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LXR AND AROMATASE KNOCK-OUT MICE: ANIMAL MODELS PROVIDING INSIGHT INTO HUMAN DISEASES

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Front Cover: Ventral prostate of Liver X receptor α knockout mouse, PAS staining.

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ABSTRACT

In our characterization of mouse strains carrying mutations in nuclear receptor and aromatase genes we have found phenotypes which resemble four human diseases: benign prostatic hyperplasia (BPH), Sjögren's syndrome (SS), amyotrophic lateral sclerosis (ALS), and exocrine pancreatic insufficiency. The work in this thesis was designed to understand the roles of LXRα, LXRβ and aromatase in development of these diseases and to investigate whether these animal models help us (1) to understand the etiology of the corresponding human diseases and (2) to develop new therapeutic approaches for the treatment of these diseases.

Paper I: Male LXRβ-/- mice develop motor neuron disease with age. Because the LXR ligand, β-sitosterol, has been implicated in ALS, we examined the possibility that LXRβ-/- mice were more susceptible than their wild type (WT) littermates to the toxic effects of β-sitosterol. Three weeks of β-sitosterol treatment of 8-month-old LXRβ-/- male mice resulted in ALS-Parkinson's like syndrome, characterized by death of the tyrosine hydroxylase-positive dopaminergic neurons in the brain and death of large motor neurons in the spinal cord. WT mice were not affected by β-sitosterol. In LXRβ-/- mice, there was activation of microglia and accumulation of intracellular protein aggregates in motor neurons, abnormalities which have been observed in ALS patients. The level of 24-hydroxycholesterol, the main excretory metabolite of cholesterol synthesized in the brain, was increased upon β-sitosterol treatment in mutant but not in WT mice. Thus loss of LXRβ renders mice more susceptible to the neurotoxic effects of β-sitosterol and we suggest that, in human populations where the incidence of ALS is unusually high, there may be genetic alterations in LXRβ gene signaling pathways.

Paper II: In this study we found that as LXRα-/- mice age, they develop smooth-muscle actin-positive stromal overgrowth around the ducts of the ventral prostate (VP) and numerous fibrous nodules growing into the ducts and causing obstruction and extreme dilatation of the ducts. We investigated whether the stromal hyperplasia in LXRα-/- mice could help in understanding the etiology of human BPH. We found proliferation in the epithelial cells but not in the accumulated stromal cells in the VP of LXRα-/- mice. Expression of the transcription repressor of E-cadherin, Snail and the intracellular mediator of TGF-β, Smad 2/3, was increased. We postulate that loss of LXRα from the prostatic epithelium results in increased epithelial proliferation and epithelial to mesenchymal transition, resulting in excessive stromal accumulation. These findings suggest that LXRα-/- mice may be a useful model to study prostatic stromal hyperplasia.

Paper III: LXRβ-/- mice are resistant to developing obesity on a high fat diet. As they age, these mice develop pancreatic exocrine insufficiency. By electron microscopy, abnormalities in the pancreatic ducts as well as in the pancreatic secretion were found.

Particularly interesting was the presence within the ducts of lamellar structures similar to those seen in cystic fibrosis. With a specific antibody we demonstrated the presence of LXRβ in the nuclei of epithelial cells lining the pancreatic ducts. We therefore searched for LXR-regulated genes that might influence the density of pancreatic secretion. We found that aquaporin-1 is an LXR-regulated gene highly expressed in the pancreatic ductal epithelium of wild type mice but not LXRβ-/- mice. Thus LXRβ plays an important role in controlling pancreatic juice secretion through the regulation of water transport, and the observed resistance to a high fat diet in these LXRβ-/- mice appears to be due to fat malabsorption secondary to pancreatic insufficiency.

Paper IV: As they age, mice which cannot synthesize estrogen (aromatase knock-out mice, ArKO mice), develop severe autoimmune exocrinopathy resembling Sjögren's syndrome (SS), which is not observed in ERα-/- or ERβ-/- mice. Many characteristics typical of human SS were found in these mice: There is B cell hyperplasia in the hematopoietic organs with severe immune cell infiltration in the salivary glands and kidneys, proteolytic fragments of α-fodrin in the salivary glands and anti-α-fodrin antibodies in the serum. When mice were raised on a phytoestrogen-free diet, there was a mild but significant incidence of infiltration of B lymphocytes in WT mice and severe destructive autoimmune lesions in ArKO mice. In age-matched WT mice fed a diet containing normal levels of phytoestrogen, there were no autoimmune lesions. These findings suggest that estrogen deficiency results in a lympho-proliferative autoimmune disease resembling SS and suggest that estrogen might have clinical value in the prevention or treatment of this disease.

LIST OF PUBLICATIONS

This Thesis is based on the following publications, referred to in the text by their roman numerals:

I. **Kim HJ**, Fan X, Gabbi C, Yakimchuk K, Parini P, Warner M, Gustafsson JA. Liver X receptor β (LXRβ): A link between β-sitosterol and amyotrophic lateral sclerosis-Parkinson's dementia. Proc Natl Acad Sci 105(6):2094-2099,2008

II. **Kim HJ**, Andersson C.L, Bouton D, Warner M, Gustafsson JA. Stromal growth and epithelial cell proliferation in ventral prostates of LXR knockout mice. Proc Natl Acad Sci, 2008, *in press*

III. Gabbi C, **Kim HJ**, Hultenby K, Bouton D, Warner M, Gustafsson JA. Pancreatic exocrine insufficiency in LXRβ-/- mice is due to a reduction in aquaporin-1 expression. Proc Natl Acad Sci 105(39):15052-15057,2008

IV. Shim GJ, Warner M, **Kim HJ**, Andersson S, Liu L, Ekman J, Imamov O, Jones ME, Simpson ER, Gustafsson JA. Aromatase-deficient mice spontaneously develop a lymphoproliferative autoimmune disease resembling Sjögren's syndrome. Proc Natl Acad Sci 24;101(34):12528-33,2004

Related paper (not included in this thesis):

1. Fan X, **Kim HJ**, Bouton D, Warner M, Gustafsson JA. Expression of Liver X receptor beta is essential for formation of superficial cortical layers and migration of later-born neurons. Proc Natl Acad Sci 105(36):13445-13450,2008

2. Shim GJ, Gherman D, **Kim HJ,** Omoto Y, Iwase H, Bouton D, Kis LL, Andersson CT, Warner M, Gustafsson JA. Differential expression of oestrogen receptors in human secondary lymphoid tissues. J Pathol 208(3):408-14,2005

To my beloved family for all their love and support.

Hyun-Jin Kim

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
Aβ	amyloid peptide
ACC	acetyl-coA carboxylase
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ALS-PDC	amyotrophic lateral sclerosis-parkinsonism dementia complex
Apo	apolipoprotein
AQP1	aquaporin 1
AQP1-/-	aquaporin knockout
ArKO	aromatase knockout
BMD	bone mineral density
BPH	benign prostatic hyperplasia
BrdU	5'-bromodeoxyuridine
CETP	cholesteryl ester transfer protein
ChREBP	carbohydrate response element binding protein
CNS	central nervous system
COX	cyclooxygenase
CYP	cytochrome P450 enzyme
CYP7A1	cholesterol 7α-hydroxylase
CYP19	aromatase
CYP46A1	cholesterol-24-hydroxylase
ER	estrogen receptor
ERα-/-	estrogen receptor α knockout
ERβ-/-	estrogen receptor β knockout
FAS	fatty acid synthase
G6Pase	glucose-6-phosphatase
GABA	gamma-aminobutyric acid
GLUT	glucose transporter
HDL	high-density lipoprotein
LDL	low-density lipoprotein
LXR	liver X receptor
LXRα-/-	liver X receptor α knockout
LXRβ-/-	liver X receptor β knockout
NPC1L1	Niemann-Pick C1-Like 1
NR	nuclear receptor
RXR	retinoid X receptor
SCD-1	stearoyl-CoA desaturase-1
SN	substantia nigra
SREBP	sterol regulatory-binding protein
SS	Sjögren's syndrome
TGF-β	transforming growth factor-β
TH	tyrosine hydroxylase
VP	ventral prostate
WT	wild type

1 INTRODUCTION

Despite major advances in medicine and pharmacology, there are a large number of serious diseases which remain untreatable. In recent years, there has been a growing appreciation that nuclear receptors (NRs) could be more widely used as targets for treatment of diseases. These receptors are ligand-activated transcription factors which regulate hormone action, lipid and glucose metabolism and proliferation. The work in this thesis involves mainly two members of the nuclear receptor superfamily (liver X receptors α and β) and an enzyme responsible for the synthesis of a key nuclear receptor ligand, estradiol-17β.

