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THE ROLE OF HYDROXYSTEROID (17-BETA) DEHYDROGENASES 2, 7 AND 12 IN MOUSE DEVELOPMENT

by

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Ylin onnellisuus elämässä on varmuus siitä,
että on rakastettu;
rakastettu omana itsenään
tai...

paremminkin itsestä huolimatta.
- Victor Hugo

To my family

ABSTRACT

Pia Rantakari: The role of hydroxysteroid (17-beta) dehydrogenase 2, 7 and 12 enzymes in mouse development (2010)
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Hydroxysteroid (17-beta) dehydrogenases (HSD17Bs) are a group of enzymes activating or inactivating the ligand signaling through the nuclear receptors. HSD17Bs catalyze the conversion between 17-keto and 17β-hydroxysteroids *in vitro*. Both androgens and estrogens are highly active in the 17β-hydroxy configuration, whereas the affinity of the 17-keto derivatives for the corresponding receptors is markedly lower. Thus, HSD17Bs are considered to regulate the balance between biologically highly active and less active sex steroids in various sex steroid dependent tissues. HSD17B enzymes belong to two families: aldo-keto reductases and ketosteroid reductases. To date, in these two enzyme families 14 different enzymes with HSD17B activity have been characterized. The enzymes possess different substrate and cofactor specificities, different tissue distribution, and different preferences for the direction of the reaction. In addition to catalyzing reaction between sex steroids, several HSD17B enzymes have been shown to be involved in other metabolic pathways as well.

Hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2) is known to catalyze the inactivation of 17β-hydroxysteroids to less active 17-keto forms, and catalyze the conversion of 20α-hydroxyprogesterone to progesterone *in vitro*. Hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7) has been shown to catalyze the conversion of both estrone to estradiol (17-ketosteroid reductase activity) and zymosterol to zymosterone (3-ketosteroid reductase activity involved in cholesterol biosynthesis) *in vitro*. HSD17B12 has been shown to be involved both in the biosynthesis of estradiol and in the elongation of the essential very long fatty acids *in vitro* and *in vivo*. In the present study, knockout mice of *Hsd17b2, 7* and *12* (HSD17B2KO, HSD17B7KO and HSD17B12KO) were used to investigate the function of mammalian HSD17B enzymes *in vivo*.

The HSD17B2KO mice exhibited fetal and perinatal mortality. The HSD17B2KO mice that survived through the fetal period were growth retarded, and showed abnormalities in the kidney and the brain, and in the end of fetal period the HSD17B2KO mice showed signs of severe hydranencephaly. Both HSD17B7KO and HSD17B12KO mice showed early embryonic lethality and, after morphological studies a reduced size of the HSD17BKO embryos was revealed. The present study showed a lack of HSD17B7 results in a blockage in the *de novo* cholesterol biosynthesis in mouse embryos *in vivo* and a defect in the development of the neuronal tissues. Morphological defects in the cardiovascular system were also observed. The HSD17B12KO mice died at E9.5 at latest, and presented severe developmental defects. Analysis of the KO embryos revealed that the embryos initiate gastrulation, but their organogenesis is severely disrupted. The amount of arachidonic acid was significantly decreased in heterozygous ES cells suggesting that, in mouse, the HSD17B12 is involved in the synthesis of arachidonic acid and is essential for normal neuronal development during embryogenesis.

There seems to be a critical developmental window, especially for neuronal development, when the embryonic expression of *Hsd17b*, particularly *Hsd17b7* and *Hsd17b12*, is required. Although the research was carried out on mice, the novel finding of this study opens up the possibility that *Hsd17b2, 7* and *12* could be the missing or defective genes behind some of the neuronal disorders in humans.

