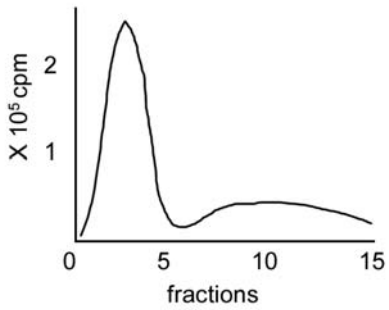

Figure 2.11 Random priming elution profile. First top representing the radio- labelled ds cDNA probe, last top representing unincorporated nucleotides.

2.6.7 Digital handling of blots

All blots were digitally scanned on an Epson™ Expression 1680 Pro, with a transparent unit for positive film, at resolution 300 dpi. The digital blots were then mounted in Adobe™ Photoshop™ 5.5. Blots representing transcripts with very weak signals (PPARα, PPARγ and some of the SCD-1s) were digitally manipulated in a way simulating 2-3 extra weeks of exposure. All lanes in same blot (whole picture file) were treated equally, conserving the semi-quantitative qualities of a Northern blot.

All the blots from Sertoli cell stimulation and feeding experiments were imported on the Epson™ Expression 1680 Pro scanner and the intensities read with Kodak Digital Science™ 1D Image Analysis Software v. 3.5. The intensity readings from the scanning of the autoradiograms were normalised to the 18S rRNA signals and the values plotted relative to the values of their respective 3 hour controls.

2.6.8 Plasmid amplification

The PPARα cDNA probe, and plasmid pCRII (Invitrogen, CA, USA) and pBabe [Morgenstern & Land, 1990] containing PPARδ and PPARγ_2, respectively, were kindly provided by Associate professor Hilde Nebb, Institute for Nutrition Research, University of Oslo. Competent *E.coli* DH5α bacteria, provided by Professor Winnie Eskild, Department of Biochemistry, University of Oslo, were transformed with PPARδ- and PPARγ_2-plasmids. The transformed bacteria were selected on the basis of ampicillin resistance and grown in LB-medium. Plasmids were isolated from the bacteria employing the QIAGEN® Maxi Kit (Qiagen, Hilden, Germany). The PPAR cDNAs were excised from the plasmids by digestion with restriction enzyme *Eco*RI. Plasmid and cDNA were separated on a 1% agarose gel with circulating 0,5×TBE buffer running buffer. Finally, the cDNA was isolated from the gel employing QIAEX®II Gel Extraction Kit (Qiagen). All pipetting of DNA samples was performed with a positive displacement pipette.

2.6.8.1 Competent E.coli bacteria

1. Plate *E.coli* DH5α on LB dishes, and incubate at 37°C over night.
2. Pick 10-12 big colonies, inoculate them in 250 mL SOC, and incubate the broth at 18°C until A_{600}=0,6. This normally takes 2 days.

3. Cool on ice for 10 min, and sediment by centrifugation; 2.500 rcf, 4°C, 10 min.
4. Remove the supernatant, and resuspend in 80 mL ice cold TB buffer for 10 min on ice.
5. Centrifuge; 2.500 rcf, 4°C, 10 min.
6. Remove the supernatant, and resuspend the cells in 10 mL ice cold TB buffer.
7. Add 10 mL TB-DMSO buffer (14% DMSO), mix, and incubate10 min on ice.
8. Freeze 200 µL aliquots of the cells directly in Eppendorf tubes standing in N_2 (liq). Store at -80°C.

1 M KCl: 7,46 g KCl
100 mL dH_2O
Autoclave

1 M NaCl: 5,84 g NaCl
100 mL dH_2O
Autoclave

2 M Glucose: 36,04 g glucose
dH_2O to 100 mL
Autoclave

SOB: 2,0 g Bacto™ Tryptone
0,5 g Bacto™ Yeast Extract
1,0 mL 1 M NaCl
0,25 mL1 M KCl
96 mL dH_2O
Autoclave

TB buffer: 1,5 g pipes
1,1 g $CaCl_2 \cdot 2H_2O$
9,3 g KCl
dH_2O to 500 mL.
Adjust pH to 6,7 with 1 M KOH.
5,3 g $MnCl_2 \cdot 4H_2O$
Sterile filter
Store at 4°C.

LB dishes: 5,0 g Bacto™ Tryptone
2,5 g Bacto™ Yeast Extract
5,0 g NaCl
450 mL dH_2O
Dissolve and adjust pH to 7,5 with 1 M NaOH.
dH_2O to 500 mL
Add 6,0 g Bacto™ Agar.
Autoclave
Cool to 55°C (1 hour, room temp.).
Dispense 27 mL of LB agar in each dish.
Remove germs and air bubbles by flaming.
Let the LB agar set over night at room temperature.
Store at 4°C, wrapped in plastic bags.

SOC: 10 mL SOB
100 µL 1 M $MgCl_2$
100 µL 1 M $MgSO_4$
100 µL 2 M glucose
Sterile filter

2.6.8.2 Transformation

1. Make a dilution series with the plasmid in TE- buffer, pH 8,0: 10,0 - 5,0 - 1,0 - 0,1 ng/µL.
2. Transform 50 µL competent *E.coli* bacteria with the plasmid by adding 1,0 mL of the different plasmid dilutions. As control use 50 µL competent *E.coli* without added plasmid.
3. Incubate the bacteria on ice for 40 min, and then at 37°C for exactly 2 min.
4. Add 450 µL SOC, and incubate the cells at 37°C for 60 min. Shake the tubes now and again.
5. Heat LB-Amp culture dishes in an oven at 37°C.
6. Plate 150 mL of the transformation solution on different LB-Amp culture dishes.
7. Flame the plating fork between the platings.
8. Let the dishes stand on the bench for 15 min to allow the transformation solution to soak in to the agar.
9. Seal the dishes with Parafilm™, and incubate them over night upside-down in an oven at 37°C.
10. When incubated, store the dishes at 4°C to avoid growth of satellite colonies.

Ampicillin (10 mg/ml): 2000 mg Ampicillin
200 mL dH_2O
Store at -20°C

LB-Amp dishes: See *2.6.8.1 Competent E.coli bacteria*
Add 2,5 mL ampicillin (10 mg/mL)
before dispensing the medium in the dishes.

SOC: See *2.6.8.1 Competent E.coli bacteria*

LB: 20,0 g Bacto™ Tryptone
10,0 g Bacto™ Yeast Extract
20,0 g NaCl
1900 mL dH_2O
Dissolve and adjust pH to 7,5 with 1 M NaOH.
dH_2O to 2000 mL
Autoclave

TE-buffer See *2.6.1 Phenol-chloroform extraction of total RNA*

2.6.8.3 Selection and cultivation

1. Count or estimate the number of colonies per dish (see tab. 2.7).
2. Harvest one free colony (without satellite colonies) from two different dishes with a flamed cell harvester.
3. Transfer the colonies to two 50 mL Falcon tubes with 10 mL LB + 75 µL ampicillin. Seal the tubes with Parafilm™, and incubate the pre-colonies in an orbital-shaking incubator at 37°C, 100 rpm.
4. Check on the cultures once per hour. When the culture(s) start to get fallow, continue the incubation for another 2 hours. The culture is now growing exponentially.
5. Mix one of the pre-cultures with 500 mL LB + 3,75 mL ampicillin in a 2 L Erlenmeyer culture flask.
6. Incubate over night at 37°C, 100 rpm.

LB: See *2.6.8.2 Transformation*

Ampicillin (10 mg/ml): See *2.6.8.2 Transformation*

ng/µL plasmid	PPARδ	PPARγ$_2$
10	~ 2500	620
5,0	~ 3200	-
1,0	0 (!)	228
0,1	202	15
0,0 (Control)	0	0

Table 2.7 Selecting clones. Number *E.coli* colonies transformed with plasmid containing PPARδ and PPARγ$_2$, respectively.

2.6.8.4 Isolation of plasmids

1. Optional: Sterilely pipette out 0,5 mL from the *E.coli* culture, and add 0,5 mL glycerol. Store at -20°C (with stock cultures the transformation step can be omitted in proceeding preparations).
2. Sediment the bacteria by centrifugation; 6.000 rcf, 4°C, 15 min, and discard the supernatant.
3. Resolve the pellet in 10 mL P1 by vortexing.
4. Lyse the cells by transferring the solution to an Erlenmeyer and adding 10 mL P2.
5. Mix thoroughly, and incubate at room temperature for 5 min.
6. Add 10 mL P3 (4°C), mix.
7. Centrifuge; 20.000 rcf, 4°C, 30 min.
8. Filter the supernatant through 4 layers of sterile gauze.
9. Equilibrate a Tip 500 column with 10 mL QBT.
10. Apply the filtered supernatant to the Tip 500 column, allow the column to empty by gravity flow, and discard the eluate.
11. Wash the column with 30 mL QC.
12. Elute the plasmid with 15 mL QF, and collect the eluate.
13. Add 10 mL isopropanol to the collected eluate, mix, and precipitate the DNA on ice for 10 min.
14. Centrifuge 15.000 rcf (minimum), 4°C, 30 min. Discard the supernatant, and remove all the isopropanol.
15. Wash the pellet with 1,5 mL cold 70% Ethanol, and transfer the pellet to an Eppendorf tube.
16. Centrifuge; 13.000 rcf, 4°C, 15 min. Remove the supernatant.
17. Resuspend the pellet in 50 mL TE buffer pH 8,0 on ice. This takes approximately 30 min.
18. Measure the DNA concentration by reading the A_{260} in an UV spectrophotometer (see fig. 2.8; DNA absorption coefficient = 20). Store the samples at -20°C.

Plasmid isolation buffers: P1, P2, P3, QBT, QC, QF and Tip 500 column all found in the QIAGEN® Maxi Kit (Qiagen).

Glycerol: 10 mL glycerol
Autoclave
Store at -20°C

2.6.8.5 Cutting plasmid with restriction enzymes

1. Digest your plasmid by setting up the following reaction: 250 µg plasmid, 750 IU restriction enzyme (*Eco*RI, 40 IU/µL) - maximum 100 µL, 75 µL 10× restriction buffer (SH), and dH_2O to 750 µL.
2. Incubate at 37°C for 3-4 hours, or over night, and stop the reaction on ice.

Restriction enzyme 40 IU/µL *Eco*RI (Sigma-Aldrich)

Restriction buffer SH (Sigma-Aldrich)

2.6.8.6 Separation of DNA fragment through agarose gel

1. Dissolve 3,0 g agarose in 300 mL 0,5×TBE electrophoresis buffer by boiling up the solution.
2. Cool to 65°C. This takes 20 min at room temperature.
3. Add 25 µg ethidium bromide (40 µL - 0,625 µg/µL).
4. Apply a comb with large wells (> 500 µL) in the electrophoresis chamber.
5. Cast the gel directly in the electrophoresis chamber, and allow it to set and polymerise for at least 45 min.
6. Pour 0,5×TBE electrophoresis buffer in the chamber, and remove the combs. The buffer should cover the gel with at least 0,5 cm.
7. Thaw the restriction products on ice, mix, and spin down.
8. Mix the DNA with loading buffer (> 0,2× µL DNA), and spin down.
9. Prepare DNA standards by adding loading buffer (> 0,2× µL DNA). Spin down the samples.
10. Incubate the samples (standards included) at 65°C for 15 min, cool on ice, and spin down.
11. Load the samples onto the gel, and wrap the electrophoresis chamber in alu-foil.
12. Run the gel at 100V. Control the separation process by illuminating the gel with an UV lamp (312 nm).
13. The DNA bands should be well separated before the electrophoresis is stopped (this can take from 2 to 18 hours).

0,5M EDTA: See *2.6.1. Phenol-chloroform extraction of total RNA*

Loading buffer: See *2.6.2 Size-fractionation of RNA **through** denaturing agarose gel*

5×TBE: 108 g Trizma® base
55 g boric acid
40 mL 0,5 M EDTA
dH_2O to 2000 mL
Autoclave

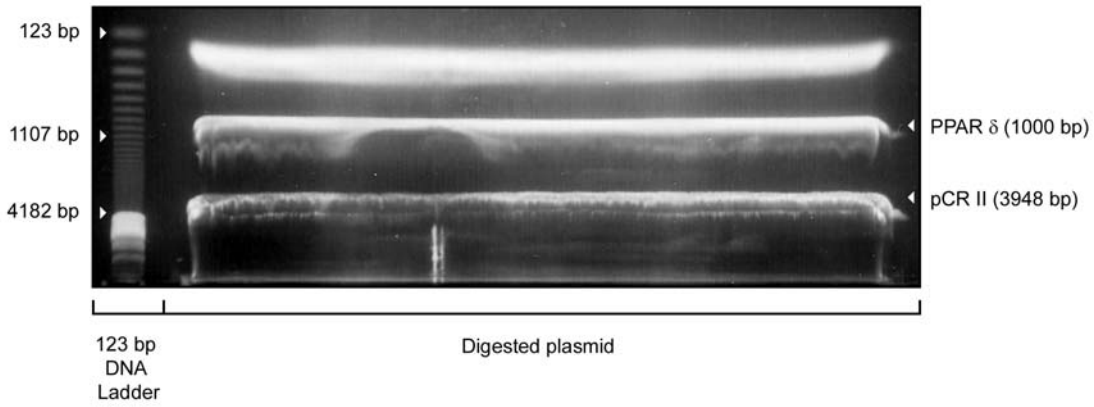

Figure 2.12 The PPARδ cDNA was excised from the plasmid by digestion with restriction enzyme *Eco*RI. Plasmid and cDNA were separated on a 1% agarose gel with circulating 0,5×TBE buffer running buffer.