1.1 KNOCK-OUT MOUSE MODELS TO STUDY HUMAN DISEASES

Recent developments in the generation and characterization of genetically engineered mouse models have resulted in improvements in understanding human diseases and suggested new targets for development of pharmaceuticals. However, extrapolation between the phenotype of genetically engineered mice and human diseases is not always straightforward. One of the points for consideration is that different mouse strains have different behavioral and phenotypic characteristics and that different mouse strains cope with imposed genetic manipulations is different ways (Rose-Hellekant, Gilchrist et al. 2002; Bianchi-Frias, Pritchard et al. 2007). One example of the effect of genetic background on introduction of a mutation into a gene is the case of the epidermal growth factor receptor (EGFR) knock-out mice (Threadgill, Dlugosz et al. 1995). EGFR deficiency on a CF-1 background results in peri-implantation death; on a 129/Sv background, homozygous mutants die at mid-gestation while on a CD-1 background, the mutants live for up to 3 weeks. In the human population, it is known that the same mutation often produces symptoms of varying severity in different individuals.

If the deletion or inactivation of a protein is not a lethal change, compensatory mechanisms will be employed by the mouse to cope with abnormalities caused by the mutation. Most of these compensatory mechanisms are not predictable. It is not surprising and should not alarm investigators if in two different laboratories, mice engineered to have a mutation in a single gene turn out to have quite different phenotypes in the two laboratories. One example of genes whose mutation could lead to distinct phenotypes in different labs or in different environments are those involved

in regulation of the immune system. Exposures to environments with different levels of stress, bacteria, and parasites should be expected to lead to different responses in these mice. Another example of the important influence of genetic background on knock-out phenotype is the SREBP-1c-/- mice. It has been reported that 50-80 % of these mice die *in utero* but the ones who survive have a compensatory over-expression of SREBP-2 (Liang, Yang et al. 2002).

1.1.1 Liver X receptor (LXR) knock-out mice

Studies of LXR knock-out mice, have greatly contributed to our understanding of the biological and physiological roles of LXRs. On a high cholesterol diet, LXRα-/- mice showed marked rapid pathological changes in their livers with hepatomegaly and fatty livers developing within a week. In LXRβ-/- mice and WT mice there were no macroscopic changes on the same diet (Peet, Turley et al. 1998; Alberti, Schuster et al. 2001). The absence of LXRα results in a block in conversion of cholesterol to bile acids, as well as an increase in dietary cholesterol uptake. Moreover, the combined deficiency of LXRα and LXRβ (LXRαβ-/- mice) results in impaired triglyceride metabolism, increased LDL and reduced HDL cholesterol levels, and cholesterol accumulation in macrophages of the spleen, lung and arterial wall. These data confirm the physiological importance of both LXRs in lipid metabolism and their protective role against the development of atherosclerosis (Schuster, Parini et al. 2002).

In the absence of LXRβ, excessive cholesterol accumulates in the Sertoli cells resulting in severe cellular disruption and dysregulation of spermatogenesis (Robertson, Schuster et al. 2005; Volle, Mouzat et al. 2007) and of epididymis structure and functions and thus male fertility (Frenoux, Vernet et al. 2004). There is no accumulation of excessive lipid droplets in Leydig cells, although serum testosterone has a strong tendency to decrease with age in LXR knock-out mice, indicating that Leydig cells without LXRs are, in fact, less functional. Conversely, in female LXRβ-/- mice there is impaired contractile function in uterus, with abnormal accumulation of neutral lipids in uterine myocytes. These changes are possibly the cause of fetal resorption in the uterine horns of LXRβ but, not in LXRα-/- mice (Mouzat, Prod'homme et al. 2007).

LXRβ-/- mice exhibit age-dependent lymphadenopathy and splenomegaly, which suggests role for LXR signaling in innate immunity. LXRα is expressed at high levels in peritoneal-derived and bone marrow-derived macrophages, whereas little or

no mRNA was detected in resting purified B and T cells. On the other hand, LXRβ is expressed in macrophages, T cells and B cells. Tontonoz and his colleagues demonstrated that LXRβ is essential for lymphoid homeostasis and antigen-driven immune responses *in vivo* (Bensinger, Bradley et al. 2008).

In LXRβ-/- mice there is reduced epidermal thickness due to decreased keratinocyte proliferation (Komuves, Schmuth et al. 2002), reduction in expression of COX-2, matrix metalloproteinase 2 (MMP2) and MMP9, all characteristics of chronologically aged human skin (Chang, Shen et al. 2008).

LXRα-/- female mice had a significant increase in bone mineral density (BMD) in cortical bone. Although loss of LXRα did not affect the number of endosteal osteoclasts in the cortical bone, these cells were less active than those in WT mice, resulting in an overall reduction in bone resorption. LXRβ-/- mice exhibited no change in BMD. There was a significant decline in the number of the trabecular osteoclasts but this was compensated for by an increase in the expression of the osteoclast markers, cathepsin K and TRACP. These mice also had an increase in alkaline phosphatase and osteocalcin levels in serum, probably indicating an increased osteoblast activity.

1.1.2 ERα and ERβ knock-out mice

In 1993, when the first ERα knock-out mouse was generated (Lubahn, Moyer et al. 1993), ERα was believed to be the only receptor responsible for mediating the hormonal effects of estradiol, and it was very surprising to most endocrinologists that loss of ERα was compatible with life. Two years after the creation of the ERα mice, ERβ was discovered (Kuiper, Enmark et al. 1996), and ERβ-/- mice and ERαβ-/- were created (Krege, Hodgin et al. 1998). Characterization of these mouse models showed that life is possible without either or both estrogen receptors, but reproductive functions were severely impaired (Couse and Korach 1999). In addition, ERα and ERβ were found to have distinct and non-redundant roles in the immune, skeletal, cardiovascular and central nervous systems. Some of the most remarkable effects of ER knock-out are on morphogenesis. Estrogen is an important morphogen, and its role in morphogenesis was evident from the mammary gland (Forster, Makela et al. 2002), uterus (Couse and Korach 1999), ovary (Couse and Korach 1999), lung (Morani, Barros et al. 2006), prostate (Weihua, Makela et al. 2001; Imamov, Morani et al. 2004) and brain (Wang, Andersson et al. 2001) of ERα and ERβ knock-out mice. In addition, estrogen via estrogen receptors was confirmed to be an important regulator of differentiation and

proliferation in general and particularly in the immune system. In the bone marrow, estrogen represses differentiation of multipotent hematopoietic stem cells into both lymphoid and myeloid lineages (Kouro, Medina et al. 2001; Medina, Garrett et al. 2001). While ERβ-/- mice develop myeloproliferative disease resembling chronic myeloid leukemia in human with pronounced splenomegaly resulting from enhanced B cell proliferation in bone marrow (Shim, Wang et al. 2003), ERα-/- mice develop autoimmune glomerulonephritis with spontaneous splenic germinal center formation (Shim, Kis et al. 2004).

1.1.3 Aromatase knock-out mice

In order to understand estrogen signaling, it was essential to determine whether loss of estrogen receptors was the same as loss of estrogen. The answer came in 1999 when the phenotype of aromatase knock-out mice (ArKO) was published (Fisher, Graves et al. 1998). ArKO mice cannot synthesize estrogen from androgen. Female ArKO mice are infertile due to disruption of folliculogenesis and a failure to ovulate (Britt, Drummond et al. 2001). At 10-12 weeks of age, there are multiple ovarian follicles arrested in the antral phase and stromal hyperplasia. By 8 months of age, there is increased B-cell lymphopoiesis (Oz, Hirasawa et al. 2001) and by 1 year the ovaries become grossly dysmorphic with numerous cystic follicles and fibrous stroma. Male ArKO mice have shortened femur length and undermineralized bone (Oz, Hirasawa et al. 2001). In addition, ArKO mice show arrested spermatogenesis, possibly due to increased number of apoptotic round spermatids, and there is Leydig cell hyperplasia (Robertson, O'Donnell et al. 1999). Upon aging, these mice develop obesity with a decrease in lean body mass, hypercholesterolemia, hyperleptinemia, and insulin resistance (Simpson 2000). Thus, studies of the ArKO mouse model have led to several insights into the multiple functions played by estrogens in the development and maintenance of fertility, lipid metabolism, and bone remodeling and immune system (Murata, Robertson et al. 2002).