Keywords: Gene targeting, Knockout, hydroxysteroid (17-beta) dehydrogenase

TIIVISTELMÄ

Pia Rantakari: Hydroksisteroidi (17-beta) dehydrogenaasi 2-, 7-, ja 12-entsyymien toiminnan selvittäminen hiiren alkionkehityksen aikana
Fysiologian osasto, Turun yliopiston tautimallinnuskeskus, Biolääketieteen laitos, Lääketieteellinen tiedekunta, Turun Yliopisto, Kiinamyllynkatu 10, 20520 Turku, Suomi

Hydroksisteroidi (17-beeta) dehydrogenaasit (HSD17B) ovat entsyymejä jotka vaikuttavat tumareseptorien aktivointiin ja inaktivointiin. HSD17B entsyymit katalysoivat inaktiivisten 17-ketosteroidien muuntumista aktiivisiksi 17β-hydroksisteroideiksi. Näiden reaktioiden oletetaan muodostavan reseptoritoimintaa edeltävän kontrollimekanismin, koska androgeeni- ja estrogeenireseptorit transaktivoivat kohdegeeninsä sitomalla 17β-asemasta hydroksyloidut steroidit voimakkaammin reseptoriin kuin 17-ketosteroidit. HSD17B-entsyymit kuuluvat joko lyhytketjuiseen dehydrogenaasi/reduktaasi perheeseen (short-chain reductase, SDR) tai aldoketoreduktaasi perheeseen (AKR). Hydroksisteroidi (17-beeta) dehydrogenaaseja on tähän mennessä identifioitu 14, joilla kaikilla on soluspesifiset ilmentymisprofiilit, substraattispesifisyydet ja säätelymekanismit. Osa HSD17B-entsyymeistä toimii steroidihormonien lisäksi myös muiden samankaltaisten molekyylien aineenvaihduntareiteissä.

In vitro -työt ovat osoittaneet, että HSD17B2-entsyymi katalysoi estradiolin ja testosteronin muuntumista ääreiskudoksissa heikompitehoisiksi hormoneiksi estroniksi ja androstendioniksi, ja entsyymi myös aktivoi 20α-hydroksiprogesteronin progesteroniksi (P). HSD17B7-entsyymin on ositettu katalysoivan *in vitro* estronin muuttumista aktiivisemmaksi estradioliksi ja sillä on havaittu olevan myös tärkeä rooli kolesterolibiosynteesin loppuvaiheessa, jossa HSD17B7 katalysoi reaktiota zymosteronista zymosteroliin. HSD17B12 on myös osoitettu katalysoivan estronin muuttumista *in vitro,* mutta sillä on myös havaittu olevan merkittävä rooli rasvahappobiosynteesissä sekä *in vitro* että *in vivo*. Tässä tutkimuksessa tehtiin *Hsd17b2, Hsd17b7* ja *Hsd17b12* poistogeeniset hiiret (HSD17B2KO, HSD17B7KO ja HSD17B12KO) joita käytettiin näiden geenien toiminnan tutkimiseen *in vivo*.

Tutkimuksissa havaittiin, että osa *Hsd17b2* poistogeenisistä hiiristä kuoli alkiokehityksen aikana. Ne HSD17B2KO hiiret jotka selviytyivät syntymään asti, olivat selkeästi kasvuhäiriöisiä, ja niillä oli rakennepoikkeavuuksia munuaisissa ja aivoissa. HSD17B2KO-hiirillä havaittiin synnynnäinen aivojen nestekierron häiriö eli hydrokefalus. Tutkimuksissa havaittiin, että *Hsd17b7*-tai *Hsd17b12*-geenin poistaminen hiireltä johtaa alkionaikaiseen kuolleisuuteen ja vakaviin rakennehäiriöihin. Tässä tutkimuksessa voitiin osoittaa että, hiiren *Hsd17b7* geenin poistaminen estää normaalin kolesterolisynteesin ja johtaa hermokudosten kehityshäiriöihin. Tutkimuksemme osoittivat, että HSD17B12KO alkioiden gastrulaatio oli normaali, mutta kudosten ja elinten erilaistuminen eli organogeneesi oli häiriintynyt ja johti alkion neuraaliputken normaalin kehityksen estymiseen. *Hsd17b12*-poistogeenisyyden voitiin myös osoittaa vaikuttavan arakidonihapon synteesiin.