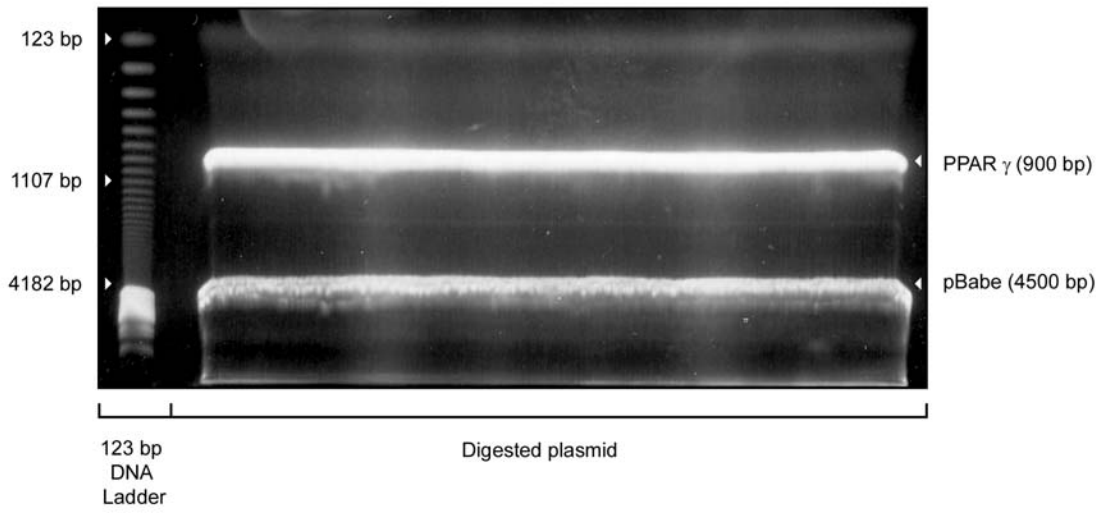

Figure 2.13 The PPARγ cDNAs was excised from the plasmids by digestion with restriction enzyme *Eco*RI. Plasmid and cDNA were separated on a 1% agarose gel with circulating 0,5×TBE buffer running buffer.

2.6.8.7 Isolation of DNA fragments from agarose gel

The protocol is dimensioned for DNA fragments with size in the range of 40 bp to 50 kb:

1. Examine the gel on UV light table (312 nm), and take a photo of the gel.
2. Use the DNA standards to identify the cDNA band.
3. Cut out the desired band. Be tough; cut as close to the band as possible (~2 mm).
4. Transfer the piece of gel to a 50 mL Falcon tube, and determine the weigh of the gel piece.
5. Add 3 mL QX1/g gel (if insert size is between 100 bp-4 kb), and mix thoroughly.
6. Calculate the mass of the DNA using the formula:
   (insert size × mass DNA on gel)/(plasmid size + insert size).
7. Vortex QIAEXII 30 sec, and add the desired volume:
   ≤ 2 µg DNA:10 µL QUIAEXII, 2-10 µg DNA: 30 µL QIAEXII, >10 µg DNA: 1 µL QIAEXII/µg DNA.
8. Incubate at 50°C for 10 min, or until the gel is dissolved. Mix every 2. min.
9. Check the colour of the reaction mixture (should be yellow). If the mixture turns orange-lilac, 3 M Sodium acetate pH 4,8 can be added until the colour turns yellow (approximately 10 µL).
10. Sediment by centrifugation; 13.000 rcf, 20°C, 30 sec.
11. The pellet should now be visible. Discard the supernatant.
12. Wash the pellet with 2×500 µL QX1, and spin down.
13. Wash the pellet with 2×500 µL PE, and spin down.
14. Discard the supernatant, and remove as much of the PE as possible.
15. Let the pellet dry under a light bulb for 30 min, and evaluate the pellet size.
16. Elute the DNA from the pellet by adding a volume of 10 mM Tris/HCl buffer equal to the pellet volume.
17. Vortex and incubate at room temperature for 5 min.
18. Centrifuge; 13.000 rcf, 20°C, 30 sec.
19. Transfer the supernatant into a new tube, and repeat 2-3 times. Save the eluates in different tubes.
20. Measure the DNA concentration by reading the A_{260} in an UV spectrophotometer (see fig. 2.8; DNA absorption coefficient = 20).

Gel extraction buffers: QX1, QIAEXII and PE all found in the QIAEX®II Kit (Qiagen)

3M Sodium acetate: See *2.6.1 Phenol-chloroform extraction of total RNA*

10 mM Tris/HCl: 0,303 g Trizma® base
200 mL dH_2O
Dissolve and adjust pH to 8,5 with conc. HCl.
dH_2O to 250 mL
Autoclave

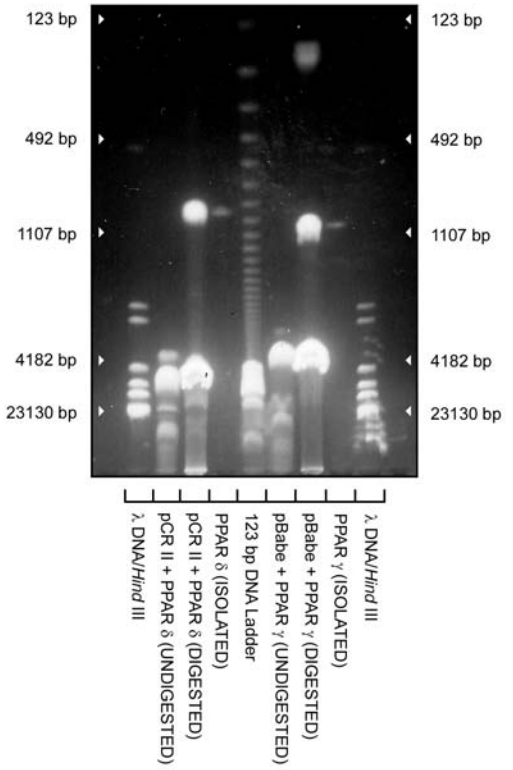

Figure 2.14 The plasmid amplification procedure results in a ds cDNA that can undergo random priming and function as a probe. Before further use, the cDNA product should be analysed on a mini-gel. The gel should be loaded with isolated plasmid, digested plasmid and isolated cDNA. The mini-gel is a 60 mL variant of the agarose gel described under *2.6.8.6 Separation of DNA fragment through agarose gel.*

2.7 LIPID EXTRACTION

Total lipids were extracted from liver and testis from rats fed fat free and standard pellet diet, respectively. The lipids were extracted by chloroform:methanol (2:1) as described previously. [Folch et al., 1957] The lipids were trans-methylated and analysed on a gas chromatograph (GC-FID) [Tran et al., 2001]. The methylated fatty acids were identified by co-chromatography with standards. The extraction and analyses were performed by the technical staff at the Institute of Clinical Biochemistry, Rikshospitalet University Hospital in Oslo.

2.8 STATISTICS

For the presentation of the lipid extraction results mean values ($\overline{x}$) and standard deviation (SD) of the different data sets were calculated. Furthermore, the different groups where compared with Students' t-test (two-tailed) using SPSS® vers. 11. The level of significance was defined as $P<0,05$.

2.8 CHEMICALS

Chemicals, enzymes, medium, kits and materials	Company	Cat.nr.
Agarose (Ultra PURE)	GibcoBRL, Invitrogen	540-5510UA
[α-^{32}P] dCTP (25 µl, 3000Ci/mmol, 250µCi)	Amersham Biosciences	AA0005
Ampicillin	Sigma-Aldrich	A-0166
Arachidonic acid (20:4(n-6))	Sigma-Aldrich	A-9673
Bacto™ Agar (DIFCO 0140-01)	Puls	214010
Bacto™ Tryptone (DIFCO 0123-17-3)	Puls	211705
Bacto™ Yeast Extract (DIFCO 0127-17-9)	Puls	212740
Bio-Rex™ RG Grade mixed Bed Resin	Bio-Rad	444-9999
Boric acid	Fluka	15663
Bovine serum albumin (BSA)	Sigma-Aldrich	A-4503
Bovine serum albumin (BSA; essential fatty acid free)	Sigma-Aldrich	A-6003
Bromophenol blue	Sigma-Aldrich	B-2555
$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich	C-5080
Casein	Laborel	-
Cell harvester (Nunc, 1µL)	Tamro	254410
Cellulose	Laborel	-
Chloroform	Fluka	25690
Collagenase	Sigma-Aldrich	C-0130
CsCl (Caesium Chloride)	GibcoBRL, Invitrogen	5507 UB
Culture dishes (Nunc, 92 mm)	Tamro	214-150350
Dexamethasone (water soluble)	Sigma-Aldrich	D-2915
$(Bu)_2$cAMP (N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate)	Sigma-Aldrich	D-0627
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich	D-5758
DNA-ladder (123 bp)	GibcoBRL, Invitrogen	15613-011
DNA-ladder (λDNA/*Hind*III)	GibcoBRL Invitrogen	10382-018
DNA template (200 µM)	MedProbe	-
DNA primer (antisense, 200 µM)	MedProbe	-
DNase	Sigma-Aldrich	DN-25
*Eco*RI	Sigma-Aldrich	R-4640
EDTA (etylenediamine tetraacetic acid dihydrate)	Sigma-Aldrich	ED2SS
Ethanol (100%, Prima)	Arcus	
Ethidium bromide (0,625 µg/µL)	*Bio*Logical, MERCK Eurolab	4.0221-10
Ficoll-400	Sigma-Aldrich	F-4575
Foetal bovine serum (FBS)	GibcoBRL, Invitrogen	10106-169
Follicle-stimulating hormone (Ovine)	Sigma-Aldrich	F-8174
Formaldehyde (37%)	Sigma-Aldrich	F-8775
Fungizone™ (Bristol-Myers SQUIBB, 50 mg/mL)	Rikshospitalets Apotek	436444
Glucose	Sigma-Aldrich	G-7021
Glycerol	Fluka	49781
Guanidine isothiocyanate	GibcoBRL, Invitrogen	15535-016
HBSS-w (Hanks' balanced salt solution with Ca/Mg)	GibcoBRL, Invitrogen	24020-091
HBSS-w/o (Hanks' balanced salt solution without Ca/Mg)	GibcoBRL, Invitrogen	14170-088
HCl (36-38%)	Fluka	84422
Hepes	Sigma-Aldrich	H-3375
Hyperfilm™ ECL	Amersham Biosciences	RPN 2114 K
Insulin	Sigma-Aldrich	I-6643
Isoamylalcohol (3-methyl-1-butanol)	Fluka	59090
Isopropanol	Fluka	59304
KCl	Sigma-Aldrich	P-4504
KOH	Sigma-Aldrich	P-5958
Lactic acid (DL-lactic Acid)	Sigma-Aldrich	L-4263
L-glutamine	GibcoBRL, Invitrogen	043-05030H
Maize starch	Gift from Prof. E. Christiansen	-
Megaprime DNA labelling system	Amersham Biosciences	RPN 1606
MEM (Eagles' minimum essential medium)	GibcoBRL, Invitrogen	21090-022

Chemicals, enzymes, medium, kits and materials	Company	Cat.nr.
2-Mercaptoethanol	Sigma-Aldrich	M-6250
Methionine	Laborel	-
$MgCl_2$ (1 M)	Sigma-Aldrich	M-1028
$MgCl_2$ (ABgene, 25 mM)	MERCK Eurolab	69613-250
$MgSO_4$ (1 M)	Sigma-Aldrich	M-3409
$MnCl_2 \cdot 4H_2O$	Sigma-Aldrich	M-9522
NaCl	Sigma-Aldrich	S-9888
$Na_2HPO_4 \cdot 7H_2O$	Fluka	71647
$NaH_2PO_4 \cdot 2H_2O$	Fluka	71505
NaOH	Fluka	71691
NAP-5 column (Sepadex™ G-25)	Amersham Biosciences	17-0853-01
NDA (2-Naphthol-3-6-disulfonic acid Disodium Salt)	Fluka	70530
NICK column (Sepadex™ G-50)	Amersham Biosciences	17-0855-01
Nucleotides (ABgene, dNTP, 10 mM)	MERCK Eurolab	69649-1
Nylon membrane (Hybridisation)	AH diagnostics	HB-OV-RM
Nylon membrane (70 µm)	Burmeister	-
Nylon membrane (100 µm)	Burmeister	-
Nylon transfer membrane (MSI)	Sigma-Aldrich	N00HYA0010
Paper tissue (20×20 cm, Cellestoff)	Rikshospitalets sentrallager	765444
PBS (phosphate-buffered saline, tablets)	Sigma-Aldrich	P-4417
PCR buffer w/o $MgCl_2$ (ABgene, 10×)	MERCK Eurolab	69613-250
Penicillin (Pan Pharma)	Rikshospitalets Apotek	505503
Percoll™	Amersham Biosciences	17-0891-01
Phenol	GibcoBRL, Invitrogen	5509 UA
Pipes (1,4-Piperazinediethanesulfonic acid)	Sigma-Aldrich	P-6757
Polaroid paper (Polapan Pro 100)	Fotophono	E1959120
Polyvinyl pyrrolidone (PVP)	Sigma-Aldrich	P-5288
Pyruvate	Boehringer Mannheim	128 147
QIAEX®II Kit	MERCK Eurolab	16047-1
QUIAGEN® Maxi Kit	MERCK Eurolab	16032-10
Restriction buffer (SH)	Sigma-Aldrich	B-3657
Salmon DNA (Non-hom., 5 mg/mL)	Sigma-Aldrich	D-9156
Salt mix	Gift from Prof. E. Christiansen	-
SDS (Sodium dodecyl sulphate)	Bio-Rad	161-0302
SLS (N-Laurylsarcosine)	Sigma-Aldrich	L-5125
Sodium acetate (trihydrate)	Sigma-Aldrich	S-8625
Sodium citrate (dihydrate)	Fluka	71404
Sterile filter (Supor Acrodisc, 25 mm, 0,2 µm)	Dan Meszansky AS	4612
Streptomycin (CEPA, 50mg/mL)	Rikshospitalets Apotek	753483
Sucrose (Dansukker)	REMA 1000	-
Syringe needle (Microlance 3, 21 GA)	Rikshospitalets sentrallager	1066
Taq polymerase (ABgene, 5 IU/µL)	MERCK Eurolab	69613-250
Testosterone	Steraloids	A6950-000
Tetradecylthioacetic acid (TTA)	Gift from Prof. em. Jon Bremer	-
Trizma® base (Tris[hydroxymethyl]aminomethane - Tris base)	Sigma-Aldrich	T-1503
Trypsin (DIFCO)	Puls	0152-131
Trypsin inhibitor	Sigma-Aldrich	T-9003
Vitamin mix	Gift from Prof. E. Christiansen	-
Whatman® paper (3 MM)	Sigma-Aldrich	Z27, 088-1

1. of February 2002

3. RESULTS

3.1 DESATURASES

3.1.1 Localisation of the desaturases

In this study, we show that in rat Δ^5- and Δ^6-desaturase are highly expressed in testis and epididymis (fig. 3.1). Liver and kidney were included as examples of tissues expressing high and low levels of Δ^5- and Δ^6-desaturase, respectively (fig. 3.1). Δ^5- and Δ^6-desaturase follow the same expression pattern in these four tissues. Interestingly, the Δ^5- and Δ^6-desaturase expression in the epididymis seems to be as high, or even higher, than in liver.

SCD1 seems neither to be expressed in testis from adult rats (91-day-old), nor in kidney, while expressed in epididymis at the same level as in liver (fig. 3.1). Although not detected in whole testis tissue, SCD1 can be observed in isolated Sertoli cells (fig. 3.2). SCD2 is expressed in testis and epididymis (fig. 3.1), whereas in kidney and liver the expression is very low or absent.