1.2 NUCLEAR RECEPTORS

The NR superfamily is composed of 48 members in man. Studies of the evolution of NRs (Laudet 1997), has led to their classification into seven well defined subfamilies (Table 1). Protein purification in the 1970s (Wrange, Carlstedt-Duke et al. 1979), and molecular cloning and sequencing of the genes encoding nuclear receptors in the1980s (Miesfeld, Okret et al. 1984; Miesfeld, Rusconi et al. 1986), permitted

comparison of their DNA and amino acid sequences. We now know that members of the nuclear receptor super family have a conserved structural and functional organization. The proteins can be described as a sequence of functional domains denoted from the N to the C terminus as A/B, C, D, E and F domains (Figure 1). The N-terminal domain (A/B) is highly variable in sequence and length and usually contains a constitutively active transactivation region (AF-1), which directly interacts with the core transcription machinery or with coregulators. The DNA-binding domain (C), which contains two zinc fingers, is highly conserved and involved in DNA-binding and receptor homo- or hetero-dimerization depends on the tissue and receptor properties. Between C and E domains, there is a flexible hinge region (D) which facilitates the three-dimensional folding and it contains the nuclear localization signaling of the receptor. The ligand-binding domain (E) is important for ligand-binding, heat shock protein dissociation, receptor dimerization, nuclear localization and interaction with transcriptional coregulators. Finally the C-terminal domain (F) is extremely variable and has been shown to contribute to the transactivation capability of the receptor, while its structure and function are not well known.

Table 1. A list of the 48 known human nuclear receptors categorized according to sequence homology.

Subfamily	Name	Abbreviation	NRNC Symbol
0	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1	DAX-1	NR0B1
	Small heterodimer partner	SHP	NR0B2
1	Thyroid hormone receptor α	TRα	NR1A1
	Thyroid hormone receptor β	TRβ	NR1A2
	Retinoic acid receptor α	RARα	NR1B1
	Retinoic acid receptor β	RARβ	NR1B2
	Retinoic acid receptor γ	RARγ	NR1B3
	Peroxisome proliferator-activated receptor α	PPARα	NR1C1
	Peroxisome proliferator-activated receptor β	PPARβ	NR1C2
	Peroxisome proliferator-activated receptor γ	PPARγ	NR1C3
	Rev-ErbA α	Rev-erbAα	NR1D1
	Rev-ErbA β	Rev-erbAβ	NR1D2
	RAR-related orphan receptor α	RORα	NR1F1
	RAR-related orphan receptor β	RORβ	NR1F2
	RAR-related orphan receptor γ	RORγ	NR1F3

Subfamily	Name	Abbreviation	NRNC Symbol
1	Liver X receptor β	LXRβ	NR1H2
	Liver X receptor α	LXRα	NR1H3
	Farnesoid X receptor	FXR	NR1H4
	Vitamin D receptor	VDR	NR1I1
	Pregnane X receptor	PXR	NR1I2
	Constitutive androstane receptor	CAR	NR1I3
2	Hepatocyte nuclear factor 4α	HNF4α	NR2A1
	Hepatocyte nuclear factor 4γ	HNF4γ	NR2A2
	Retinoid X receptor α	RXRα	NR2B1
	Retinoid X receptor β	RXRβ	NR2B2
	Retinoid X receptor γ	RXRγ	NR2B3
	Testicular receptor 2	TR2	NR2C1
	Testicular receptor 4	TR4	NR2C2
	Human homologue of the drosophila tailless gene	TLX	NR2E1
	Photoreceptor cell-specific nuclear receptor	PNR	NR2E3
	Chicken ovalbumin upstream promoter-transcription factor I	COUP-TF1	NR2F1
	Chicken ovalbumin upstream promoter-transcription factor II	COUP-TF2	NR2F2
	V-erbA-related gene	EAR 2	NR2F6
3	Estrogen receptor α	ERα	NR3A1
	Estrogen receptor β	ERβ	NR3A2
	Estrogen-related receptor α	ERRα	NR3B1
	Estrogen-related receptor β	ERRβ	NR3B2
	Estrogen-related receptor γ	ERRγ	NR3B3
	Glucocorticoid receptor	GR	NR3C1
	Mineralocorticoid receptor	MR	NR3C2
	Progesterone receptor	PR	NR3C3
	Androgen receptor	AR	NR3C4
4	Nerve growth factor IB	NGFIB	NR4A1
	Nuclear receptor related 1	NURR1	NR4A2
	Neuron-derived orphan receptor 1	NOR1	NR4A3
5	Steroidogenic factor 1	SF1	NR5A1
	Liver receptor homolog-1	LRH1	NR5A2
6	Germ cell nuclear factor	GCNF	NR6A1

To date, it has not been possible to determine the tertiary structure of intact full-length nuclear receptor. However structures of the ligand binding (Bledsoe, Stewart et al. 2004) and DNA-binding (Dahlman-Wright, Wright et al. 1992) domains have provided valuable information about how the receptor interacts with DNA and how ligand binding influences receptor structure and function (Kuiper, Enmark et al. 1996).

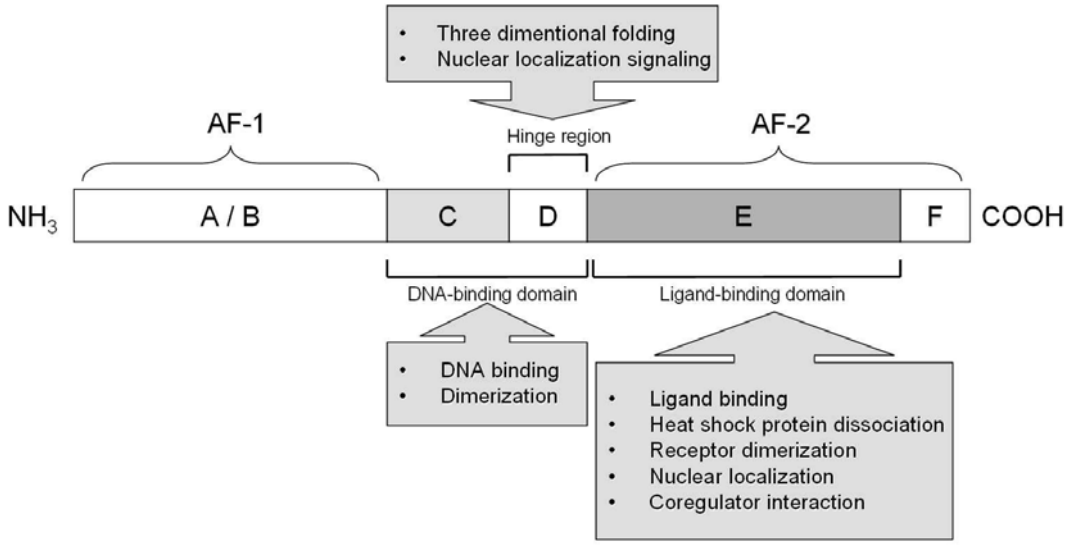

Figure 1. Schematic illustration of the structural and functional domains of nuclear receptors.

1.2.1 LXRs in general

LXRβ was the first discovered orphan receptor in our group and was named "orphan receptor 1" (OR-1) (Teboul, Enmark et al. 1995). In 1995, its ligand was not known but its response element on DNA (LXRE), was identified as a direct hexanucleotide repeat (AGGTCA) separated by four nucleotides (DR4) (Peet, Janowski et al. 1998), and it was found to form obligatory heterodimers with RXR (Wiebel and Gustafsson 1997). Upon analysis of the promoter of LXRβ, NFkB and seven potential Ets-protein-binding sites were found in the LXRβ promoter, suggesting an important physiological function in the haematopoietic and immune systems (Feltkamp, Wiebel et al. 1999). Later in 1997, the Mangeldorf group was working with a receptor cloned from the liver and called liver X receptor (LXR). They discovered that the ligand for this receptor was an oxysterol (Janowski, Willy et al. 1996; Lehmann, Kliewer et al. 1997). This receptor turned out to be similar to OR-1 and it became clear that two receptors are paralogues in the subfamily of nuclear receptor superfamily (Teboul, Enmark et al. 1995). This subfamily is now called the liver X receptors (LXRs) and

consists of LXRα (NR1H3) and LXRβ (NR1H2; formerly OR-1) (Janowski, Grogan et al. 1999; Yang, McDonald et al. 2006).

LXRα mRNA is expressed in metabolically active tissues such as the liver, pancreas, prostate, small intestine, macrophages, kidney, adrenal gland and adipose tissue, while LXRβ mRNA is quite evenly distributed throughout the body with particularly high levels in the developing brain (Willy, Umesono et al. 1995; Auboeuf, Rieusset et al. 1997; Fan, Kim et al. 2008). LXRα and LXRβ have about 77% amino acid sequence identity in both DNA-binding and ligand-binding domains.