Tämän tutkimuksen tulokset osoittivat, että tutkittujen HSD17B-entsyymien ilmentyminen on tärkeää erityisesti hermokudosten kehittymiselle hiiren alkionkehityksen aikana.

Avainsanat: Poistogeeniset hiiret, Hydroksisteroidi (17-beeta) dehydrogenaasi

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ABBREVIATIONS

Hormones

A-diol	androstenediol, 5-androstene-3β,17β-diol
3αA-diol	5α-androstane-3α,17β-diol
A-dione	androstenedione, 4-androsten-3,17-dione
5αA-dione	5α-androstan-3,17-dione
ADT	androsterone
3βA-diol	5α-androstane-3β,17β-diol
DHEA	dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one
DHEA-S	dehydroepiandrosterone sulfate
DHT	5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one
E1	estrone, 3-hydroxy-1,3,5 (10)-estratriene-17-one
E2	estradiol, 1,3,5 (10)-estratriene-3,17β-diol
P	progesterone, 4-pregnene-3,20-dione
T	testosterone, 17β-hydroxy-4-androsten-3-one

Others

AA	arachidonic acid
ACC1	acetyl-CoA carboxylase 1
ADH	alcohol dehydrogenase
AKR	aldo-keto reductase
ALDH	aldehyde dehydrogenase
AR	androgen receptor
ARE	*cis*-acting mRNA destabilizing AU-rich element
AVE	anterior visceral endoderm.
BAC	bacterial artificial chromosome
cDNA	complementary DNA
DHA	docosahexaenoic acid
CNS	central nervous system
Cre	Cre recombinase
CYP26	cytocrome P450 retinoic acid hydroxylase
CRABP	cellular retinoic acid binding protein
dNTP	deoxynucleoside triphosphate
Dre	Dre recombinase
E	embryonic day
EFA	essential fatty acid
ER	endoplastic reticulum
ES	embryonic stem cell
Esr1	estrogen receptor-1 (α)
Esr2	estrogen receptor-2 (β)
FAS	fatty acid synthase
GATA-4	GATA-binding protein 4
TGC	trophoblast giant cells
GFP	green fluorescent protein

GGTC	German Gene Trap Consortium
GLC	gas-liquid chromatography
GCs	glycogen cells
GT	gene trap
HE	heterozygous
HO	homozygous
HSD17B	hydroxysteroid (17-beta) dehydrogenase
AKR1C1	aldo-keto reductase family 1 member C1 (20α-HSD)
KAR	3-ketoacyl-CoA reductase
ICI	a steroidal estrogen antagonist
ICM	inner cell mass
IRES	internal ribosome entry site
KO	knockout
Lefty2	left-right determination factor 2
LIF	leukemia inhibitory factor
MDR	medium chain alcohol dehydrogenase / reductase
MEF	mouse embryonic fibroblast
NAD^+	nicotinamide-adenine dinucleotide
$NADP^+$	nicotinamide-adenine dinucleotide phosphate
Neo	neomycin resistance gene
PCR	polymerase chain reaction
PE	parietal endoderm
Ptch	human homolog 1 of Drosophila patched receptor
PUFA	polysaturated fatty acid
pDEST	gateway destination vector
PECAM-1	platelet/endothelial cell adhesion molecule 1
pENTR	gateway entry vector
Pgk1	phosphoglycerol kinase 1
RA	retinoic acid
RAR	retinoid receptor
RBP	cellular retinol binding protein
RDH	retinol dehydrogenase
PRAP	prolactin receptor-associated protein
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
SDR	short-chain dehydrogenase/ reductase
Shh	sonic hedgehog
siRNA	small interfering ribonucleic acid
Sp	spongiotrophoblast cells
SREBP	sterol regulatory element-binding protein
SSR	site-specific recombinase
TG	transgenic
UPAT	unbiased gene trap
VE	visceral endoderm
VEGF	vascular endothelial growth factor
VLCFA	very-long-chain fatty acid
WT	wild type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, to which the text refers by their respective Roman numerals (I-III).