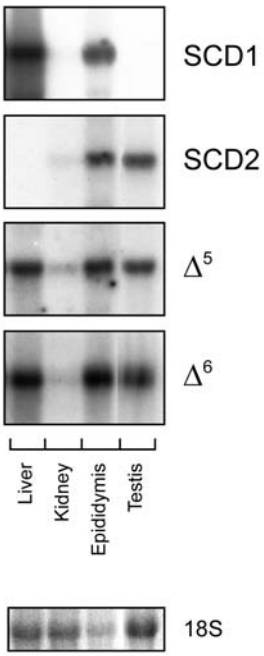

Figure 3.1 Expression of SCD1 and SCD2, Δ^5- and Δ^6-desaturase in different tissues from adult rat. Total RNA (20 µg/lane) was extracted from liver, kidney, epididymis and testis from 91-day-old rats. The ethidium bromide-stained 18S rRNA signal is shown in the lower panel. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). All lanes are from same blot. Each blot is representative for 3 individual experiments.

The desaturase expression pattern among liver, testis and kidney in juvenile rats (35-day-old; standard pellet diet) can be viewed in figure 3.7. In general the same observations can be done at this age. Notice that the liver and testis lanes in figure 3.7 contain 10 µg of total mRNA, while the kidney lanes contain 20 µg.

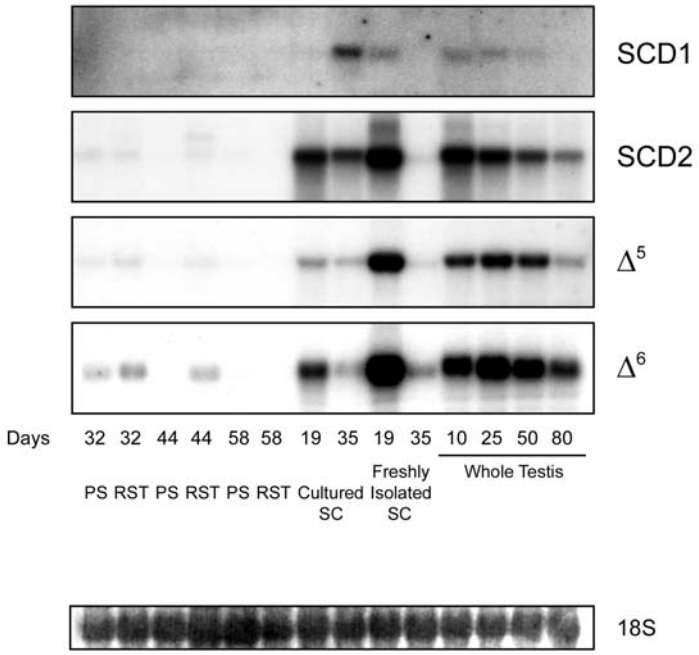

Figure 3.2 Expression of SCD1 and SCD2, Δ^5- and Δ^6-desaturase in separated testicular cells and whole testis tissue from rats of different ages. Total RNA was extracted from whole testis (10-, 25-, 50- and 80-day-old rats), enriched germ cell suspension (PS - pachytene spermatocytes and RST - round spermatids; 32-, 44- and 58-day-old rats), freshly isolated preparations enriched in Sertoli cells, and Sertoli cells cultured for 5 days (19- and 35-day-old rats). Each lane contains 20 µg of total RNA. The ethidium bromide-stained 18S rRNA signal is shown in the lower panel. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). Each blot is representative for 2 individual experiments.

In figure 3.2 the cellular localisation of SCD1, SCD2, Δ^5- and Δ^6-desaturase in the testis can be seen. Once again, Δ^5- and Δ^6-desaturase follow the same expression pattern. The highest levels of expression can be observed in freshly isolated preparations enriched in Sertoli cells. Variations in the desaturase expression among the Sertoli cell preparations may reflect the varying amount of germ cell impurities and the physiological activity of the Sertoli cells in the various preparations. Δ^5- and Δ^6-desaturase are far lower expressed in all the different germ cell fractions than in the Sertoli cells. The age-dependent decrease in the desaturase

signals seen in whole testis tissue confirms this cellular localisation (fig. 3.2). It is unknown whether the very weak signals observed in the germ cells fractions represent desaturase mRNA from germ cells or from contaminating cell types, e.g. Sertoli cells.

A more detailed age-curve is presented in figure 3.3. Here it can be seen that the signal intensity of both Δ^5- and Δ^6-desaturase correlates with the "Sertoli cell profile" of the developing testis in figure 1.9. The highest level of Δ^5- and Δ^6-desaturase mRNA is seen between day 15 and day 20, which coincides with the onset of spermatogenesis. At this age the Sertoli cells constitute the major cell fraction (>65%; fig. 1.9). On day 25 the first haploid cells appear, and as they become more abundant, the desaturase signals and the relative amount of Sertoli cells decrease proportionally, until levelling off between day 40 and day 50. The strong desaturase expression seen on day 30 is partly due to overloading of the lane.

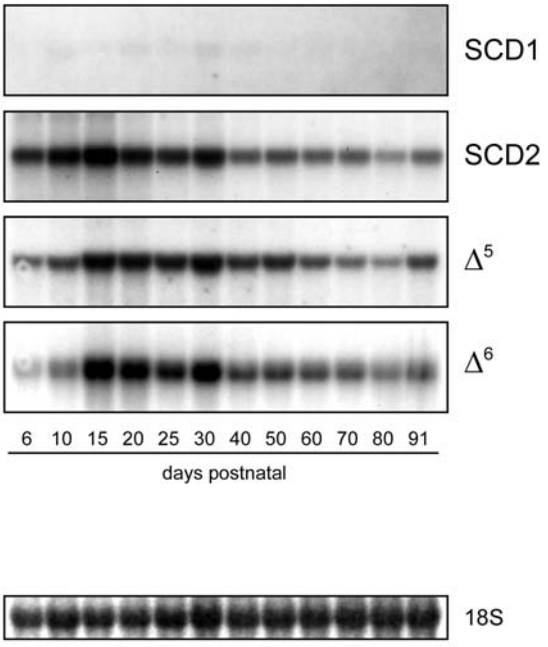

Figure 3.3 Expression of SCD1 and SCD2, Δ^5- and Δ^6-desaturase in whole testis tissue from rats of different ages. Total RNA (20 µg/lane) was extracted from whole testis tissue of rats from 6- to 91-days of age. The ethidium bromide-stained 18S rRNA is shown in the lower panel. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). Each blot is representative for 4 individual experiments.

SCD1 is expressed in testis, but at very low levels. The SCD1-blots in figure 3.2 and 3.3 are results of more than 3 weeks exposure, compared to approximately 1 week for the other genes. SCD1 is expressed in Sertoli cells, with the most pronounced signals observed in cultured Sertoli cells from 35-day-old rats. In germ cells the expression is close to absent. This finding is confirmed through repeated experiments (n=2). As for Δ^5- and Δ^6-desaturase,

the SCD1 expression pattern is confirmed by the age-curve in figure 3.2. In figure 3.3, however, the SCD1 signals are too weak to further analyse the age-dependent expression. SCD2 is much higher expressed in testis than SCD1 (fig. 3.1) and follows essentially the same distribution as the other desaturases (fig. 3.2), with a slightly different age-dependent expression (figs. 3.2 and 3.3). Compared to what is observed for Δ^6-desaturase, the SCD2 expression between day 6 and 15 is relatively higher compared to the maximum level at day 15.

3.1.2 Hormonal regulation of the desaturases

Foetal bovine serum

To evaluate how the isolation and culturing conditions influence on the desaturase gene expression before starting the stimulation experiments, Sertoli cells were cultivated in Minimum Essential Medium (MEM) with or without 10% foetal bovine serum (FBS) on day 3 and 4 of the isolation procedure (fig. 3.4). Furthermore, the same cultures were grown in the presence or absence of 10% FBS for 3, 6 and 12 hours on day 5, representing the hormonal stimulation interval (fig 3.4). All cultures had been cultivated in MEM with 10% FBS from day 0 to day 2. As can be seen in figure 3.4 all of the four desaturase mRNA levels seem to be at highest at t=0, when the cells are grown in the absence of FBS on day 3 and 4. This regime represents the isolation procedure used in our experiments (see *2.4 Preparation of Sertoli cells*).

Δ^5- and Δ^6-desaturase seem to be unaffected by the composition of the media on day 5, as the levels of expression just show minor differences between + and – FBS at a given point of time. However, a time-dependent downregulation of both Δ^5- and Δ^6-desaturase can be observed when comparing the signals at e.g. 3 hours (–) with 12 hours (–). This effect can also be seen in figure 3.5 (control cultures), though not so pronounced.

SCD1 mRNA level is upregulated by FBS, while SCD2 seems to be downregulated at least after 6 and 12 hours (fig. 3.4). The time-dependent variations of SCD1 and SCD2 mRNA are less evident. However, a small upregulation of SCD2 from 3 to 12 hours can be seen in figures 3.5 and 3.8 (control cultures; MEM without FBS).

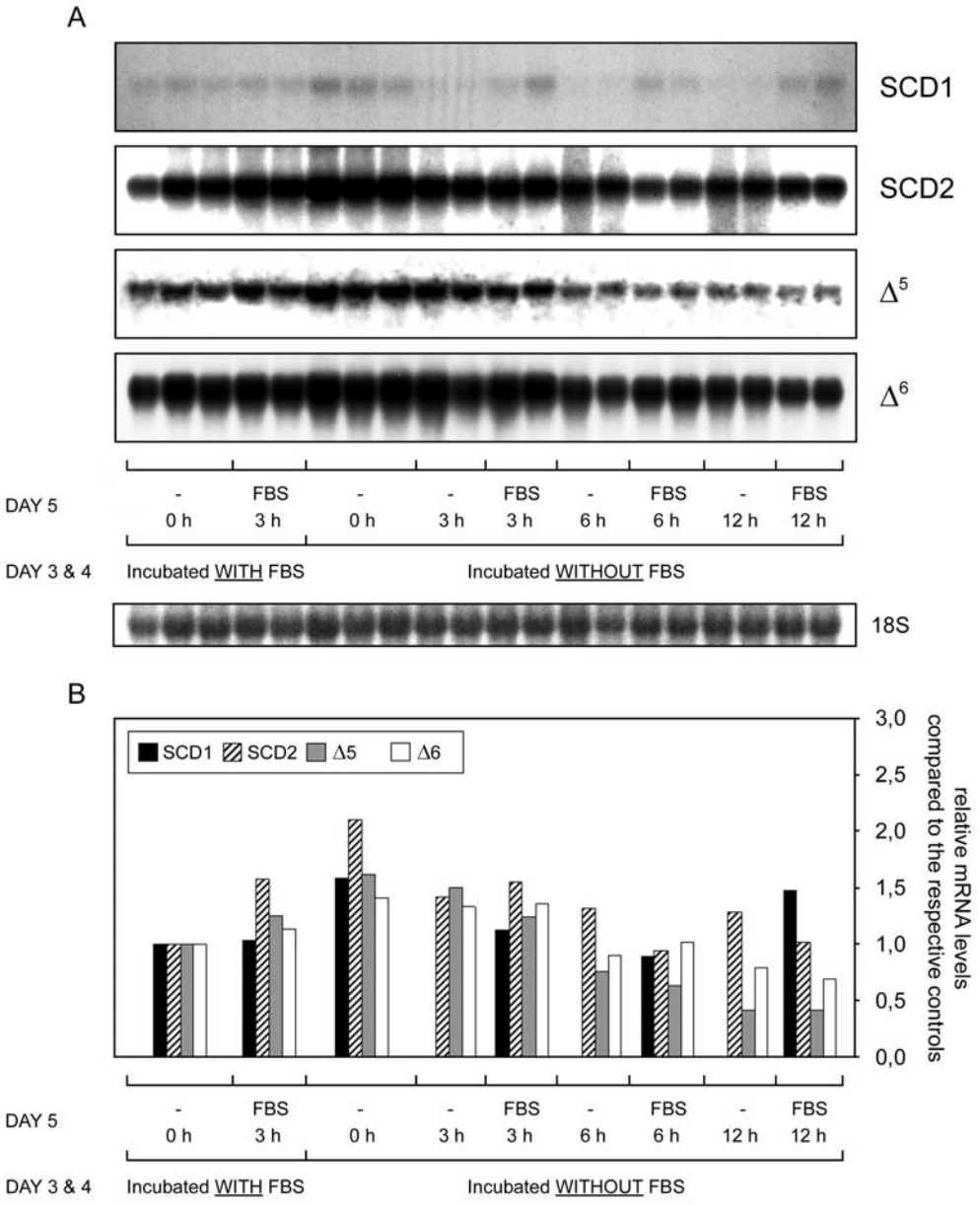

Figure 3.4 Regulation of SCD1 and SCD2, Δ^5- and Δ^6-desaturase mRNA in rat Sertoli cells in response to foetal bovine serum. Sertoli cells from 19-day-old rats were isolated, plated and grown in Minimum Essential Medium (MEM) with 10% foetal bovine serum (FBS) from day 0 to day 2. The cultures were then grown in MEM with or without 10% FBS on day 3 and 4. On day 5 after plating the medium was changed again, and the cells were grown in presence (+) or absence (-) of FBS for 3, 6 or 12 hours. Each lane contains 20 μg of total RNA (A). The duplicates and triplicates represent two or three separate cultures, respectively. The ethidium bromide-stained 18S rRNA signal is shown below the blots. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). The SCD1 blot in this figure is the result of a 3-week exposure. The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of the t=0 cultures, grown with FBS on day 3 and 4 (B).