LXR-RXR heterodimers become activated upon binding of LXR and/or RXR agonists (Peet, Janowski et al. 1998). The endogenous ligands for LXRs are oxysterols which are oxidized derivatives of cholesterol including 22-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, 20(S)-hydroxycholesterol and 27-hydroxycholesterol, but cholesterol itself is not a ligand (Janowski, Willy et al. 1996; Forman, Ruan et al. 1997; Lehmann, Kliewer et al. 1997; Janowski, Grogan et al. 1999; Fu, Menke et al. 2001). Recently, high concentration of glucose and D-glucose and phytosterols, particularly β-sitosterol, were reported to be activators of LXRs (Plat, Nichols et al. 2005; Mitro, Mak et al. 2007). The synthetic agonists T0901317 (Schultz, Tu et al. 2000) and GW3965 (Collins, Fivush et al. 2002), activate both LXR subtypes with approximately equal potency and efficacy.

1.2.2 LXRs in cholesterol and phytosterol metabolism

LXRs exert many of their functions by regulating specific target genes involved in metabolism of cholesterol and other sterols (Peet, Turley et al. 1998; Repa, Turley et al. 2000). Cholesterol is an essential component of cell membranes of all animal tissues, but its highest level is in the central nervous system. It plays a central role in many biochemical processes, since it is the precursor of steroid hormones, vitamin D and bile acids. Vertebrates synthesize cholesterol de novo and absorb cholesterol from the diet. Most tissues can synthesize cholesterol but the rate of synthesis is regulated by cholesterol most of which is obtained from the diet. Overall cholesterol homeostasis in the body is regulated by the liver which excretes cholesterol in the form of bile acids but, because high levels of cholesterol are toxic, most cells have the capacity to excrete cholesterol in a process called reverse cholesterol transport.

LXRs are involved in all aspects of cholesterol homeostasis: they regulate reverse cholesterol transport, conversion of cholesterol into bile acids, and cholesterol

absorption/excretion in the intestine. LXRα regulates two pathways of cholesterol excretion: first, by enhancing conversion of cholesterol to bile acids, by increasing the expression of CYP7A1, cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classical pathway of bile acid biosynthesis (Peet, Turley et al. 1998); second, by promoting the transcription of protein involved in transport of cholesterol out of cells. The major transport proteins are apolipoprotein E (Laffitte, Repa et al. 2001) and several members of a family of transporter proteins called ATP-binding cassette (ABC) transporters, such as subtype G5 and G8 that transport cholesterol from the hepatocytes to the bile canaliculi (Repa, Berge et al. 2002; Yu, York et al. 2003)

ABC transporters facilitate removal from cells of various endogenous and foreign substances (Venkateswaran, Repa et al. 2000; Kennedy, Venkateswaran et al. 2001; Sabol, Brewer et al. 2005). ABCA1 (Costet, Luo et al. 2000; Repa, Turley et al. 2000) is a key protein in the reverse cholesterol transport, leading to enhanced efflux of free cholesterol. This cholesterol is transferred to apoA-I, resulting in the formation of mature, cholesterol ester containing high density lipoprotein (HDL) whose main function is cholesterol trafficking between tissues.

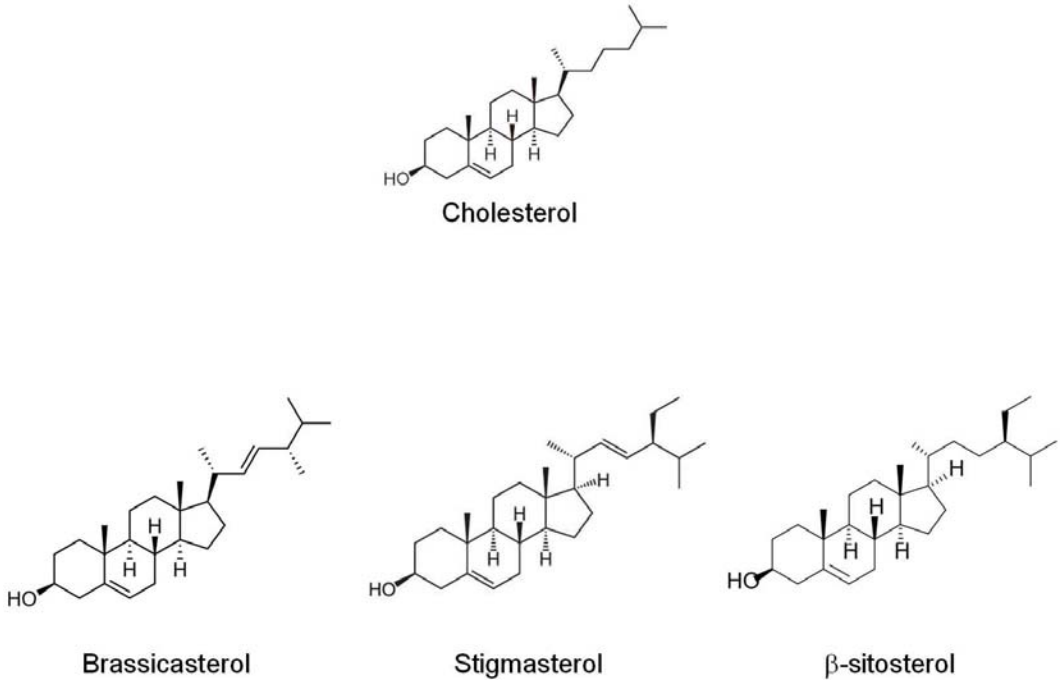

Figure 2. Structure of cholesterol and phytosterols

Mutations in ABCA1 have been identified as the genetic defect in Tangier's disease, which is characterized by extremely low levels of HDL cholesterol, and accumulation of cholesterol in tonsils, spleen, liver, brain and spinal cord (Bodzioch, Orso et al. 1999; Brooks-Wilson, Marcil et al. 1999; Lawn, Wade et al. 1999; Rust,

Rosier et al. 1999). Patients with this disease present with disorders of the nervous system, enlarged tonsils, splenomegaly, hepatomegaly, and higher susceptibility for developing premature atherosclerosis. ABCG5/8 heterodimers, on the other hand, are important for the hepatic biliary secretion of cholesterol and for sterol absorption from the gut. Mutation in either of the monomers causes sitosterolemia, which is characterized by hyperabsorption and decreased biliary excretion of plant sterols (sitosterol, campesterol, stigmasterol, brassicasterol and ergosterol), tendon and tuberous xanthomas.

Phytosterols are present in plant oils, particularly in nuts, and avocados. The structure of phytosterols is very similar to that of cholesterol (Figure 2). They differ from cholesterol only by an additional ethyl-group (sitosterol) or methyl-group (campesterol) at the C24-position, or by an additional double bond at the C22-position (brassicasterol, stigmasterol). However, these small structural differences between cholesterol and phytosterols lead to very different metabolic fate for phytosterols in animals. Both cholesterol and phytosterols are absorbed by intestinal enterocytes via Niemann-Pick C1-Like1 (NPC1L1) transporter (Yang, Yu et al. 2004). Cholesterol, but not phytosterols, is esterified by acyl-CoA:cholesterol O-acyltransferase 2 and incorporated into chylomicrons. Phytosterols remain unesterified and are transported back to intestinal lumen by ABCG5/8 transporters (Jansen, Lutjohann et al. 2006).

1.2.3 LXRs in lipid and fatty acid metabolism

The body's reserves of carbohydrate are stored in the liver and muscle as glycogen while fat reserves are stored in adipose tissues and fatty acid esters (Murray RK, Granner DK et al. 2003). After a meal high in carbohydrates, many genes encoding enzymes involved in the lipogenesis are transcriptionally induced in liver. These include: sterol regulatory-binding protein-1c (SREBP-1c), Acetyl-CoA carboxylase (ACC), Fatty acid synthase (FAS) and Stearoyl-CoA desaturase-1 (SCD-1) (Tabor, Kim et al. 1998; Magana, Koo et al. 2000; Osborne 2000). SREBP-1c, which turns on the lipogenesis, is an LXR-regulated gene. LXR activation increases the synthesis of fatty acids and triglycerides by regulating a number of lipogenic genes, such as SREBP-1c (Schultz, Tu et al. 2000), FAS, SCD-1 (Horton, Goldstein et al. 2002; Joseph, Laffitte et al. 2002) and ACC (Peet, Turley et al. 1998). All the three latter genes (FAS, SCD-1, ACC) have been shown to be directly regulated by SREBP-1c (Shimano 2001). In addition, LXR is able to directly stimulate transcription of FAS and ACC (Zhang, Yin et al. 2001; Joseph, Laffitte et al. 2002). At the same time,

activation of LXR also influences lipoprotein metabolism through the control of modifying enzymes such as lipoprotein lipase (LPL) (Zhang, Repa et al. 2001), cholesteryl ester transfer protein (CETP) (Luo and Tall 2000) and phospholipid transfer protein (PLTP) (Laffitte, Joseph et al. 2003).