I **Pia Rantakari**, Leena Strauss, Riku Kiviranta, Heidi Lagerbohm, Jenni Paviala, Irma Holopainen, Seppo Vainio, Pirjo Pakarinen, Matti Poutanen (2008). Placenta defects and embryonic lethality resulting from disruption of mouse hydroxysteroid (17-beta) dehydrogenase 2 gene. *Mol Endocrinol*. 3:665-75

II Heli Jokela, **Pia Rantakari**, Tarja Lamminen, Leena Strauss, Roxana Ola, Aino-Liisa Mutka, Helena Gylling, Tatu Miettinen, Pirjo Pakarinen, Kirsi Sainio, Matti Poutanen (2010). Hydroxysteroid (17-beta) dehydrogenase 7 activity is essential for fetal *de novo* cholesterol synthesis and for neuroectodermal survival and cardiovascular differentiation in early mouse embryos. *Endocrinology, in press*

III **Pia Rantakari**, Heidi Lagerbohm, Mika Kaimainen, Jukka-Pekka Suomela, Leena Strauss, Kirsi Sainio, Pirjo Pakarinen, Matti Poutanen (2010). Hydr oxysteroid (17-beta) dehydrogenase 12 is essential for mouse organogenesis and embryonic survival. *Endocrinology, in press*

In addition, unpublished data is included. The original publications have been reproduced with the kind permission of the copyright holders.

1. INTRODUCTION

Among the methods for functional studies of novel genes, gene targeting is straightforward and commonly used in mouse (Glaser et al. 2005). This method is based on gene disruption via homologous recombination between an endogenous gene and exogenous transfected DNA. However, using the gene targeting method is still largely an art. Especially the construction of targeting vectors is complicated and requires a great deal of labor.

Cells in the developing embryo must combine complex signals from the genome and environment to make decisions about their behavior or fate. The ability to genetically modify the mouse genome by removing or replacing a specific gene has improved our ability to understand the fundamental biology of the decision-making process, and how these decisions may go wrong during abnormal development. Various human diseases have been mimicked in the mouse and the signaling pathways between human and mouse have been shown to be conserved (Thyagarajan et al. 2003). Many genes are known to be involved in mouse embryogenesis, but much is still unknown, regarding the regulatory hierarchy governing the developmental events, and many more genes are yet to be discovered to be important to normal embryonic development.

Hydroxysteroid (17-beta) dehydrogenases (HSD17Bs) are a group of enzymes activating or inactivating the ligand signaling through the nuclear receptors. HSD17Bs catalyze the conversion between 17-keto and 17β-hydroxysteroids *in vitro*. Both androgens and estrogens are highly active in the 17β-hydroxy configuration, whereas the affinity of the 17-keto derivatives for the corresponding receptors is markedly lower. Thus, HSD17Bs are considered to regulate the balance between biologically highly active and less active sex steroids in various sex steroid dependent tissues (Luu-The et al. 1995, Labrie et al. 1997, Moeller and Adamski 2009). HSD17B enzymes belong to two families: aldo-keto reductases and ketosteroid reductases (Peltoketo et al. 1999). To date, in these two enzyme families 14 different enzymes with HSD17B activity have been characterized. The enzymes possess different substrate and cofactor specificities, different tissue distribution, and different preferences for the direction of the reaction (Labrie et al. 1997, Labrie et al. 2000, Luu-The 2001). In addition to catalyzing reaction between sex steroids, several HSD17B enzymes have shown to be involved in other metabolic pathways as well (He et al. 1999, Baes et al. 2000, Zhongyi et al. 2007, Moeller and Adamski 2009). Among HSD17B enzymes, *Hsd17b2* and *Hsd17b7* are shown to be expressed during the mouse development, suggesting an essential role of these enzymes in normal mouse development. *Hsd17b2* is expressed in several epithelial cell types in developing mouse embryo (Mustonen et al. 1998a), and the expression of *Hsd17b*7 in mouse embryo is reported mainly in the brain and in the heart (Marijanovic et al. 2003, Shehu et al. 2008). In addition to expression in mouse embryo, *Hsd17b2* and *Hsd17b7* are strongly expressed in the mouse placenta (Mustonen et al. 1997a, Nokelainen et al. 2000).