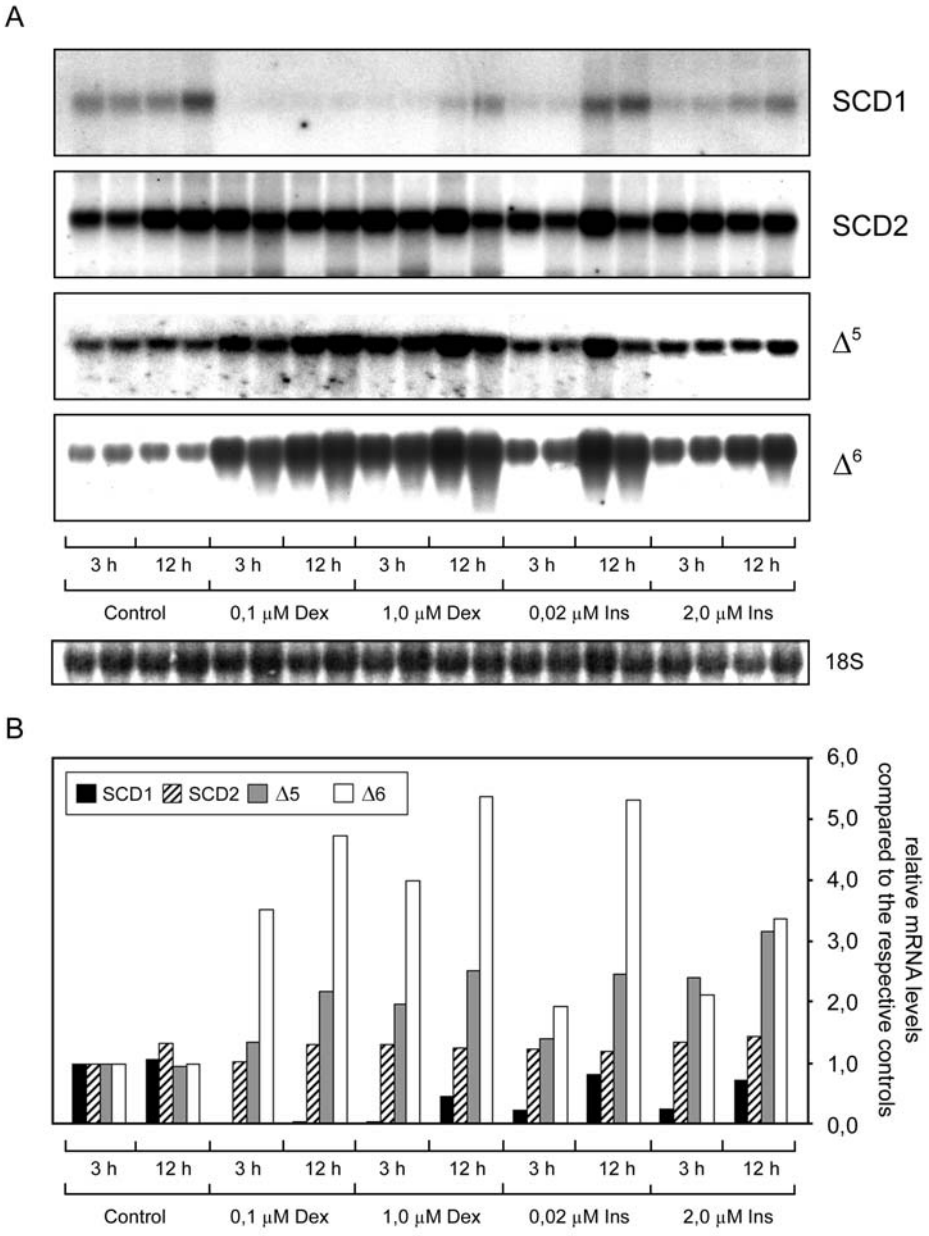

Figure 3.5 Regulation of SCD1 and SCD2, Δ^5- and Δ^6-desaturase mRNA in rat Sertoli cells in response to dexamethasone and insulin. Sertoli cells were isolated from 19-day-old rats. Stimulation experiments started on day 5 after plating. The cells were treated with dexamethasone (0,1 μM and 1,0 μM) and insulin (0,02 μM and 2,0 μM) for 3 and 12 hours. Each lane contains 20 μg of total RNA (A). The duplicates represent two separate cultures. The ethidium bromide-stained 18S rRNA signal is shown below the blots. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). The SCD1 blot in this figure is the result of a 3-week exposure. The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of their respective 3 hour controls (B).

Dexamethasone and insulin

Dexamethasone and insulin upregulate both Δ^5- and Δ^6-desaturase in Sertoli cells (fig. 3.5). This finding is verified in other experiments (figs. 3.8 and 3.9). The Δ^5- and Δ^6-desaturase signals are slightly higher when the cells are stimulated with 1,0 μM dexamethasone than with 0,1 μM (fig.3.5). When looking at the insulin-stimulated Sertoli cells, the most marked upregulation of Δ^6-desaturase is seen with the lowest concentration (0,02 μM) at 12 hours (fig 3.5). However, when repeated (fig. 3.9), approximately no difference is seen between the Δ^6-desaturase mRNA levels in Sertoli cells treated with 0,02 μM and 2,0 μM insulin at 12 hours. For Δ^5-desaturase, the mRNA expression is strongest at the highest concentration of insulin (figs. 3.5 and 3.9). Furthermore, in all three experiments the induction of Δ^5-desaturase by dexamethasone and insulin is weaker than for Δ^6-desaturase (fig. 3.5, 3.8 and 3.9).

The SCD1 mRNA level is downregulated both by dexamethasone and insulin, as observed after 3 hours (fig. 3.5) For both hormones at both concentrations, a small upregulation of SCD1 can be observed from 3 to 12 hours, bringing the SCD1 gene expression towards the control levels. The time-dependent dexamethasone effect on the SCD1 expression is confirmed in figure 3.8. In figure 3.9, all cultures were treated with dexamethasone and insulin for 12 hours, hence no downregulation is observed. The regulation of SCD2 in response to dexamethasone and insulin is less pronounced than for the other desaturases (fig. 3.5). However, through repeated experiments a weak upregulation of SCD2 mRNA has been observed following both dexamethasone and insulin treatment (figs. 3.8, 3.9 and 3.5 - high concentrations, 3 hours).

Testosterone and follicle-stimulating hormone

The blots in figure 3.6 show the Sertoli cell response to testosterone, FSH and $(Bu)_2$cAMP with respect to Δ^5-desaturase, Δ^6-desaturase, SCD1 and SCD2. All four desaturases are upregulated by FSH. This effect is confirmed through $(Bu)_2$cAMP-stimulation, which propagates an even more pronounced upregulation. The most dramatic effect is seen with SCD1. SCD1 mRNA turns from almost non-detectable to a high expression when the Sertoli cells are stimulated with FSH (1,0 IU/mL). Normalisation of the blot indicates a more than

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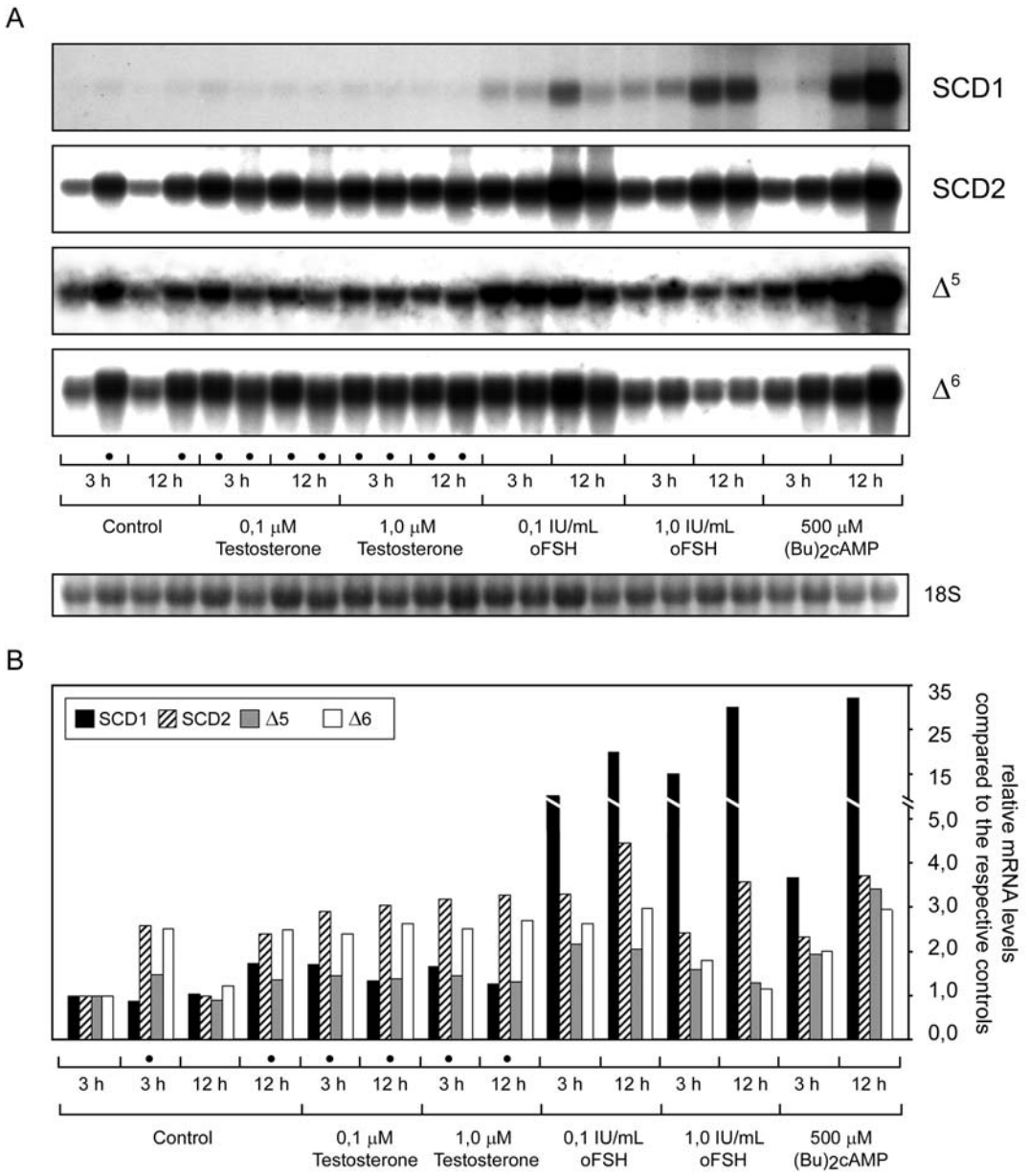

Figure 3.6 Regulation of SCD1 and SCD2, Δ^5- and Δ^6-desaturase mRNA in rat Sertoli cells in response to testosterone, FSH and $(Bu)_2$cAMP. Sertoli cells were isolated from 19-day-old rats. Stimulation experiments started on day 5 after plating. The cells were treated with testosterone (0,1 μM and 1,0 μM), FSH (0,1 IU/mL and 1,0 IU/mL) and $(Bu)_2$cAMP (500 μM) for 3 and 12 hours. All lanes marked with • correspond to cell cultures that contained a final ethanol concentration of 0,001%, necessary for dissolving testosterone. Each lane contains 20 μg of total RNA (A). The duplicates represent two separate cultures. The ethidium bromide-stained 18S rRNA signal is shown below the blots. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). The SCD1 blot in this figure is the result of a 2-week exposure. The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of their respective 3 hour controls without ethanol (B).

30× upregulation. The Δ^5-desaturase , Δ^6-desaturase and SCD2 mRNA FSH responses are most pronounced with 0,1 IU/mL FSH. By comparing the testosterone-treated Sertoli cells cultures with the control cultures added ethanol, it seems that none of the desaturases is regulated by testosterone. On the other hand, ethanol (0,001%) seems to upregulate all the four desaturases

3.1.3 Effect of essential fatty acid deficiency on the desaturase expression

The fatty acid profile in whole liver and testis from 35-day-old rats fed a standard pellet and a fat free diet, respectively, for 14 days is shown in table 3.1. The testis profile differs somewhat from the liver profile in the animals fed the standard pellet diet. Stearic acid, 18:0, linoleic acid, 18:2(*n*-6), and DHA, 22:6(*n*-3), are more dominant in the normal liver compared to the normal testis. On the other hand, Osbond acid, 22:5(*n*-6), constitutes 10,4 % of the fatty acids in testis, while in liver it is non-detectable.

When feeding the rats a fat free diet, the lipid composition changes in both liver and testis. Interestingly, the changes follow a similar pattern in both organs. There is a drop in the arachidonic acid, 20:4(*n*-6) content from 16,0 % to 7,9 % in liver and from 13,9 % to 9,5 % in testis. Furthermore, a decrease in the 18:2(*n*-6) and 22:6(*n*-3) can be observed. The decrease in 22:6(*n*-3), however, is only significant in liver. At the same time a compensatory effect is seen in the 18:1(*n*-7/9) group, in both liver and testis. Mead acid, 20:3(*n*-9), which function as kind of a marker for essential fatty acid deficiency, appears in both tissues when feeding the animals a fat free diet (liver; 5,8 %, testis; 3,4 %). By definition essential fatty acid deficiency is attained when the ratio Mead acid to arachidonic acid is higher than 0,4 in liver [Holman, 1968]. As can be seen, this limit was exceeded (tab. 3.1). Thus, essential fatty acid deficiency was induced. In testis this ratio was 0,36.

The desaturase gene expression's response to EFAD conditions can be viewed in figure 3.7. In liver Δ^5- and Δ^6-desaturase and both the SCDs are upregulated as a result of a fat free diet. The most pronounced upregulation is seen with Δ^6-desaturase followed by SCD1 and Δ^5-desaturase.

	Liver		Testis	
Fatty acids	Standard pellet	Fat free diet	Standard pellet	Fat free diet
16:0	22.9 ± 1.0	23.8 ± 4.5	24.4 ± 0.6	24.0 ± 1.6
18:0	16.6 ± 0.6	14.2 ± 0.5 [a]	5.4 ± 0.1	5.2 ± 0.3
18:1(*n*-7/9)	13.4 ± 1.6	26.1 ± 1.9 [a]	10.8 ± 0.3	16.4 ± 1.8 [a]
18:2(*n*-6)	14.9 ± 1.5	2.3 ± 0.9 [a]	4.9 ± 0.4	1.3 ± 0.3 [a]
20:3(*n*-9)	nd	5.8 ± 0.5	nd	3.4 ± 0.6
20:4(*n*-6)	16.0 ± 0.7	7.9 ± 1.3 [a]	13.9 ± 0.3	9.5 ± 0.4 [a]
22:5(*n*-6)	nd	1.1 ± 0.1	10.4 ± 1.0	6.6 ± 0.6 [a]
22:6(*n*-3)	9.9 ± 0.4	5.4 ± 1.0 [a]	3.9 ± 0.5	3.5 ± 0.4
Other FA	6.2 ± 1.1	13.5 ± 2.4 [a]	26.3 ± 2.0	30.1 ± 4.0
20:3/20:4	0.0 ± 0.0	0.75 ± 0.18 [a]	0.0 ± 0.0	0.36 ± 0.08 [a]

Table 3.1 Fatty acid composition of whole liver and testis from rats fed standard pellet diet and fat free diet. Six 21-day-old Wistar rats (littermates) were split into two groups of three and fed a standard pellet diet and a fat free diet, respectively (see *2.2 Animals – Dietary studies* for diet composition). The regimes comprised 14 days of feeding, before the rats were killed by CO_2-asphyxiation. Liver and testis were dissected from the rats, and total lipids were extracted by chloroform:methanol (2:1). The lipids were trans-methylated and analysed on a gas chromatograph. Explanations to the fatty acids see *1.1.2 Metabolism of polyunsaturated fatty acids.* All fatty acid values given in mol % ± standard deviation. N=3, nd=non-detectable and 18:1(*n*-7/9) equals the total of 18:1(*n*-7) and 18:1(*n*-9). [a] $P<0.05$

Furthermore, SCD2 signals appear in the EFAD liver as compared to the non-detectable mRNA levels in normal liver (figs. 3.1 and 3.7). Interestingly, none of the desaturases are induced in response to EFAD conditions in whole testis tissue, as opposed to what is seen in liver (fig 3.7). Due to the low expression of SCD1 in testis and the limited availability of animals in this experiment, making isolation of Sertoli cell impossible, any effects of EFAD on the SCD1 expression can not be excluded.