Cha and Repa reported that LXR also regulates hepatic lipogenesis through transcriptional regulation of a novel protein called carbohydrate response element-binding protein (ChREBP) (Cha and Repa 2007). The mouse ChREBP gene promoter contains an LXR response element at about 2.4 kbp, upstream of the transcriptional start site and LXR agonists increase ChREBP mRNA in livers of WT but not LXRαβ-/- mice. In addition, Uyeda and his colleagues have suggested that ChREBP can enhance transcription of many lipogenic genes independently of SREBP-1C (Iizuka, Bruick et al. 2004). Recently, one group has reported that glucose is required for ChREBP functional activity (Denechaud, Bossard et al. 2008), and another group has reported that glucose is an agonist of LXR (Mitro, Mak et al. 2007). More studies are needed for the understanding of the physiological significance of these observations.

1.2.4 LXRs in Glucose metabolism

Besides their well established role in cholesterol and lipid homeostasis, LXRs are also recognized as important regulators of glucose homeostasis. This role of LXR is not surprising since the physiological regulation of lipid metabolism in closely linked to that of carbohydrate metabolism. Laffitte et al. showed that LXR agonists improve glucose tolerance in a mouse model of diet-induced insulin resistance (Laffitte, Chao et al. 2003). Activation of LXR indirectly suppresses gluconeogenic enzymes (e.g. phosphoenol pyruvate carboxykinase (*PEPCK*), glucose-6-phosphatase (*G6Pase*)) in the liver, while LXR ligands induce expression of the insulin-sensitive glucose transporter *glut4* by directly binding to LXR response element in its promoter (Dalen, Ulven et al. 2003; Laffitte, Chao et al. 2003). These positive effects on hepatic and peripheral insulin sensitivity are mediated by LXRα (Commerford, Vargas et al. 2007). LXR ligands have also proven effective in treatment of obese mice (ob/ob), diabetic mice (db/db) and insulin-resistant Zucker rats (fa/fa), which have insulin resistance and type II diabetes, supporting the potential of LXR agonists for the treatment of diabetes (Cao, Liang et al. 2003; Chisholm, Hong et al. 2003; Grefhorst, van Dijk et al. 2005). Furthermore, activation of LXRs in pancreatic β-cells stimulates insulin biosynthesis and secretion (Efanov, Sewing et al. 2004).

1.2.5 Cholesterol metabolism in the central nervous system

In humans, the CNS accounts for only 2% of body weight, while it contains 25% of the cholesterol present in the whole body pool. In rodents, not only in the embryonic period, but also in the first few weeks after birth during differentiation and development of the CNS, the cholesterol required for brain growth apparently comes exclusively from *de novo* synthesis in the brain. There is little, if any, uptake of cholesterol from plasma into the brain and, unlike the liver, peripheral levels of cholesterol do not influence total brain cholesterol level (Quan, Xie et al. 2003). When mice are fed a high cholesterol diet, there is no change in the concentration or rate of synthesis of cholesterol in any region of CNS, while there is elevated total cholesterol and a 7-fold increase in lipoprotein cholesterol concentration in the plasma and liver (Quan, Xie et al. 2003). The reason for the tight regulation of the brain cholesterol is probably the impermeability of the blood-brain barrier (BBB) to cholesterol from outside of CNS.

In the adult brain, despite its abundance, the synthesis of cholesterol is very low. This is because of the efficient recycling of brain cholesterol. The half-life of the bulk of brain cholesterol has been estimated to at least 5 years (Bjorkhem, Lutjohann et al. 1998). During early development and maturation, the CNS produces enormous amounts of cholesterol and excess cholesterol has to be excreted. In humans the major hydroxylated cholesterol excreted from the CNS is 24(S)-hydroxycholesterol (Bjorkhem, Lutjohann et al. 1998). The enzyme responsible for the synthesis of this oxysterol, cholesterol-24-hydroxylase (CYP46A1), is expressed essentially only in the brain, exclusively in neurons, not glial cells or myelin (Lund, Guileyardo et al. 1999). Compared to other tissues the ratio of 24(S) to 27(S) -hydroxycholesterol in CNS is high.

1.2.6 LXRs in the developing central nervous system

Information regarding LXRα and LXRβ mRNA expression in the CNS has come from studies in developing and adult rodent brain (Kainu, Kononen et al. 1996; Zhang and Mangelsdorf 2002; Annicotte, Schoonjans et al. 2004). These studies showed that the LXRβ receptor was expressed throughout the brain at a level higher level than that of the liver while LXRα receptor appeared to be much less represented in the CNS (Zhang and Mangelsdorf 2002). Recently, in studies from our group, the function of LXRβ in the developing brain has been partly unveiled (Fan, Kim et al.

2008). LXRβ protein is widely expressed in the mouse brain at later embryonic stages, and the expression pattern in the cerebral cortex is developmentally regulated. Studies on LXRβ-/- mice suggest that LXRβ is involved in lamination of the cerebral cortex and is essential for the migration of late-generated neocortical neurons to their appropriate position in the cortex (Fan, Kim et al. 2008).

1.2.7 LXR in the aging central nervous system

As they age, LXRβ-/- male mice develop impaired motor coordination associated with lipid accumulation and loss of large motor neurons in the spinal cord, with several neuropathological features reminiscent of the chronic motor neuron disease, amyotrophic lateral sclerosis (ALS) (Andersson, Gustafsson et al. 2005). LXRβ may be required for excretion of excessive sterols and lipids from the spinal cord and its malfunction may cause neuronal loss due to sterol overload in the large motor neurons in spinal cord. In the LXRαβ-/- mice, several severe abnormalities have been observed in the brain, including accumulation of lipid droplets in ependymal cells lining the ventricles, neuronal loss, and astrocyte proliferation particularly in the substantia nigra and globus pallidus (Wang, Schuster et al. 2002). These data suggest that one or both of LXRs may be required to excrete excess sterols and lipids from the CNS to plasma.

1.2.8 The possible involvement of LXR and sterols in human ALS

Amyotrophic lateral sclerosis (ALS) (Andersson, Gustafsson et al. 2005) is a neurodegenerative disease characterized by selective loss of motor neurons in the spinal cord, brain stem and motor cortex. Since the first description in 1869, the pathogenesis of ALS still remains elusive (Charcot 1869). Mutation in cooper/zinc superoxide dismutase 1 (SOD1) has been identified as one of the causes for rare familial ALS (FALS). The development and analysis of SOD1 mutant transgenic mice has led to some insights into the role of reactive oxygen species in ALS. While 20-30% of FALS cases are associated with a mutation of SOD1, more than 90-95% of ALS cases are sporadic, not showing any known genetic traits. The causes of sporadic ALS are not clear, and may be multifactorial. Two promising lines of investigation are *(i)* abnormalities in the immune system and *(ii)* neurotoxicity due to excessive levels of glutamate (Chrissandra J. Zagami 2008).

ALS-PDC is a chronic neurodegenerative disorder highly prevalent in the native Chamorro population of Guam Island in the western pacific. Clinically Guamanian ALS is indistinguishable from sporadic ALS. There have been a number of hypotheses put forward to explain the unusually high incidence of this disorder in Guam. Most of these hypotheses pointed to environmental neurotoxic factors (Plato, Galasko et al. 2002; Plato, Garruto et al. 2003). Intensive investigations to find the factors responsible for ALS-PDC have been conducted (Khabazian, Bains et al. 2002; Cox, Banack et al. 2003; Miller 2006). Many studies have focused on neurotoxins, β-D-glucoside, and β-methylamine-alanine (BMAA), which are found in Cycad seeds used in the diet in Guam (Khabazian, Bains et al. 2002)

There is growing evidence that this complex disease is, in fact, a multisystem disorder. In particular, recent studies revealed that two thirds of patients with ALS present with a stable high energy expenditure which correlates with survival. In ALS animal models there is reduced adiposity and hypermetabolism. An increase in the lipid content of the diet offers neuroprotection and extends survival in these animal models, whereas restricting calorie intake exacerbates the motor symptoms (Dupuis, Corcia et al. 2008).