The objective of the present study was to gain information for the significance of HSD17B enzymes during the mouse development. We studied the *in vivo* consequences of loss of function in *Hsd17b2, Hsd17b7* and *Hsd17b12* genes in mouse. Gene specific knockout mouse models were general either through the gene targeting or genetraps.

2. REVIEW OF THE LITERATURE

2.1. Mouse early development

The early development of mouse involves the decisions of which cells will give rise to extra-embryonic membranes and which give rise to the embryo proper (Theiler 1989). The same general mechanisms regulate the development of both extraembryonic and embryonic cell types and similar genes regulate the cell differentiation. Development of the extraembryonic structures directly impacts on the morphogenesis within the embryo, because of alternative cell-fate decisions and direct tissue interactions (Perry 1981, Cross et al. 1994).

2.1.1. Preimplantation development

The fertilized egg divides and develops as it moves along the oviduct to the uterus. In mice this process takes 4-5 days. The first cleavage during the journey produces two identical cells. The lengths of the cell cycle phases are considerably different between 1- and 2-cell stage mouse embryos. The S phase increases from 4 h to 7 h from the pronuclear stage to the 2-cell stage, whereas the duration of G2 and M increases from 8 h to nearly 12 h (Streffer et al. 1980). The extended cycle in mouse embryos may be related to one of the major events of preimplantation development, the embryonic genome activation. The earliest developmental changes of mouse embryo are under maternal post-transcriptional control (Renard et al. 1994) and the activation of the mouse embryonic genome occurs at the late 2-cell stage (Goddard and Pratt 1983, Kidder 1992). The embryonic genome is activated in two phases: a limited activation occurs between 18 and 21 h after conception and a major activation is taking place between 26 and 29 h after fertilization (Flach et al. 1982). The change in protein synthesis of preimplantation mouse embryo occurs at the same time with the activation of the embryonic genome (Pratt et al. 1983, Clegg and Pikó 1983, Giebelhaus et al. 1983).

Compaction is the first event in morphogenesis and cellular differentiation. During compaction, the cells become polarized and the tight junctions are formed between them (Becker, Leclerc-David and Warner 1992). At compaction two different cell populations are formed and previously pluripotent cells become embryonic or trophoblast stem cells and the developmental potential is regulated by cell position in the compacted morula. Cells remaining in contact with the outside are destined to form the trophectodermal lineage, whereas the inner cells are destined to become embryonic stem cells forming the inner cell mass (ICM) (Gardner and Beddington 1988) (Figure 1). During the time of compaction the mouse embryo switches from a dependence on the pyruvate and lactate to a metabolism based on glycolysis (Biggers 1971, Houghton et al. 1996). Embryo culture experiments have shown that glucose cannot support early embryo development before the 8-cell stage (Leese and Barton 1984, Houghton et al. 1996). The switch to need of glucose may be caused by the increased energy demand around the time of compaction, as protein synthesis increases when the blastocyst cavity is formed (Flach et al. 1982, Wang and Latham 1997). In addition, glucose provides important pentose moieties for nucleic acid synthesis and is required for phospholipid and non-essential amino acid biosynthesis (Johnson et al. 2003).