The vacant upregulation in testis becomes even more interesting, when observing the effects in another metabolically peripheral tissue, the kidney. Here, like in liver, an upregulation of all the four desaturases is seen when turning from standard pellets to a fat free diet. The strength of induction, on the other hand, follows an opposite trend of what is seen in liver, with SCD1 being the most upregulated gene and Δ^6-desaturase the least upregulated (fig. 3.7).

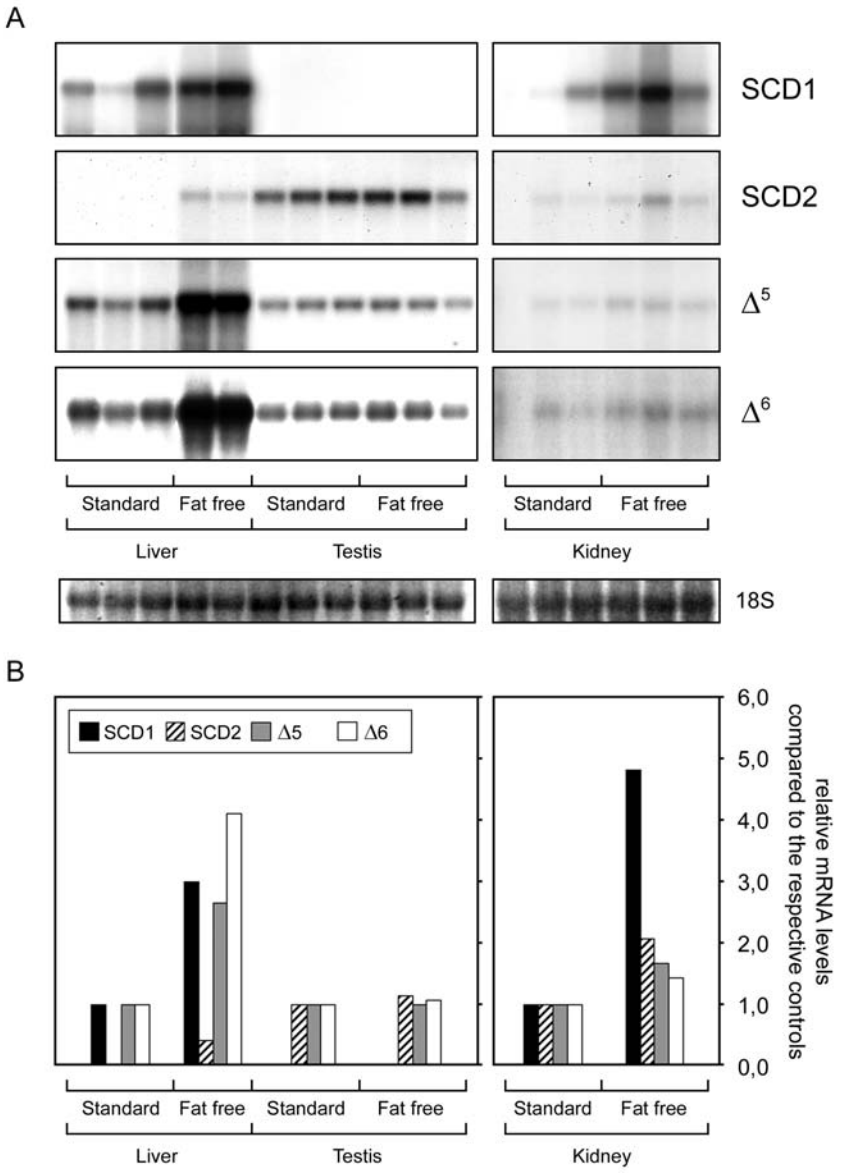

Figure 3.7 Regulation of SCD1 and SCD2, Δ^5- and Δ^6-desaturase mRNA in response to a fat free diet. Six 21-day-old Wistar rats (littermates) were split into two groups of three and fed a standard pellet diet and a fat free diet, respectively (see *2.2 Animals – Dietary studies* for diet composition). The regimes comprised 14 days of feeding, before the rats were killed by CO_2-asphyxiation. Liver, testis and kidney were dissected from the rats, and total RNA was extracted the tissues. Each lane contains either 10 µg (liver and testis), or 20 µg (kidney) of total RNA. Lane 1-6-12, 2-7-13 etc. up to 5-11-17 represent different tissues from the same animal (A). The ethidium bromide-stained 18S rRNA signal is shown below the blots. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of their respective "standard pellet diet"-controls (B). Liver SCD2-signals were plotted relative to standard pellet testis.

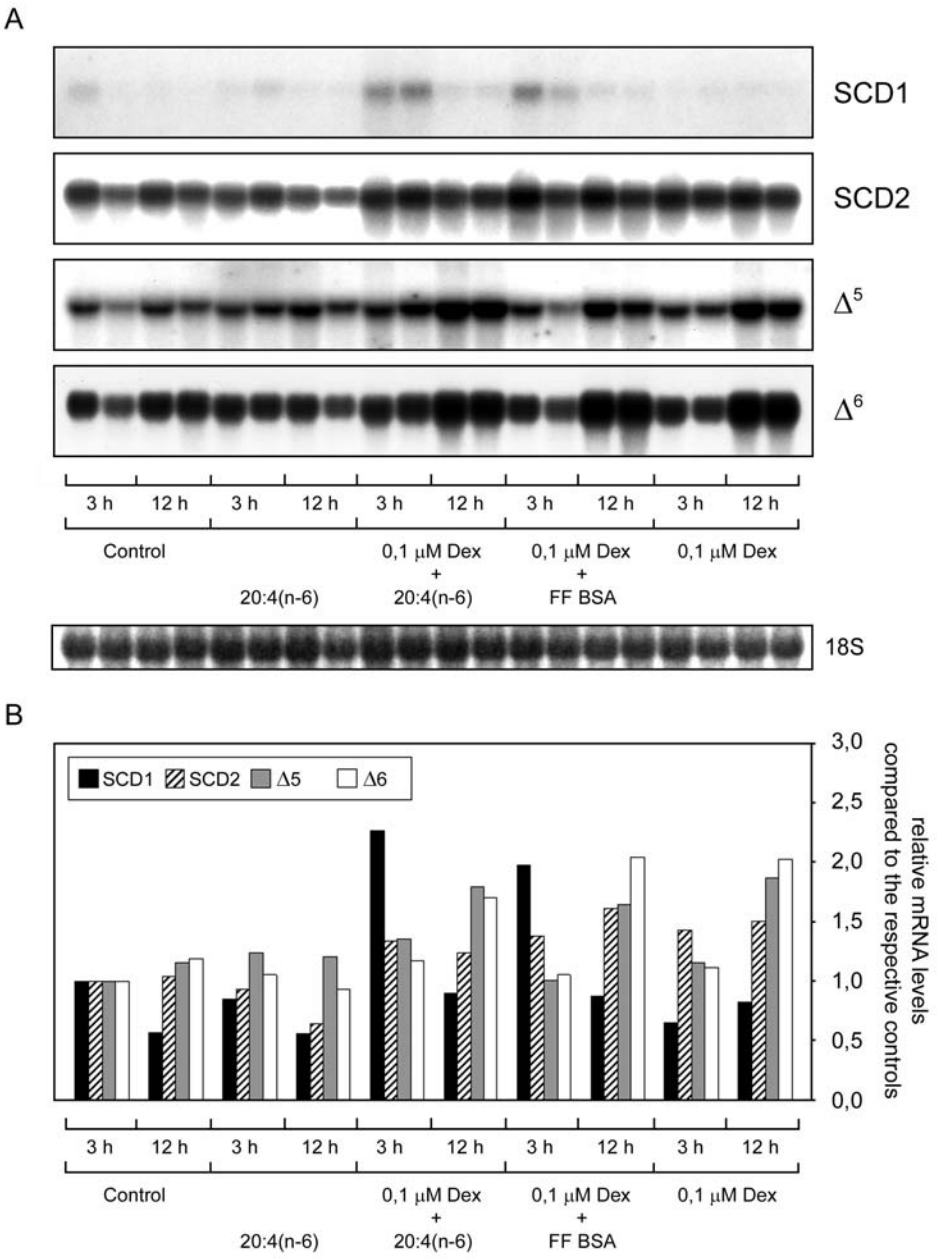

Figure 3.8 Regulation of SCD1 and SCD2, Δ^5- and Δ^6-desaturase mRNA in rat Sertoli cells in response to arachidonic acid and dexamethasone. Sertoli cells were isolated from 19-day-old rats. Stimulation experiments started on day 5 after plating. The cells were treated with arachidonic acid (20:4(*n*-6); 50 μM), dexamethasone (Dex; 0,1 μM) and fatty acid free bovine serum albumin (FF BSA; 0,072% w/w), singly or in combination, for 3 and 12 hours. Each lane contains 20 μg of total RNA (A). The duplicates represent two separate cultures. The ethidium bromide-stained 18S rRNA signal is shown below the blots. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). The SCD1 blot in this figure is the result of a 3-week exposure. The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of their respective 3 hour controls (B).

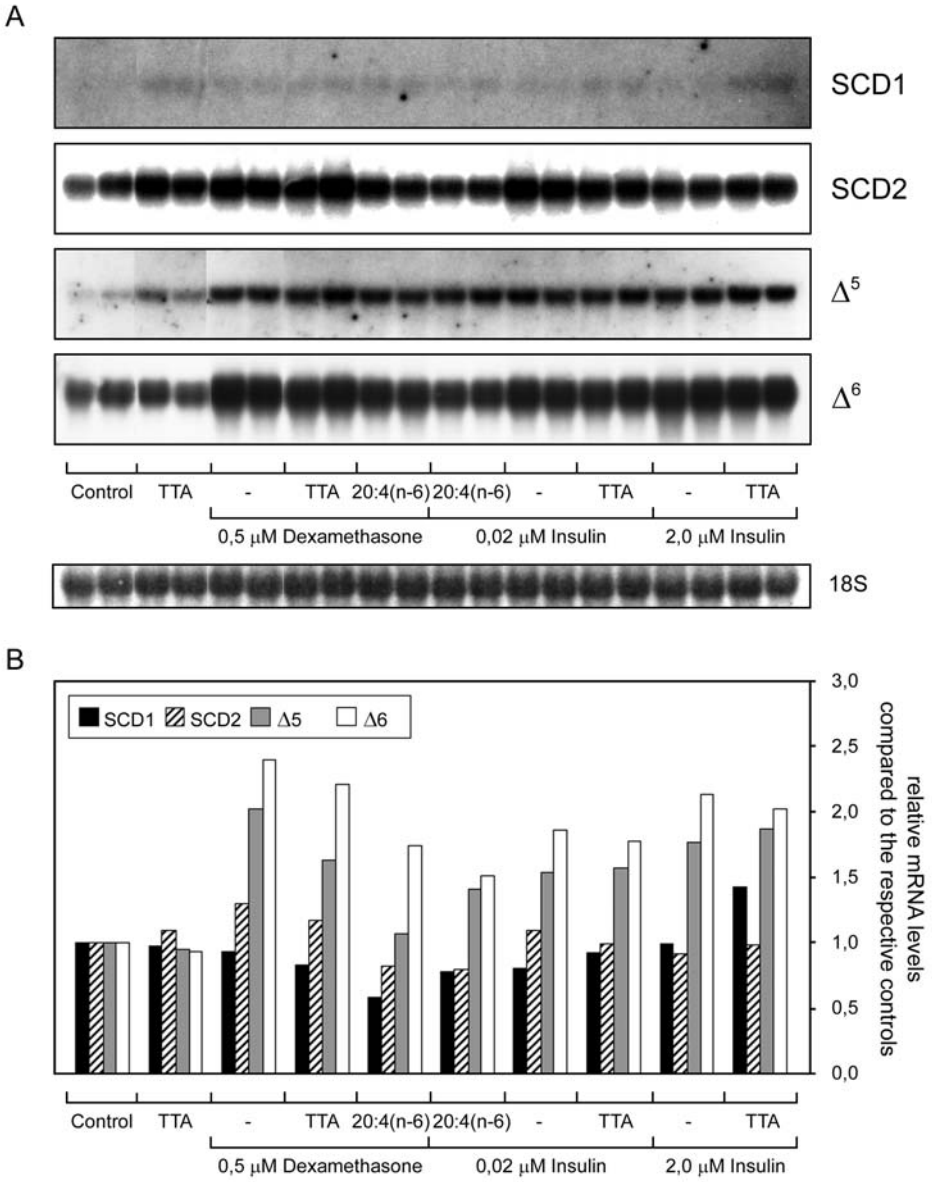

Figure 3.9 Regulation of SCD1 and SCD2, Δ^5- and Δ^6-desaturase mRNA in rat Sertoli cells in response to arachidonic acid, TTA, dexamethasone and insulin. Sertoli cells were isolated from 19-day-old rats. Stimulation experiments started on day 5 after plating. The cells were treated with arachidonic acid (20:4(*n*-6); 50 μM), tetradecylthioacetic acid (TTA; 50 μM), dexamethasone (0,5 μM) and insulin (0,02 μM and 2,0 μM), singly or in combination, for 12 hours. Each lane contains 20 μg of total RNA (A). The duplicates represent two separate cultures. The ethidium bromide-stained 18S rRNA signal is shown below the blots. SCD1: Stearoyl-CoA desaturase 1 (5,9 kb transcript), SCD2: Stearoyl-CoA desaturase 2 (5,9 kb transcript), Δ^5: Δ^5-desaturase (4,1 kb transcript) and Δ^6: Δ^6-desaturase (3,8 kb transcript). The SCD1 blot in this figure is the result of a 3-week exposure. The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of their respective 3 hour controls (B).