1.2.9 Cholesterol and LXRs in Alzheimer's disease (AD)

The involvement of cholesterol in neurodegenerative diseases, in particular, Alzheimer's disease (AD), is a subject of intense investigation at present. Cholesterol-lowering drugs including LXR agonists are proposed as a target drugs to treat degenerative neuronal disorders. AD is characterized by progressive neuronal loss and cognitive impairment upon aging. Pathologically, AD is characterized as regional accumulation of amyloid peptide (Aβ) and neurofibrillary tangles, involved in diverse inflammatory processes and cholesterol metabolism (Ball 1977). Many epidemiologic studies suggest an association between plasma cholesterol and risk for AD. In addition, treatments with cholesterol-lowering drugs, such as statins, have been reported to lower the incidence and prevalence of AD. Some studies suggest an association between plasma 24(S)-hydroxycholesterol level and risk and severity of AD (Bjorkhem, Lutjohann et al. 1998). Clinical studies have shown that there are elevated plasma levels of 24(S)-hydroxycholesterol in patients with AD and vascular dementia patients (Lutjohann, Papassotiropoulos et al. 2000; Papassotiropoulos, Lutjohann et al. 2000). Because more than 90% of plasma 24(S)-hydroxycholesterol comes from the brain, plasma levels of the oxysterol may reflect brain cholesterol turnover.

There is growing evidence that LXR agonists can protect against AD. These drugs act by both suppression of plaque-related inflammatory response and increasing Aβ clearance (Zelcer, Khanlou et al. 2007; Jiang, Lee et al. 2008). In particular, activation of LXRs inhibits the microglia-mediated inflammatory response and functionally inactivates the promoters of proinflammatory genes (Zelcer and Tontonoz 2006). Suppression of the inflammatory response is not limited only to AD.

In a mouse experimental brain injury model, LXR agonists induce a protective anti-inflammatory pathway in the brain with decreased expression of pro-inflammatory genes and reduced NF-kB transcriptional activity (Morales, Ballesteros et al. 2008; Sironi, Mitro et al. 2008). In addition to its anti-inflammatory actions, several studies have demonstrated an important role of LXRs in the function of the choroid plexus (Wang, Schuster et al. 2002; Fujiyoshi, Ohtsuki et al. 2007). In Alzheimer's disease, there is thickening of the basement membrane of the choroid plexus and epithelial atrophy (Serot, Bene et al. 2003). Similar abnormalities in the choroid plexus have been observed in LXRαβ-/- mice (Wang, Schuster et al. 2002).

1.2.10 LXRs in prostatic disease

Cholesterol has long been known to be important in the physiology of the prostate and defects in cholesterol homeostasis have been implicated in diseases of the prostate (Hager, Solomon et al. 2006). The most common prostate problem in men over 50 years of age is BPH.

BPH is characterized by progressive hyperplasia of glandular and stromal tissues around the urethra, resulting in compression of the neck of the urinary bladder and urethral canal, which interferes the normal flow of urine. Despite the prevalence of BPH, its etiology remains a mystery. By the age of 70, more than 80% of men suffer from this disorder.

Several theories of its pathogenesis have been proposed. The suggested "culprits" include: androgen receptor, estrogen receptor α and β, and TGF-β via Smad pathways. It appears that growth factors and downstream molecules are overexpressed in BPH (De Miguel, Royuela et al. 1999; Pollan, Benghuzzi et al. 2003). These are same growth factors that promote stromal and epithelial growth and induce epithelial-mesenchymal transformation (EMT) (Boyer, Valles et al. 2000). During EMT, there is loss of E-cadherin expression and a gain of more mesenchymal properties. In human BPH there is increased expression of the mesenchymal marker, vimentin, in

hyperplastic epithelium (Fraga, True et al. 1998). TGF-β, through activation of the transcription factors, Smad 2/3, plays an important role in EMT. It induces expression of Snail, which is a suppressor of E-cadherin expression (Hajra, Chen et al. 2002).

There are several treatment options for BPH; however no completely effective treatment for BPH exists. The mainstay of therapies is the combination of 5α-reductase inhibitors, which control the levels of 5α-dihydrotestosterone (DHT), and alpha-blockers, to regulate adrenergic tone. In some patients surgery, transurethral resection of the prostate (TURP), is the only effective intervention. However current surgical treatments are associated with risk of urinary incontinence and current pharmacological intervention is not always effective. In men with BPH, many clinical trials suggest that phytotherapy (use of plants and herbs) improves lower urinary tract symptoms and increases quality of life. Extracts of the plant contain a mixture of phytosterols such as β-sitosterol, campesterol, and stigmasterol, as well as flavonoids and other compounds. Phytosterols are LXRα and LXRβ agonists with EC_{50} at 30-150nM, β-sitosterol being a more potent agonist than campesterol or stigmasterol (Plat, Nichols et al. 2005). This selective binding of β-sitosterol to LXR, might explain why the most popular plant supplement used in BPH relief is saw palmetto (*Serona repens*), which contains high levels of β-sitosterol. Saw palmetto has been tested in multiple trials and these have been reviewed recently (Wilt, Ishani et al. 2002).

1.3 AROMATASE AND ESTROGEN SIGNALING

1.3.1 Aromatase

A single gene, CYP19 (aromatase), encodes the key enzyme responsible for estrogen biosynthesis. Aromatase converts C19 androgens to aromatic C18 estrogens through three consecutive hydroxylation reaction steps (Murray RK, Granner DK et al. 2003) (Figure 3). Disruption of the aromatase gene effectively eliminates estrogen production in the entire body. In the normal body, the ovary is the source of estrogen which regulates breast, brain, uterine, and immune functions while the testis, adipose tissue, skin, and hypothalamus produce small levels of estrogen which function in a paracrine manner.

Inactivating mutations of the aromatase gene are very rare in the human population. In the cases described, there is a single base-pair change resulting in amino acid substitution or premature stop codons. In most cases, the affected mother presents with virilization in the third trimester of pregnancy. Affected female newborns have

pseudohermaphroditism with clitoromegaly and hypospadias. The cause of these abnormalities in pregnancy is the inability to convert fetal dehydroepiandrosterone (DHEA) to estrogen in the placenta and subsequent conversion of androgens to estrogens in the periphery. Affected male newborns present with tall stature secondary to failed epiphyseal fusion. They also have delayed bone age, osteopenia, and undermineralization, which can be corrected with the addition of estrogen, highlighting estrogen's critical role in men as well as women (Simpson 2000).