The zona pellucida, a protective membrane, prevents the egg from implanting in the oviduct wall. By the time the egg reaches the uterus, it has undergone several cell divisions to form a blastocyst (Figure 1). Just before the implantation, the blastocyst is an asymmetric ball of cells surrounding a fluid-filled cavity. Between embryonic days 4.5-5 (E4.5-E5), the blastocyst hatches from the zona pellucida and is ready for implantation (Cross et al. 1994, Georgiades et al. 2002).

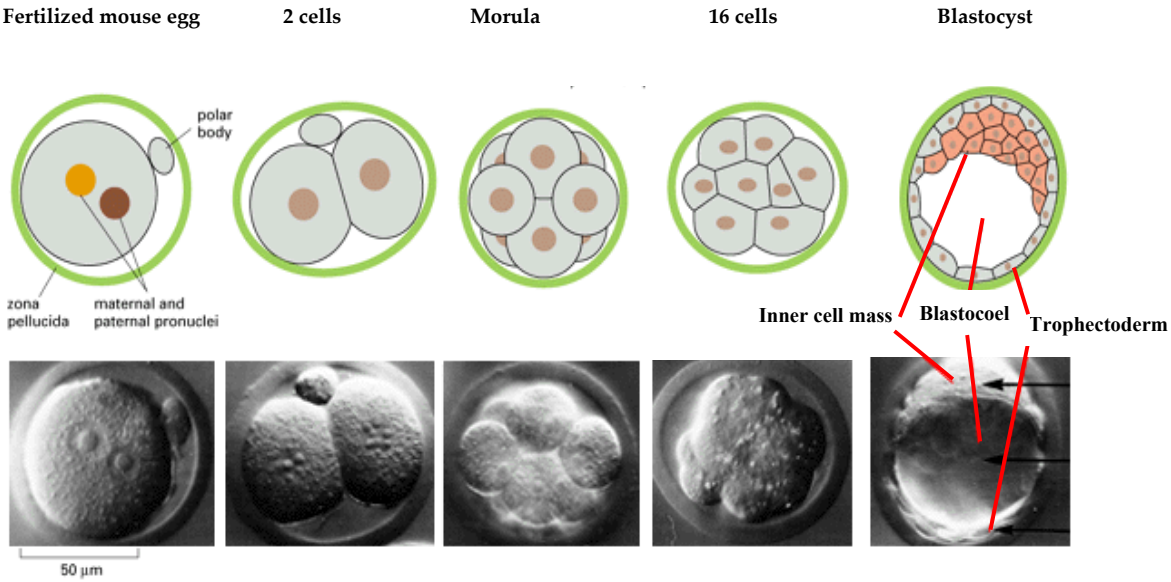


Figure 1. The preimplantation stages of mouse development, modified from Martin (Martin 1980).

2.1.2 Embryonic and extraembryonic development after implantation

The mouse blastocyst implants into the uterus before the end of E5 and continues the development by forming an egg cylinder (Tam and Behringer 1997, Rossant and Tam 2009). At this stage, trophoblast cells that overlie the ICM (polar trophectoderm) continue to proliferate and, after implantation, the polar trophectoderm gives rise to the extraembryonic ectoderm and the ectoplacental cone (Copp 1979) (Figure 2). The rapidly proliferating polar trophectoderm also includes the trophoblast stem cells (Oda et al. 2006, Quinn et al. 2006). Trophoblast cells that are not overlying the ICM (mural trophectoderm) stop dividing and become mitotically arrested, but continue to go through rounds of DNA synthesis to become polyploid trophoblast giant cells (Cross 1998, Cross 2000, Rossant and Cross 2001). The cells of the ICM give rise to the entire embryo proper as well as to mesodermal and endodermal components of the placenta and fetal membranes (Nagy et al. 1990, Nagy et al. 1993). The primitive endoderm emerges from the ICM in late blastocysts, and later transforms into the extraembryonic parietal (PE) and visceral endoderm (VE) (figure 2). The migratory parietal endoderm comes to underlie the primary trophoblast giant cell layer, together forming the parietal yolk sac layer (Theiler 1989).