3.1.4 Metabolic regulation of the desaturases

In figures 3.8 and 3.9 the effect of endogenous and synthetic fatty acids on the desaturase expression in Sertoli cells with can be viewed. The results obtained here are quite complex, however some effects can be emphasised. The most marked decrease in Δ^5- and Δ^6-desaturase and SCD2 mRNA levels is seen when the cells are treated with 20:4(n-6) or TTA in combination with 0,5 μM dexamethasone or 0,02 μM insulin for 12 hours, as compared to dexamethasone and insulin alone (fig. 3.9). 20:4(n-6) seem to be the most effective fatty acid in downregulating the expression of the same genes. When stimulating the Sertoli cells with 20:4(n-6) (fig. 3.8) or TTA (fig. 3.9) alone, only 20:4(n-6) shows a downregulating effect. This is best observed for Δ^6-desaturase and SCD2 after 12 hours treatment (fig. 3.8). The Δ^5-desaturase expression diverges somewhat from this pattern, especially when the Sertoli cells are treated with fatty acids in combination with low concentration dexamethasone (0,1 μM) or high concentration insulin (2,0 μM).

The changes in SCD1 mRNA levels are even more difficult to interpret, mainly because of their low doses and inverse response to dexamethasone and insulin compared to the other desaturases (fig 3.5). Any effects of 20:4(n-6) seem to disappear within 12 hours. This is most easily observed in figure 3.8. In contrast to the other desaturases SCD1 is upregulated by bovine serum albumin (BSA) which functioned as the fatty acid carrier protein in these experiment. This effect is most pronounced after treating the cells with hormones and fatty acids-BSA for 3 hours (fig. 3.8). This observation can probably explain the effects seen when treating Sertoli cells with foetal bovine serum (FBS; fig. 3.4). Here the SCD1 expression is upregulated in response to FBS, which contains BSA.

3.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

3.2.1 Essential fatty acid deficiency and PPARs

The expression patterns of the peroxisome proliferator-activating receptors in whole liver, testis and kidney are presented in figure 3.10. PPARα, −δ and -γ are expressed in testis from

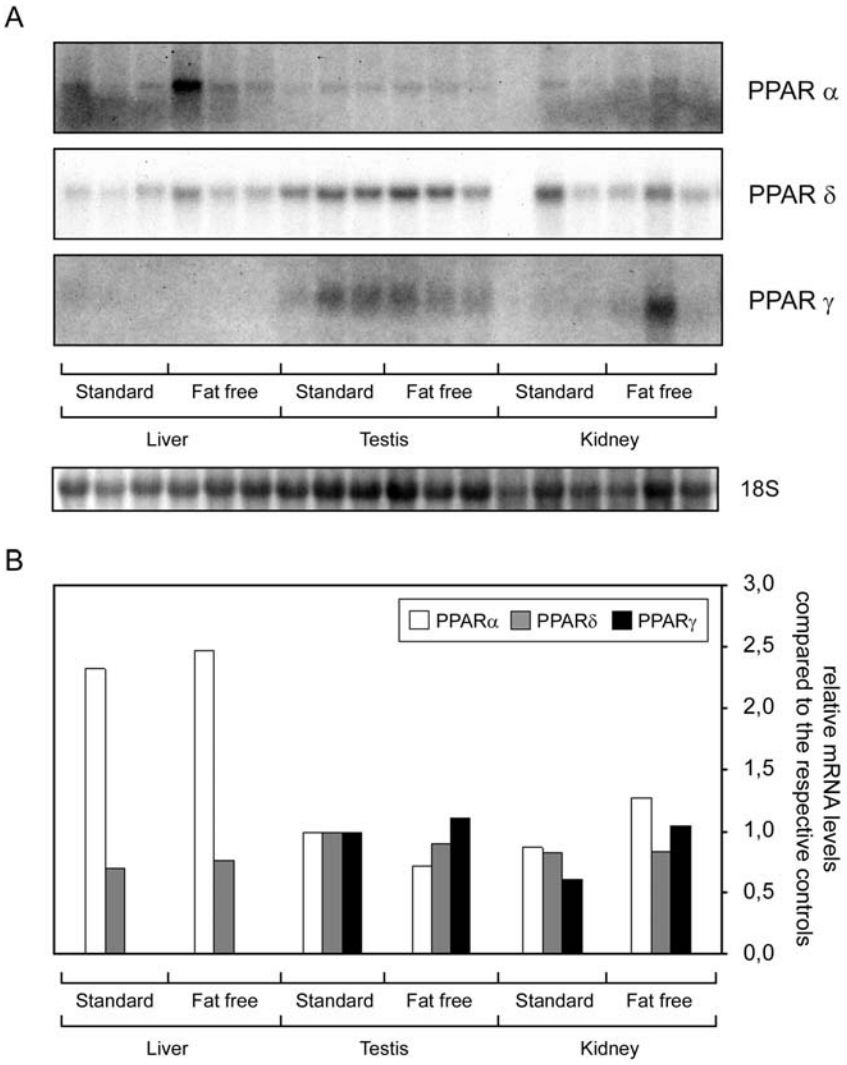

Figure 3.10 Regulation of PPARα, PPARδ and PPARγ mRNA in response to a fat free diet. Six 21-day-old Wistar rats (littermates) were split into two groups of three and fed a standard pellet diet and a fat free diet, respectively (see *2.2 Animals – Dietary studies* for diet composition. The regimes comprised 14 days of feeding, before the rats were killed by CO_2-asphyxiation. Liver, testis and kidney were dissected from the rats, and total RNA was extracted the tissues. Each lane contains 20 µg of total RNA. Lane 1-7-13, 2-8-14 etc. up to 6-12-18 represent different tissues from the same animal (A). The ethidium bromide-stained 18S rRNA signal is shown in the lower panel. PPARα: Peroxisome Proliferator-Activating Receptor α (11,5 kb transcript), PPARδ: Peroxisome Proliferator-Activating Receptor δ (3,9 kb transcript) and PPARγ: Peroxisome Proliferator-Activating Receptor γ (2,5 kb transcript). The lower graph represents intensity readings from the scanning of the autoradiograms, normalised to the 18S rRNA signals, and plotted relative to the values of standard pellet testis (B).

standard pellet-fed rats, virtually in the same levels as in kidney. In liver from standard pellet-fed rats, however, PPARα are highly expressed, more than in testis and kidney. PPARδ is expressed in normal liver, while PPARγ on the other hand, is not detectable (fig. 3.10). Notice that all lanes in these blots contain 20 µg of total mRNA in contrast to what is the case in figure 3.7 in which the liver and testis lanes contain 10 µg.

In response to the fat free diet, PPARα seems to be slightly upregulated in liver and kidney, while downregulated in testis. The PPARδ mRNA level seems to be unaffected by this diet in all three organs, while PPARγ is upregulated in testis and kidney (fig. 3.10) However, more experiments should be done to confirm these findings.

3.2.2 Localisation of the PPARs

In figure 3.11 the cellular localisation of PPARα, –δ and -γ in the testis can be seen. PPARα shows a gene expression pattern quite similar to Δ^5- and Δ^6-desaturase, in that the Sertoli cell is the cell type with the highest expression. The highest levels of PPARα mRNA can be observed in freshly isolated preparations enriched in Sertoli cells from 19-day-old animals (fig 3.11). PPARα is not detectable in any of the different germ cell fractions, and variations in the PPARα expression among the Sertoli cell preparations may reflect the varying amount of germ cell contaminating these preparations. The age-dependent decrease in the PPARα signals seen in whole testis tissue, confirms this cellular localisation (fig. 3.11).

PPARδ is expressed in both Sertoli cells and germ cells, with the highest expression in round spermatids (RST; fig 3.11). As a consequence of this, an altered age-dependent expression-pattern can be seen in whole testis tissue. On day 25 the first haploid cells appear (fig. 1.9), and as they become more abundant, the PPARδ signals increase proportionally, until levelling off around day 50 (fig 3.11).

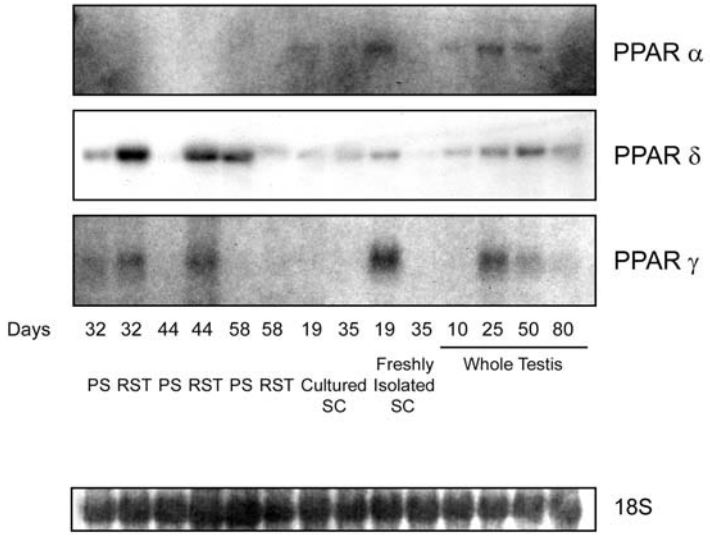

Figure 3.11 Expression of PPARα, PPARδ and PPARγ in separated testicular cells and whole testis tissue from rats of different ages. Total RNA was extracted from whole testis (10-, 25-, 50- and 80-day-old rats), enriched germ cell suspension (PS - pachytene spermatocytes and RST - round spermatids; 32-, 44- and 58-day-old rats), freshly isolated preparations enriched in Sertoli cells and Sertoli cells cultured for 5 days (19- and 35-day-old rats). Each lane contains 20 μg of total RNA. The ethidium bromide-stained 18S rRNA signal is shown in the lower panel. PPARα: Peroxisome Proliferator-Activating Receptor α (11,5 kb transcript), PPARδ: Peroxisome Proliferator-Activating Receptor δ (3,9 kb transcript) and PPARγ: Peroxisome Proliferator-Activating Receptor γ (2,5 kb transcript).

PPARγ is, like PPARδ, also expressed in both Sertoli and germ cells, but the Sertoli cell seems to be the cell type expressing the highest levels of PPARγ mRNA. Once again the age-dependent decrease in expression confirms that the Sertoli cell is the dominant contributor to the PPARγ mRNA pool in whole testis (fig 3.11).

4. DISCUSSION

4.1 DESATURASES

4.1.1 Localisation of the desaturases

In this paper we show for the first time to our knowledge, that SCD1 and SCD2 are expressed in testis. When comparing liver and testis from adult rats, the testis expresses far lower levels of SCD1 mRNA than liver, while the opposite distribution is seen with SCD2. Earlier reports reviewed by Ntambi describe large differences in expression and regulation between the SCDs [Ntambi, 1995]. SCD1 and SCD2 are both expressed in a tissue-specific manner and regulated differently in various tissues in response to hormones, fat free diets and PUFA-supplements (see *4.1.3 Effect of essential fatty acid deficiency on the desaturase expression*). Some tissues also express these desaturases constitutively. Previously published papers on sperm lipids, point in the direction that a functional sperm is dependent on a balanced composition of *n*-3, *n*-6 and *n*-9 fatty acids [Connor et al., 1998; Zalata et al., 1998; Ollero et al., 2001]. Spermatozoa enriched in *n*-9 fatty acids may turn out to be non-functional, e.g. unable to undergo the acrosome reaction. As shown in this thesis, SCD2 is responsive to *in vitro* hormonal treatment of testicular cells, indicating that there is a need for regulating the access of *n*-9 acids in testis.

Furthermore, we have shown that all four desaturases are highly expressed in epididymis from sexually mature rats. Such distribution has not been described for other tissues. Interestingly, the epididymal expression of all of the four desaturases is as high, or higher, than the hepatic expression. Two possible explanations could be given for this pronounced desaturase expression. Either the PUFAs are desaturated in epididymis as a step in the maturation process of the spermatozoa, or the high-order PUFAs are synthesised to secure vital epididymal cells. Studies on ram sperm indicate an increase in total DHA (22:6(*n*-3)) content from 14% to 25% in the spermatozoa, when passing through the epididymis [Nolan & Hammerstedt, 1997]. However, the increase in 22:6(*n*-3) is correlated with a selective loss of sperm phospholipids throughout the epididymis, resulting in an elevated proportion of choline plasmalogen-bound 22:6(*n*-3) during this passage [Jones, 1998].

A co-expression of both stearoyl-CoA desaturases in a single tissue has so far not been described. Our observations in epididymis, as viewed in figure 3.1, are therefore interesting, and the physiological role of the strong epididymal expression of both SCD1 and SCD2 should be further studied. The epididymis is, as emphasised in the introduction, an organ concerned with both maturation and storage of spermatozoa, built up by at least three distinct functional and histological parts. The question whether or not the two SCDs also are co-expressed in caput, corpus and cauda epididymis, and in their different cell types, is now addressed in new experiments.

Our previous studies on Δ^6-desaturase activity indicated that the enzyme activity is much higher in Sertoli cells than in germ cells [Retterstøl et al., 2001]. In this study we confirm these findings by demonstrating much higher Δ^6-desaturase mRNA levels in Sertoli cells, than in germ cell. Not surprisingly, Δ^5-desaturases follows the same expression pattern as Δ^6-desaturase. Furthermore, SCD1 and -2 are also predominantly expressed in the Sertoli cells. These findings, combined with the knowledge that germ cells are especially rich in PUFAs, more than the Sertoli cells [Beckman & Coniglio, 1979], suggest that the PUFAs are modified in the Sertoli cells before transported to the germ cells. A lipid transport between the Sertoli and the germ cell was postulated as early as 1888 by Von Ebner, who was the first to confirm the presence of lipid droplets in the Sertoli cells [Ebner, 1888]. The hypothesis has been refined and tested several times, but until today, it has not been supported by data from experiments designed for such studies [Beckman & Coniglio, 1979; Marzouki & Coniglio, 1982; Retterstøl et al., 2001].

4.1.2 Hormonal regulation of the desaturases

Foetal bovine serum

As can be viewed in figure 3.4, the isolation and cultivation conditions inflect on the expression of all the desaturases. In general, the isolation procedure described by Dorrington and co-workers [Dorrington et al., 1975], employed by us and numerous others studying Sertoli cells, results in the highest desaturase expression on day 5 at time 0. From this point the expression of the different desaturases takes on different trends: The Δ^5- and Δ^6-desaturase

expression declines, while the SCD1 expression is abruptly downregulated, and then follow a more constant expression pattern (fig. 3.5), as do SCD2.