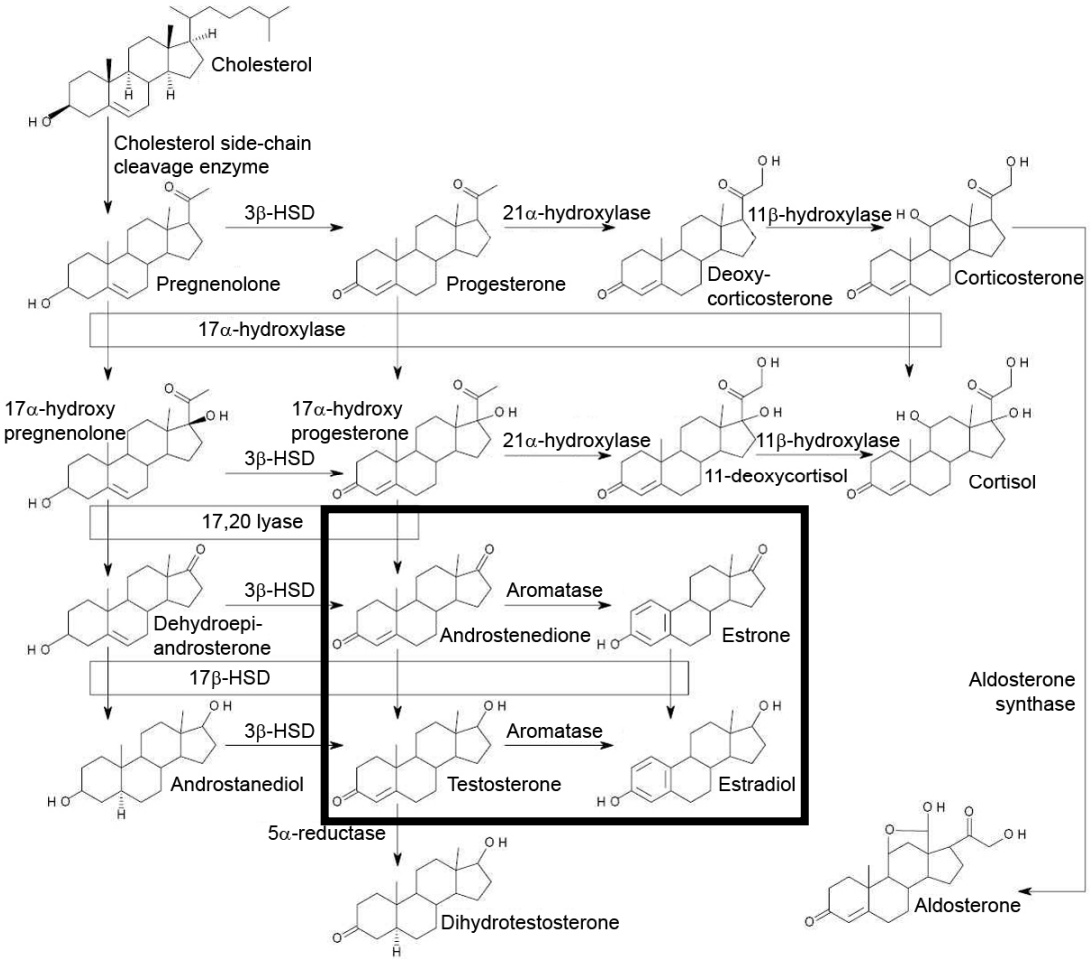

Figure 3. Biosynthesis of sex hormones

In cancers of the breast and endometrium, aromatase appears to be enhanced local synthesis of estrogen which sometimes makes growth of these cancers independent of ovarian estrogen. Aromatase inhibitors are used clinically for the treatment of breast cancer. Clinical trials have shown that anastrozole, an aromatase inhibitor, results in fewer uterine and vascular side effects than Tamoxifen but that anastrozole use is associated with an increase in incidence of joint pain, osteoporotic fractures (Howell, Cuzick et al. 2005) and Sjögren's syndrome (Laroche, Borg et al. 2007).

1.3.2 Estrogens in Autoimmune disease: Sjögren's syndrome (SS)

Estrogen is thought to be the most important factor regulating sex differences in the immune system. Several chronic inflammatory diseases, such as multiple sclerosis, systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) are classified as autoimmune diseases. These diseases are characterized by inflammatory cell infiltrates in affected organs and high levels of autoantibodies in serum. Both SLE and SS are female-dominant diseases (Whitacre 2001).

SS is a chronic, incurable autoimmune exocrinopathy that occurs 10 times more frequently in women than in men. The symptoms of primary SS are inflammatory cell infiltrates in salivary gland and lacrimal gland, manifested by xerostomia (dry mouth), keratoconjunctivitis sicca (dry eye), and other extraglandular abnormalities. SS is characterized by initial infiltration of exocrine glands with T cells, the presence of B cell hyperactivity, including germinal center formation and the presence in serum of various autoantibodies such as rheumatoid factors, anti-Ro (SS-A), anti-La (SS-B) and anti-α-fodrin autoantibodies (Haneji, Nakamura et al. 1997; Ishimaru, Saegusa et al. 1999; Bolstad, Wassmuth et al. 2001; Gottenberg, Busson et al. 2003). The treatment is mostly symptomatic and immunosuppressive drugs are only given in case of severe and life-threatening organ involvement.

1.4 MALABSORPTION SYNDROME

Malabsorption syndrome is a clinical condition arising from an abnormality in digestion or absorption of nutrient from the diet across the gastrointestinal tract. This can be manifested by some combination of symptoms like diarrhea, steatorrhea, malnutrition, weight loss and anemia. Many diseases are associated with this syndrome and can be classified into three broad categories depending on their etiology: *(i)* alterations of the digestive process due to deficit of enzymes and bile acids such as in chronic pancreatitis, autoimmune pancreatitis, cystic fibrosis, and cholestatic liver diseases; *(ii)* alterations in uptake and transport due to a damage and/or physical diminution of absorptive surface such as in gluten sensitive enteropathy, Crohn's disease, and autoimmune enteropathy; *(iii)* microbial causes such as bacterial overgrowth and parasitosis (Owens and Greenson 2007). The major cause of defective intraluminal digestion is pancreatic exocrine insufficiency due to chronic pancreatitis and cystic fibrosis. In industrialized countries, the incidence of chronic pancreatitis is about 3.5-10 per 100,000 inhabitants. About 70-80% of cases are related to long-term

alcohol abuse while 10-30% of cases represent idiopathic pancreatitis for which the etiology is still unknown (Witt 2003). A large number of mutations in genes coding for serine protease 1 (PRSS1), serine protease inhibitor Kazal type 1 (SPINK1), or the cystic fibrosis transmembrane conductance regulator (CFTR) have been described to be involved not only in the pathogenesis of pancreatitis but also, working in concert with other genetic and environmental factors, in the susceptibility to this disease (Witt, Apte et al. 2007).

2 AIMS OF THE STUDY

The overall aim of this thesis was to characterize the tissues of LXR and aromatase knock-out mouse strains and elucidate the role of LXRα, LXRβ and aromatase in the development of four specific human diseases.

The specific aims were:

I. To examine whether LXRβ-/- mice are more susceptible to the toxic effects of β-sitosterol, which has been implicated in amyotrophic lateral sclerosis (ALS)

II. To investigate the role of LXRα in stromal overgrowth in the prostate.

III. To investigate the reason for the resistance to obesity in LXRβ-/- mice.

IV. To investigate the immune phenotype of mice which are chronically estrogen depleted.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Previous studies have reported that as they age male LXRβ-/- mice develop motor neuron disease. In this study, we examined whether LXRβ-/- mice are more susceptible to the toxic effects of β-sitosterol, which is a known LXR ligand and is implicated as a causative agent in ALS of the Guam population.

At 5 and 8 months of age, motor disability in β-sitosterol-treated LXRβ-/- mice was measurable by the rotor rod test. At 16 months of age, 3 weeks administration of β-sitosterol caused severe paralysis and symptoms typical dopaminergic dysfunction in LXRβ-/- mice, while it had no observed toxicity in WT mice. Daily consumption of β-sitosterol led to the death of large motor neurons in the lumbar region of the spinal cord and loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons in the substantia nigra (SN).

We hypothesized that loss of LXRβ might affect LXR target genes, ABCG5/8 and NCP1L1, which are needed for the elimination of β-sitosterol from the body. However, levels of ABCG5/8 and NCP1L1 were not affected by loss of LXRβ and/or treatment with β-sitosterol nor were there changes in plasma levels of β-sitosterol and cholesterol. It appears that LXRα is sufficient for regulation of cholesterol and β-sitosterol from the diet.

Further investigation into the mechanism behind the cell death occurring in large motor neurons in lumbar region of the spinal cord and TH-positive dopaminergic neurons in SN in LXRβ-/- mice, led us to the conclusions that there was more activated microglia in the SN of LXRβ-/- mice but not in the spinal cord. In the large motor neurons of the spinal cord of LXRβ-/- mice, there were aggregates of ubiquitin and TDP43 (a low-molecular-weight neurofilament mRNA-binding protein that is co-localized with ubiquitin in inclusions in motor neurons in ALS). Similar inclusions were seen in the cytoplasm of β-sitosterol-treated LXRβ-/- mice but there were too few large motor neurons left in the spinal cord of these mice to study this phenomenon.

Although many studies have shown that high cholesterol level in the brain is associated with neuronal diseases such as Alzheimer's disease, appropriate levels of cholesterol are part of a healthy microenvironment for neurons and are essential for maintenance of many signal pathways like synaptic vesicle turnover, calcium-

dependent neurotransmitter release, signaling of GABA and glutamate in CNS. The cholesterol content of LXRβ-/- mouse brains was significantly higher than in WT mice. β-sitosterol administration caused a decrease in cholesterol contents in the brains of both LXRβ-/- and WT mice. In β-sitosterol-treated LXRβ-/- mouse brains, there was a significant increase in the concentration of 24-hydroxycholesterol, the metabolite which is excreted from the brain. We interpret these results to mean that in the absence of LXRβ, activation of LXRα by β-sitosterol leads increased excretion of cholesterol from the brain and that the neuronal toxicity is due to the depletion of cholesterol from the brain. Because β-sitosterol caused reduction in brain cholesterol in WT mice, we speculate that prolonged use of LXR ligands may lead to cholesterol depletion from neurons and neurotoxicity even when both LXR receptors are functional.

3.2 PAPER II

In this study, we found that LXRα is strongly expressed in the luminal and basal cells of prostatic epithelium. Histological examination of the VP of LXRα-/- mice revealed smooth-muscle actin-positive stromal overgrowth and prostatic ductal hyperplasia with numerous fibrous nodules pushing into the epithelium. The proliferation index, measured with the proliferation markers, Ki67 and BrdU, revealed epithelial and stromal proliferation in the fibrous nodules. However there was no evidence of proliferation in the dense stroma surrounding prostatic ducts.