Gastrulation begins at E6.5 and the basic body plan is determined (Tam and Behringer 1997). During the mouse development from implantation to gastrulation, the cell number increases almost 100-fold (Snow and Bennett 1978). Gastrulation begins in the embryonic ectoderm layer and gives rise to the three germ layers of the mouse embryo, the ectoderm that forms the skin and the nervous system; the mesoderm that generates the skeletal system and internal organs; and the endoderm that gives rise to the gut and associated organs (Theiler 1989, Kaufman and Bard 1999). In addition, gastrulation gives rise to extraembryonic mesoderm that forms the amnion, visceral yolk sac and allantois, and to extraembryonic ectoderm that forms the chorion (Perry 1981, Cross 1998). In mammals, the generation of the three-dimensional body axes (anterior-posterior, dorsal-ventral, and left-right) is essentially linked to the creation of different cell lineages during gastrulation (Yamaguchi 2001).

Hematopoiesis in the early embryo initiates at two independent sites, the yolk sac and in the para-aortic splanchnopleura, that later include the developing aorta, gonads, and mesonephros (Godin et al. 1995, Medvinsky and Dzierzak 1996, Palis et al. 1999). The haematopoiesis starts at the extraembryonic visceral yolk sac (Boucher and Pedersen 1996, Palis and Yoder 2001) and the first angioblasts appear around E7.0-7.5. After that they migrate, aggregate, proliferate and eventually differentiate to form a vascular plexus through the process called vasculogenesis (Breier 1999). Several mouse genes have been shown to have a role in normal angiogenesis. One of the best studied is the family of the vascular endothelial growth factors (VEGF) (Li and Behringer 1998, Ferrara et al. 2003).