The time-dependent expression patterns of the four desaturases differ, but comparing all treated cultures with their respective time controls eliminates the uncertainty introduced by this variation. Based on the results from this experiment, we chose to employ the standard isolation and cultivation protocol for Sertoli cells [Dorrington et al., 1975] in the subsequent experiments.

Finally, the time-dependent downregulation of Δ^5- and Δ^6-desaturase expression, could be interpreted as the result of a "from *in vivo* to *in vitro*" situation. The lowered mRNA levels may be a result of the cells starving from the lack of one or several factors and/or metabolites. This is further discussed in the following chapters.

Dexamethasone and insulin

Through repeated experiments we have found that dexamethasone and insulin upregulate Δ^5- and Δ^6-desaturase gene expression in Sertoli cells. Concerning the stearoyl-CoA desaturases the picture is more complex. SCD1 mRNA is downregulated by dexamethasone and insulin, while SCD2 is upregulated both hormones. The fact that SCD2 is several-fold higher expressed in testis than SCD1, suggests that SCD2 is responsible for the Δ^9-desaturase activity in this organ.

Interestingly, the regulation of the desaturases in testicular cells is opposite of what is found in liver. De Gómez Dumm *et al.* reported as early as 1979 that dexamethasone depresses the Δ^5- and Δ^6-desaturase activity in liver microsomes, while inducing the Δ^9-desaturase activity [de Gómez Dumm et al., 1979]. However, this was an *in vivo* study on female rat liver microsomes measuring desaturase activity. Disregarding the experimental conditions, the different regulation observed may reflect the roles liver and testis play in the fatty acid metabolism; the liver provides various tissues with fatty acids, while the testis has a great demand for PUFAs. This fact could, from a physiological point of view, explain the differences seen in liver and testis with respect to the regulation of Δ^5- and Δ^6-desaturases.

In 1986 Marra *et al.* reported effects similar to de Gómez Dumm *et al.* When adding liver microsomes stimulated *in vivo* with dexamethasone to unstimulated liver microsomes *in vitro*, the Δ^5- and Δ^6-desaturase activity decreased [Marra et al., 1986]. This group addressed the origin of this inhibition to a dexamethasone-induced, trypsin labile, loosely bound microsome factor. This factor has to our knowledge not been further characterised, nor have studies been performed on Δ^5- and Δ^6-desaturase gene expression in dexamethasone stimulated liver. This opens for more than one explanation for the different findings in liver and testis. Either there exist liver specific Δ^5- and Δ^6-desaturase inhibitors, or the increased Δ^5- and Δ^6-desaturase transcripts in Sertoli cells do not result in increased protein activity, due to repressor actions.

When looking at regulation of the stearoyl-CoA desaturase expression in dexamethasone-treated testicular cells, both de Gómez Dumm and Marra have described similar trends in liver. At transcriptional level we observe an upregulation SCD2 in Sertoli cells, while the two other groups found an increased Δ^9-desaturase activity in liver microsomes [Marra et al., 1988]. Marra and co-workers further suggested that a dexamethasone-induced protein was responsible for this activation.

Concerning the insulin effect on the desaturases, the presented results are in accordance with earlier results published on liver. We find that insulin upregulates Δ^5- desaturase, Δ^6-desaturase and the net stearoyl-CoA desaturase gene expression in rat Sertoli cells. Brenner and co-workers have studied diabetic rats, and found sevenfold lower Δ^6-desaturase mRNA in liver when compared to normal rats. After 24-hours insulin administration, the Δ^6-desaturase mRNA level increased eightfold [Rimoldi et al., 2001]. In an earlier study they found impaired hepatic Δ^5- and Δ^9-desaturase activities in addition to impaired Δ^6-desaturase activity in diabetic rats [Brenner et al., 2001]. These activities could be restored by insulin treatment.

Δ^6-desaturase is generally accepted as rate-limiting in the formation of arachidonic acid, 20:4(*n*-6), and eicosapentaenoic acid, 20:5(*n*-3), from linoleic acid, 18:2(*n*-6), and linolenic acid, 18:3(*n*-3), respectively [Brenner, 1977]. It is therefore interesting that the level of Δ^6-desturase mRNA is more strongly upregulated than Δ^5-desaturase mRNA by both insulin and dexamethasone. After all these genes reside in reverse orientation on chromosome 11 in the human genome, separated by less than 11.000 base pairs head to head (5' to 5') [Cho et al.,

1999b]. This opens up for the possibility that transcription of Δ^5- and Δ^6-desaturase is governed by the same regulatory sequences.

Testosterone and follicle-stimulating hormone

In this study we show that all four desaturases are upregulated by FSH in Sertoli cells, while testosterone does not seem to influence the desaturase gene expression. These results are in accordance with observations done by Marzouki and Coniglio in the 80's [Marzouki & Coniglio, 1984; Coniglio et al., 1988]. They showed that the testis of hypophysectomised male rats converted less [1-^{14}C] 20:3(*n*-6) into [1-^{14}C] 20:4(*n*-6) and [1-^{14}C] 22:4(*n*-6), concluding that hypophysectomy, hence loss of gonadotropin action, inhibited the Δ^5-desaturase activity in testis [Marzouki & Coniglio, 1984]. They further showed that treatment with testosterone did not affect the metabolism of any of the [^{14}C] substrates in testis [Marzouki & Coniglio, 1984]. No such studies, with respect to Δ^6- or Δ^9-desaturase activity, have been reported. Coniglio and co-workers also showed that the elongation of PUFAs in testis was impaired by hypophysectomy, and that the elongation activity was restored with FSH treatment, but not with testosterone [Coniglio et al., 1988].

Our study shows that the induction of Δ^5- and Δ^6-desaturase by FSH is more rapid than the induction of the stearoyl-CoA desaturases. Whether or not this reflects the involvement of different transactivators is not known. The induction of the stearoyl-CoA desaturases obtained with $(Bu)_2$cAMP indicates that increased cAMP level is involved in the FSH-dependent upregulation of all four desaturases. When comparing the Δ^5-desaturase, Δ^6-desaturase and SCD2 signals in freshly isolated preparations enriched in Sertoli cells with the signals in cultured Sertoli cells (fig. 3.2), there is a marked difference. The highest levels of desaturase mRNA are observed in the fresh Sertoli cell preparations, even though more germ cells are present in these preparations than in cultured Sertoli cells. Obviously, there must be factors, paracrine or endocrine, or metabolites present in the cell preparations that are lost upon culturing. FSH could be one of these factors.

The upregulation of all the four desaturases by ethanol in Sertoli cells is difficult to explain. However, two recent studies in hepatoma cells shows that ethanol when metabolised to acetaldehyde, inhibits the transcriptional and DNA-binding activity of PPAR-α and activates SREBP-1a and -1c [Galli et al., 2001; You et al., 2002]. By inhibiting alcohol dehydrogenase,

hence oxidation of ethanol to acetaldehyde, these effects are abolished. Both PPARα, when bound to PUFAs, and SREBP-1c are known to activate transcription of SCD1 [Ntambi, 1999] and Δ^5- and Δ^6-desaturase [Matsuzaka et al., 2002]. Furthermore, in refed state, SREBP-1c is the dominating transcription factor of those above-mentioned. SREBP-1c has been shown to be undetectable in whole testis tissue [Shimomura et al., 1997], however, this does not rule out the possibility that SREBP-1c is present at low levels in one or more testicular cell type e.g. the Sertoli cells. In fact, data presented in this work point in that direction (see *3.1.4* and *4.1.4 Metabolic regulation of the desaturases*). Thus, when ethanol is added to the Sertoli cells, an activation of SREBP-1c may be the dominating effect. This could explain the desaturase upregulation observed.

FSH seems to be a much more important hormone for the Sertoli cells with respect to the desaturation-/elongation apparatus than testosterone. Although the level of androgen receptor (AR) is age-dependent with highest level in the adult rats [Shan et al., 1995], expression of the receptor has been demonstrated in cultured Sertoli cells from 19-day-old animals [Ree et al., 1999]. In the male, FSH receptors are known to be localised only in the testis, and only on the Sertoli cells. In this way FSH acts as an organ-specific regulator. When combining the results from this paper and the above-mentioned papers of Mazouki *et al.* and Coniglio *et al.*, one can speculate if FSH acts as a key hormone for the whole desaturation-/elongation apparatus in testis. It is well known that many, if not most, of the parameters of Sertoli cell function are increased as a result of FSH stimulation [Griswold, 1993]. In our opinion, this links the desaturation-/elongation apparatus to the Sertoli cell functions.

In 1979 Clausen and co-workers published a paper where the onset of spermatogenesis is correlated with plasma FSH levels [Clausen et al., 1979]. Plasma FSH level increases approximately 5 times from day 19 to day 35 in the male rat. At the same time, this interval covers both the onset of the spermatogenesis and the appearance of the first haploid cells. When comparing the age-dependent desaturase blot (fig. 3.3) with the cellular composition of the rat testis (fig. 1.9), there is an obvious difference between the two curves between day 15 and day 40. In this interval the desaturase signals in whole testis tissue are higher than expected from the cellular composition, with respect to Sertoli cells. When taking into account the increase in plasma FSH levels occurring around this age, and the fact that FSH upregulates the gene expression of the desaturases, this difference gets a plausible

explanation: In the developing testis, FSH enhances the desaturase expression in the Sertoli cells to meet the demand for PUFAs in order to secure a functional spermatogenesis.

4.1.3 Effect of essential fatty acid deficiency on the desaturase expression

When feeding sexually immature rats a fat free diet for 14 days, the lipid composition changes in both liver and testis. As shown in table 3.1, these changes follow a similar pattern. The content of fatty acids belonging to the *n*-3 and *n*-6 families drops, while the total *n*-9 content increases in both organs. Mead acid, 20:3(*n*-9), appears in both testis and liver, hence the 20:3(*n*-9)/20:4(*n*-6) ratio increases. Only in liver this ratio exceeds the 0,4-limit that indicates EFAD conditions [Holman 1968]. However, the 20:3(*n*-9)/20:4(*n*-6) ratio in testis equals 0,4 (0,36±0,08) after 14 days of fat free diet. Similar ratios have previously been observed in fat-starved testis, but not discussed in this context [Marzouki & Coniglio, 1982].

The question arises: Was essential fatty acid deficiency induced in the testis? To address this question, essential fatty acid deficiency has to be defined.

The traditional way of defining EFAD is rooted in observations of secondary effects, e.g. metabolic and physiological changes. Studies on essential fatty acid deficiency have been performed in a number of species including rodents, bovines, primates and humans, as reviewed by Holman [Holman 1968]. Independent of the species, the same physiological changes have been observed in animals fed fat free diets; determatitis, roughened hair, lung changes, impaired reproduction in both sexes, impaired litter growth, and increased skin permeability. Furthermore, the duration and the onset of the fat free regime have been shown to affect the severity of the different physiological changes. The changes in metabolic rates and the lipid profiles in different cells and tissues have also been determined [Peluffo et al., 1976; Marzouki & Coniglio, 1982; Retterstøl et al., 1995; Stoll & Spector, 1995; Melin & Nilsson, 1997]. On the metabolic level the changes seem to be affected by the onset of the fat free regime, while the duration seems to be less important. One example can be given; the 20:3(*n*-9)/20:4(*n*-6) ratio in rat hart reaches a plateau after 60 days of fat free diet [Holman, 1968].

When combining the observations of metabolic and physiological changes, definitions like the 0,4-limit appeared. But as the molecular basis for these alterations has been elucidated, the complexity of EFAD-conditions has grown. It has long been known that desaturase activity is increased in liver as a result of fat free diets [Ntambi, 1995; Melin & Nilsson, 1997]. However, it was not until 1999 when Cho and co-workers cloned Δ^5- and Δ^6-desaturase, that these observations was explained by increased desaturase mRNA levels [Cho et al., 1999a]. Efforts made trying to understand the dietary regulation of the desaturases at the transcriptional level, followed naturally from these studies [Ntambi, 1999; Matsuzaka et al., 2002] (*see 1.1.4.1 Peroxisome proliferator-activated receptors* and *1.1.4.2 Sterol regulatory element-binding proteins*)

Now matter how complex this picture now looks (fig 1.5) the 0,4-limit has survived and is still frequently referred to. This is first of all due to the fact that this limit has been derived from studies in liver, which continues to be the organ of main interest, and secondly because the ratios calculated from different tissues are almost identical [Holman, 1968]. The problem arises when the "hard science rules" are tried projected into more odd biological systems, like the testis. It can not be ignored that the rise in the content of Mead acid, 20:3(*n*-6), is a result of increased availability of stearic acid, 18:1(*n*-9), just as much as lowered *n*-3/*n*-6 desaturase substrates. Furthermore, an increase in the 18:1(*n*-9) content is dependent on increased stearoyl-CoA desaturase transcription. Thus, it is difficult to interpret essential fatty acid deficiency in an organ not responding to fat free diets with regard to desaturase expression, when the EFAD definition is founded on this response.

As can be seen in fig 3.7, SCD1 and SCD2 are upregulated in liver and kidney in response to the fat free diet. Furthermore, Δ^5- and Δ^6-desaturase are upregulated, especially in liver, but also in kidney. In testis, however, none of the desaturases is upregulated. One might question if the lipid changes in the testis were dramatic enough to induce desaturase upregulation, and further speculate if essential fatty acid deficiency was induced in this organ. However, two important observations contradict these explanations. First of all, the 20:3(*n*-9)/20:4(*n*-6) ratio in testis was 0,4, hence at least a small upregulation of stearoyl-CoA desaturase should be expected. Furthermore, in kidney, which is another metabolically peripheral tissue, an upregulation of the SCD-1 gene expression was observed. The 20:3(*n*-9)/20:4(*n*-6) ratio in kidney from this experiment is not known, but the lipid profile analysis is in progress.

At this point, we are not able to fully explain the differences seen between these tissues. We have therefore extended this part of the study and started new feeding experiments. In these experiments the duration of the diet intervention is extended to 4 weeks. Hopefully, we will then be able to increase the 20:3(*n*-9)/20:4(*n*-6) ratio in testis to approximately 0,75, which was the case in liver after 2 weeks. At present, we hypothesise that the desaturases in testis are insensitive to essential fatty acid deficiency, and furthermore, that the vacant desaturase response is due to differences on the transcription factor level.