Because the stroma was not proliferating, we concluded that the defect in these mouse prostates was due to stromal accumulation rather than proliferation. We hypothesized that the dense stroma in LXRα-/- mice arose from the epithelium in a process called Epithelial-Mesenchymal-Transformation (EMT). To test this hypothesis, we checked the VP for markers of EMT, such as Snail, Smad 2/3 and E-cadherin. Interestingly, there was an increase in nuclear expression of Snail and Smad 2/3 indicating enhanced TGF-β signaling, while expression of E-cadherin was reduced or absent in prostatic epithelial cells of LXRα-/- mice. Surprisingly, upon chronic treatment of WT mice for 3 months with the LXR agonist T2320, or treatment of LXRβ-/- mice with β-sitosterol, LXRα was down-regulated and, and the VP looked very similar to those of LXRα-/- mice. We interpret these data to mean that chronic administration of LXR agonists in mice results in down-regulation of LXRα and that the dense stroma in these VP probably does arise from epithelial cells via EMT process.

3.3 PAPER III

In this study, we found that LXRβ-/- mice had pancreatic exocrine insufficiency. These mice showed reduced serum levels of amylase and lipase, reduced proteolytic activity in feces, chronic inflammatory infiltration and in the pancreatic ductal epithelium, an increased apoptosis without compensatory proliferation. Electron microscopic studies of the LXRβ-/- pancreas showed the dilated ducts with dense intra-ductal laminar structures characteristic of cystic fibrosis. The pancreatic ductal epithelium expressed LXRβ and in the absence of LXRβ, there was loss of expression of the water channel, aquaporin1 (AQP1), which plays a key role in trans-cellular fluid transport. In the inter-intralobular pancreatic ducts it seems to be responsible for pancreatic juice formation. Interestingly, AQP1-/- mice resemble LXRβ-/- mice in that, when fed with a high-fat diet, they are resistant to weight-gain, develop steatorrhea and have a decreased concentration of amylase and lipase in the pancreatic fluid. It seems that defective secretion of water in the deficiency of AQP1 in the pancreatic ducts leads to a modification in the composition of pancreatic juice that damages the pancreatic epithelia and finally leads to exocrine insufficiency.

To evaluate whether AQP1 is an LXR target gene, we treated WT mice with LXR agonist, T2320, for 7 days. T2320 significantly increased the levels of AQP1 mRNA. However it is not known whether LXRβ binds directly to the promoter of AQP1; AQP1 5' flank has multiple LXRE half sites, but further studies are required to analyze whether any of them is functional in the regulation of this gene.

In the case of the human pancreas, it is known that there is expression of AQP1 in the intralobular and interlobular ducts, but the role of AQP1 in pancreatic insufficiency and malabsorption has not yet been studied.

3.4 PAPER IV

There is a higher incidence of many autoimmune diseases, including Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE), in women than in men. Estrogen is thought to be the most important factor regulating sex differences in the immune system. In this study, we investigated whether chronic depletion of estrogen leads to autoimmune diseases. For these studies we used ArKO mice, which cannot synthesize estrogen.

As ArKO mice age, they spontaneously exhibit signs of autoimmunity with B cell hyperplasia in the bone marrow, spleen and blood. In addition, there was severe

destructive B lymphocyte infiltration in the salivary glands of ArKO mice, which is typically found in human SS. WT mice littermates cohabiting with the knock-out mice were normal. It has been well studied that bone marrow is an estrogen-regulated organ and in particular, estrogen modulates B lymphopoiesis. Interestingly, after ovariectomy in mice, there is a significant increase in the number of pro/pre B lymphocytes in bone marrow. In ERα-/- mice, there is a slight increase in cellularity of bone marrow, but a decrease in the number of pro/pre B lymphocytes and mature B lymphocytes in bone marrow. In contrast, in ERβ-/- mice, there is an increase in pro/pre B lymphocytes. Thus in a mouse, with respect to B lymphopoiesis, loss of ERβ mimics loss of estrogen. One other characteristic of estrogen deficiency is splenomegaly, which is caused by enhanced B lymphopoiesis, and also observed in ArKO mice in this study.

SS is regarded as an autoimmune disease characterized by infiltration of CD_4+/CD_{45}RO+ T cells and also by the presence of B cell hyperactivity and various autoantibodies. The percentage of the T cell population in ArKO mice was not markedly changed, but the absolute number of T cells was increased, as judged from 2- to 4- fold higher splenic cellularity of ArKO mice. The involvement of T cells in SS phenotype of ArKO mice remains to be investigated.

There was also mild but significant nephropathy as evidenced by the presence of proteinuria in ArKO mice. Renal protective effects of estrogen have been demonstrated *in vivo* and *in vitro*. Interestingly, in glomerular sclerosis-prone mice, estrogen treatment results in a reduced prosclerotic response. In contrast, ovariectomy accelerates its progression.

Long-term phytoestrogen-free diet induced a mild but significant B lymphocyte infiltration even in WT mice, and severe destructive autoimmune lesions in ArKO mice. In age-matched WT mice fed a diet containing normal levels of phytoestrogens, there were no autoimmune lesions in salivary gland and kidney. These results indicate that phytoestrogens may help to prevent the development of SS. If phytoestrogens do offer protective effects in humans, the implication would be that ERβ is involved in this protection, and therefore, it may be suggested that ERβ-selective agonists could be useful in prevention or treatment of autoimmune exocrinopathies.

In this study, we have demonstrated that estrogen deficiency spontaneously leads to an SS-like phenotype in ArKO mice. Some studies have shown the involvement of estrogen in SS. We have found no evidence of SS-like phenotype in ERα-/- mice but have found that ERβ is the predominant ER in mouse and human

salivary glands. Clearly further studies are needed for further understanding of the role or ERs in salivary glands.

4 CONCLUSION AND PERSPECTIVES

The studies in this thesis have provided some insights into the functional roles of LXRα, LXRβ and aromatase in human diseases. We have found that LXRα-/- mice develop prostatic stromal overgrowth; LXRβ-/- mice develop motor neuron disease and pancreatic exocrine insufficiency; and ArKO mice develop autoimmune disease resembling Sjögren's syndrome.

Our studies on the effects of β-sitosterol on the CNS of LXRβ-/- mice suggest that LXRβ is essential for protection against the neurotoxic effects of this phytosterol and lead us to suggest that dysfunction of LXRβ could be a genetic predisposition that leads to the development of ALS-PDC in the Guam population.

Study of the ventral prostates of LXRα-/- mice has shed new light on the role of LXRα regulating epithelial-stromal communication in ventral prostate. Furthermore, it leads us to suggest that dysregulation of LXRα predisposes to development of BPH in men and that LXRα agonists may play a role in treatment of this disorder. However, the optimal dosing schedules for such pharmaceuticals have to be investigated because long-term treatment with LXR agonist to WT mice causes down-regulation of LXRα and leads to stromal overgrowth.

LXRβ-/- mice are resistant to obesity when they are fed a high fat diet. We found that this resistance to obesity is caused by fat malabsorption due to pancreatic insufficiency. The main problem appears to be loss of expression of AQP1 in the pancreatic ducts. The result is a viscous pancreatic secretion and a destruction of the ducts. We suggest that LXR dysfunction should be investigated as a factor predisposing to idiopathic chronic pancreatitis in the human population.

Studies on ArKO mice reveal that long term deficiency of estradiol leads to autoimmune disease. The salivary glands of ArKO mice resemble those of patients with Sjögren's syndrome (massive immune invasion, α-fodrin autoantibodies and destruction of the epithelium). Dietary phytoestrogens protect against salivary gland destruction and lead us to suggest that ERβ-selective agonists could be potential therapeutic agents in treatment of autoimmune diseases.

Further studies are required to investigate why, in the absence of LXRβ, neurons are more sensitive to dietary β-sitosterol. Since LXRβ is expressed very early in the development of the brain and spinal cord, it is possible that neurons

are developmentally not well programmed to protect themselves against neurotoxins. Another unexplained observation is the degeneration of the large motor neurons in spinal cords that was observed in male, not female, mice. It is possible that female resistance is due to the neuro-protective effects of estrogen but it may also be that testosterone confers some increased sensitivity to neurotoxins.

The novel finding that AQP1 is an LXRβ- regulated gene leads to the question of whether loss of AQP1 is associated with development of motor neuron disease and loss of dopaminergic neurons in the brain. It is possible that loss of AQP1 leads to abnormal composition of cerebrospinal fluid which would predispose to neuronal death.

It is my hope that these studies have provided some insight into the etiology of some quite devastating human diseases and suggested novel targets for the treatment of these diseases.

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사랑하는 가족이 저를 응원하고 있다는 것이 그 무엇보다도 큰 힘이 되었습니다.
감사합니다. 사랑해요. ☺

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