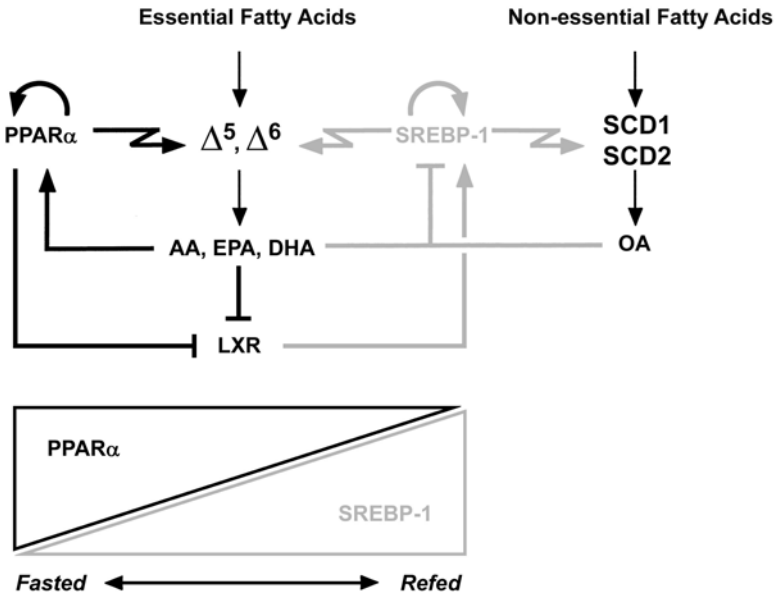

Figure 4.1 Single dietary regulation of the desaturases by PPARα in testis. The absent SREBP-1c expression in testis as compared to liver (see fig. 1.5) could be the reason for the apparent testis insensitivity to fat free diets. For details, see *1.1.4.1 PPARs & 1.1.4.2 SREBPs*. Δ^5 and Δ^6: Δ^5- and Δ^6-desaturase, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, OA: oleic acid.

This hypothesis is already supported by the observations done by Shimomura and co-workers who showed that SREBP-1c is undetectable in testis in contrast to liver and kidney [Shimomura et al., 1997]. This leaves the testis with a low responding PUFA-regulated desaturase expression as compared to other organs studied (fig. 4.1). Under refed, essential fatty acid sufficient condition, the Δ^5- and Δ^6-desaturases and especially the SCDs in the testis seem to be unresponsive to changes in the PUFA-pool. This trend is also observed *in vitro* at cellular level in this thesis (fig. 3.8 and 3.9) and is further discussed in the following section.

4.1.4 Metabolic regulation of the desaturases

When treating Sertoli cells with arachidonic (20:4(*n*-6)) or tetradecylthioacetic acid (TTA), either singly or in combination with dexamethasone or insulin, only a weak to non-existing

downregulation of the desaturases can be observed (figs. 3.8 and 3.9). This makes a great contrast to what is seen in whole liver and hepatic cells. Matsuzaka and co-workers have shown a dramatic downregulation of Δ^5- and Δ^6-desaturase gene expression in liver, increasing in strength with increased PUFA-content in the diet [Matsuzaka, et al., 2002]. In a very recent study, Tang and collaborators confirm these findings and extend the evidence by demonstrating dose-dependent downregulation of Δ^6-desaturase expression by 20:4(*n*-6) in hepatocytes [Tang et al., 2003]. Furthermore, they show that the fatty acid-controlled downregulation of the desaturases increases with the degree of fatty acid unsaturation.

Both in the whole liver and in hepatocytes, the downregulation observed for Δ^5- and Δ^6-desaturase is much more outspoken than in Sertoli cells in response to e.g. 20:4(*n*-6). When treating hepatocytes with 50 μM arachidonic acid, the Δ^6-desaturase expression is lowered to less than ¼ of what is seen in the control cultures [Tang et al., 2003]. In Sertoli cells the desaturase mRNA levels are not even halved (figs. 3.8 and 3.9).

Concerning stearoyl-CoA desaturases 1, the same dramatic downregulation as for Δ^5- and Δ^6-desaturase, has been observed in liver. When supplementing fat free diets with increasing amounts of 20:4(*n*-6), 18:2(*n*-6) and 18:3(*n*-3), the SCD1 expression in liver is repressed in a dose-dependent manner [Ntambi 1992; Kim et al., 2002]. Furthermore, the degree of desaturation has been related to the repression, as 20:4(*n*-6) was more potent than 18:2(*n*-6) in downregulating the SCD1 expression [Clarke & Jump, 1993]. In contrast, earlier reports state that SCD2 expression in rodent liver is constitutively expressed with regards to dietary PUFAs [Kaestner et al., 1993]. In this work, the previous observations done in liver are supported by our *in vivo* dietary studies (fig. 3.7). However, when comparing the liver results with the ones obtained in testis, the discrepancy still is evident. Only a very small downregulation of SCD2 is observed in Sertoli cells when the cells are treated with arachidonic acid, whereas no downregulation of SCD1 is observed.

As mentioned, the differences seen between liver and testis both *in vivo* and *in vitro* regarding dietary PUFA responsiveness, could very well be explained by the vacant testicular expression of SREBP-1c in contrast to the high expression in liver and kidney [Shimomura et al., 1997]. However, it should not be ignored that a very weak downregulation of Δ^5-, Δ^6-desaturase and SCD2 in response to both arachidonic and tetradecylthioacetic acid was

observed in Sertoli cells. The fact that the net TTA effect is desaturase repressive, indicates that some nuclear mediator of desaturase repression must be present, also in testis. After all, TTA is a strong PPARα-agonist [Spydevold & Bremer, 1989], stronger than 20:4 (n-6), inducing desaturase expression and activity. Furthermore, PPARα is expressed in the refed, essentially fatty acid sufficient Sertoli cell (see *4.2.2 Localisation of the PPARs*).

If SREBP-1c is the nuclear mediator of desaturase repression in testis, it must be present in very low doses, presumably in the Sertoli cells. A low SREBP-1c expression in the Sertoli cells could be undetectable in adult whole testis tissue [Shimomura et al., 1997], like SCD1 (figs 3.1, 3.2 and 3.6), but still contribute to some extent. An alternative explanation could be that the low but existent negative desaturase response in testis is governed by a novel tissue-specific transcription factor interacting with PUFAs. The discovery of SREBP-2gc could be given as an example [Wang et al., 2002].

The apparent testicular unresponsiveness to alterations in dietary PUFAs needs to be studied further, and the molecular mechanisms must be elucidated. Severe essential acid deficiency leads to spermatogenic arrest [Holman, 1968]. However, the secondary changes taking place in the window between severe EFAD and the normal state, should be given more attention. The lipid profile of isolated epididymal spermatozoa from EFAD rats is therefore now being analysed by our group.

4.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS

4.2.1 Essential fatty acid deficiency and PPARs

The PPAR-response to fat free diet is difficult to interpret, mainly because of methodological problems. Due to sensitivity limitations, Nothern blot analysis is not the optimal method for studying transcripts expressed at very low levels, e.g. transcription factors. At least for the PPARs, the Northern autoradiogram signals are just above the limit of detection. Therefore, small inter-individual variations can generate significant bias. Both PPARα and -δ/β expression have been shown to vary substantially between individual rats of the same strain [Escher et al., 2001].

Though non-significant, the slight upregulation of PPARα in liver and kidney in response to essential fatty acid deficiency is interesting. Knowing that PPARα induces Δ^5- and Δ^6-desaturase [Matsuzaka et al., 2002], and that the same genes are upregulated under EFAD conditions (fig. 3.7), one could speculate if the increased level of PPARα mRNA adds to the SREBP-1c-governed induction of Δ^5- and Δ^6-desaturase. Furthermore, the small downregulation of PPARα in the EFAD testis (fig 3.10) could explain how an abolished desaturase repression, seen in PUFA-treated Sertoli cells (figs. 3.8 and 3.9), would disappear in whole testis tissue from fat free diet-fed rats (fig. 3.7). In sum, what could be the case in liver and kidney during EFAD, is a combined SREBP-1c/PPARα induction of the desaturases. In testis on the other hand, the very small SREBP-1c induction and the equally small PPARα repression could balance each with respect to target gene transactivation.

As far as we know, no studies on EFAD and PPARs have been published. However, a PUFA-mediated auto-regulation of PPARα in hepatocytes is reported [Valmaseda et al., 1999; Steineger et al., 1994; see figs 1.5 and 4.1]. Since a reduced intracellular PUFA pool in general would lead to repression of PPARα expression, the induction of PPARα seen in EFAD liver and kidney (fig. 3.10; if proved significant), must be PUFA-independent.

4.2.2 Localisation of the PPARs

In this thesis we show that all three PPARs are expressed in testis and kidney from standard pellet fed rats, while liver mainly expresses PPARα and -δ (fig. 3.10). These general observations confirm the expression pattern in these three types of tissues described earlier [Braissant et al., 1996; Escher et al., 2001]. However, at this age (35-day-old), the differences in the PPAR expression between the tissues are rather small. In fact, only the hepatic PPARα expression is significantly higher compared to the other tissues. This similarity in expressional level is not observed in adult animals (>9-week-old); at this age the expression of both PPARα and -δ/β are several-fold higher in liver and kidney than in testis [Braissant et al., 1996; Escher et al., 2001]. However, when taking a closer look at the testicular localisation of the PPARs (fig. 3.11), these differences get a plausible explanation. As can be seen for PPARα especially, but also for PPARγ, the main site of expression is the Sertoli cell. The expression in germ cells is very low to absent. This confirms the *in situ* hybridisation

findings of Brassaint [Braissant et al., 1996] and the immunocytochemistry work on PPARα by Schultz [Schultz et al., 1999]. As for the desaturases, this leads to an age-dependent decrease in the PPARα and -γ expression in whole testis tissue as the germ cells proliferate (fig 1.9), lowering the relative expression from day 35 to day 70 as compared to liver and kidney.

Brassaint and co-workers have earlier stated that PPARδ/β is the most abundantly transcribed of the three receptors in testis [Braissant et al., 1996]. Even though Northern blot does not open up for semi-quantitative analyses between two or more transcripts, our results seem to support the observations by Brassaint. More interestingly, we show that PPARδ is highly expressed in germ cells, higher than in Sertoli cells, a finding which has not been reported by others. In fact Brassaint and co-workers have shown the opposite, and stated that PPARδ/β is absent in rat germ cells, from spermatogonia to spermatozoa [Braissant et al., 1996]. These diverging results are difficult to explain. Even though our results are preliminary, and need additional support, they strongly indicate PPARδ expression in germ cells. Combined with the fact that the role of PPARδ still is somewhat enigmatic, these findings will be given more attention.

Like the desaturases, PPARα and -γ are mainly expressed in the Sertoli cells. This can not be coincidental. PPARα controls numerous genes related to lipid metabolism, including PUFA desaturation. In a recent paper, Steven Clarke's group has in fact located a PPAR response element (PPRE) -385/-373 bp up-stream of the human Δ^6-desaturase gene [Tang et al., 2003]. PPARα mRNA and protein have been shown to be regulated in a stage-specific manner in the Sertoli cell nucleus, with the highest expression in stages II-VI and XIII-I of the seminiferous epithelial cycle [Shultz et al., 1999; see *1.2.1.2 The spermatogenesis*]. This suggests that PPARα expression is controlled by FSH in the testis. Schultz and co-workers further show that this is in fact the case [Shultz et al., 1999]. By that, new light is thrown over the FSH-controlled cAMP-dependent upregulation of all the four desaturases in the Sertoli cell shown in this work. The induction of the desaturases in response to FSH could result from a combined primary FSH-cAMP-CREB action and a secondary FSH-PPARα-ligand action.

PPARγ regulates the transcription of many genes involved in glucose and lipid homeostasis and insulin responsiveness. Indeed we show in this study, that the Sertoli cell is responsive to

insulin treatment, leading to Δ^5-, Δ^6-desaturase and SCD2 upregulation. Furthermore, PPARγ and -α are also known to regulate the expression of fatty acid transport protein (FATP) [Martin et al., 1997]. If modified PUFAs are transported from the Sertoli cells to the germ cells, FATP would be a likely candidate in mediating this transfer (*see 1.3 Polyunsaturated fatty acids in testis and epididymis*).

The testicular distribution of the PPARs is not yet fully established. Though preliminary, our results show both a co-expression of PPARα and the desaturases in the Sertoli cells, a possible link to intra-testicular transport of PUFAs, as well as a novel PPARδ expression in germ cells. This inspires to future studies concerning the regulation and physiological significance of the PPARs in testis.

5. CONCLUSION

In this thesis we have shown that Δ^5- and Δ^6-desaturase and stearoyl-CoA desaturase 2 (SCD2) are highly expressed in rat testis, whereas the stearoyl-CoA desaturase 1 (SDC1) expression is far lower. Furthermore, the Sertoli cell seems to be the main site of expression for all the four desaturases, while the expression in the germ cells is low to absent. Since germ cells are more enriched in PUFAs than Sertoli cells, this raises the question of a lipid transport. By employing a two-compartment culture assembly, where germ cells are grown in direct contact with Sertoli cells, and radio-labelled PUFAs, any intercellular transport may be possible to trace.

The expression of the peroxisome proliferator-activating receptors, which use PUFAs as ligands, were demonstrated in testicular cells. PPARα and -γ are mainly expressed in the Sertoli cells, following the expression pattern of the desaturases, while PPARδ expression is most pronounced in the germ cells.

Dexamethasone and insulin upregulate the three major desaturases in the Sertoli cell; Δ^5- and Δ^6-desaturase and SCD2. Interestingly, liver and testis differ with regard to the dexamethasone response. Further studies are needed to elucidate if these differences reside on the transcriptional, translational or post-translational level. Furthermore, FSH induces all four desaturases in Sertoli cells from sexually immature rats. Testosterone on the other hand does not seem to affect the desaturase expression.

In contrast to what is seen in liver, the desaturases in testis are not upregulated during essential fatty acid deficiency (EFAD) conditions. We postulate that the vacant testicular response results from the lack of certain transcription factors. SREBP-1c, which has been shown to be undetectable in the rat testis, is a likely candidate. To proceed in this matter extended dietary studies are needed, preferentially in combination with SREBP-1c knock-out liver models. If the testis unresponsiveness to altered PUFA supply is supported, a new definition of EFAD, which at present is founded on SCD induction, is needed.

All the four desaturases show strong expression in epididymis. Such strong expression might be crucial for some of the membrane-associated events taking place in this tissue. To gain

further knowledge about the interaction between sperm and epididymal cells, we have started profiling the lipid content of spermatozoa extracted from essential fatty acid deficient and sufficient epididymal subsections, respectively. Understanding the mechanisms controlling male reproductive function is crucial for diagnosis and treatment of male infertility. Therefore, to elevate the above-mentioned problems to the human model would be the ultimate goal.

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