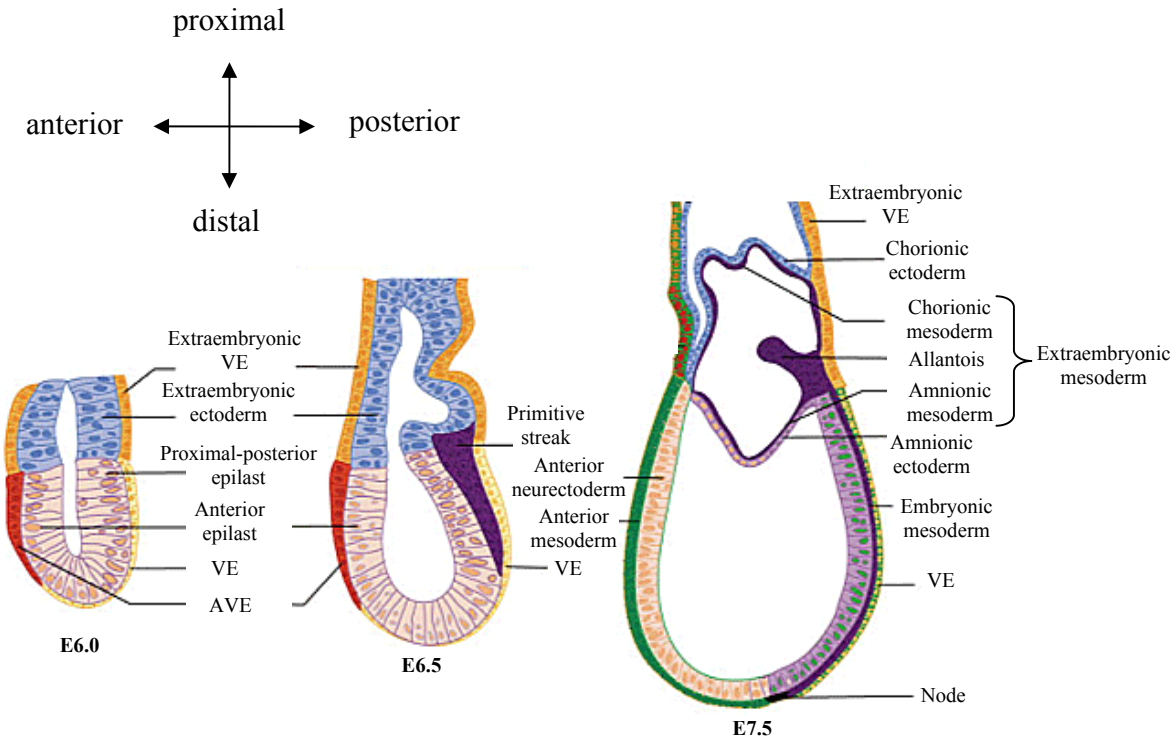


Figure 2. Schematic representation of mouse development up to E7.5. Modified from Lu et al. (Lu et al. 2001). Abbreviations: VE, visceral endoderm; AVE, anterior visceral endoderm.

By late E7.5, an allantoic bud starts to grow and push against the mesodermal side of the chorion and makes contact with the chorion. There are many mouse mutants that show defects in the fusion of allantois and chorion and this is the most common cause of embryonic lethality during mid-gestation (Rossant and Cross 2001). A properly fused allanto-chorion is visible by E8-9. Thereafter, the chorion begins to fold to form the villi, creating a space into which the fetal blood vessels grow from the allantois (Cross et al. 2003). At this time, the chorionic trophoblast cells begin to differentiate into two labyrinth cell types; multinucleated syncytiotrophoblast cells, formed by the fusion of trophoblast cells, surround the fetal endothelium of the capillaries and a mononuclear trophoblast cell type lines in the maternal blood sinuses. Together the trophoblast and fetal vasculature generate extensively branched villi of the labyrinth (Figure 3.), which become larger and more extensively branched until birth (E18.5–19.5) (Watson and Cross 2005, Cross et al. 2006). There are multiple distinct trophoblast cell types within the labyrinth. If the labyrinth is not appropriately vascularized, the placental perfusion is impaired, resulting in poor oxygen and nutrient diffusion (Adamson et al. 2002). The spongiotrophoblast layer forms the middle layer of the placenta between the outermost giant cells and the innermost labyrinth layer. The function of the spongiotrophoblast layer is unknown, but it probably has a structural role and it also produces several layer-specific secreted factors. Improper formation of the spongiotrophoblasts leads to embryonic lethality in mouse (Tanaka et al. 1997). At E8.5, the embryo has elongated, the head fold has been formed in the anterior end and the somites have started to develop (Theiler 1989). The embryonic turning occurs around E8.5 of gestation pulling the amnion and yolk sac around the embryo. Around E9, when the three germ layers have been established the organogenesis begins. The cells are interacting with one another and are reorganized to produce the tissues and organs (Theiler 1989).

By E11, due to the concerted action of giant cell layer, spongiotrophoblast layer and underlying chorio-allantoic layer (Figure 3), the essential nutrition is established for the developing embryo (Watson and Cross 2005). All these structures are clearly organized and a functional placenta is formed. At E12, a new variety of cells, glycogen cells, start to differentiate from the spongiotrophoblasts. The function of glycogen cells is unknown, but they are the only cell types which continue to invade into the decidua even in the very late stages of gestation (Kaufman and Bard 1999, Enders and Carter 2004). As the embryonic development proceeds, the placenta continues to grow in size and gains weight until E14.5, after which the rate of placental weight gain mainly stops. Typically, a normal placenta weighs around 100mg towards the end of gestation and supports the fetus which weighs around ten times the placenta itself. This indicates the efficiency of exchange occurring through this organ, which is essential for the developing fetus (Kaufman and Bard 1999, Watson and Cross 2005).

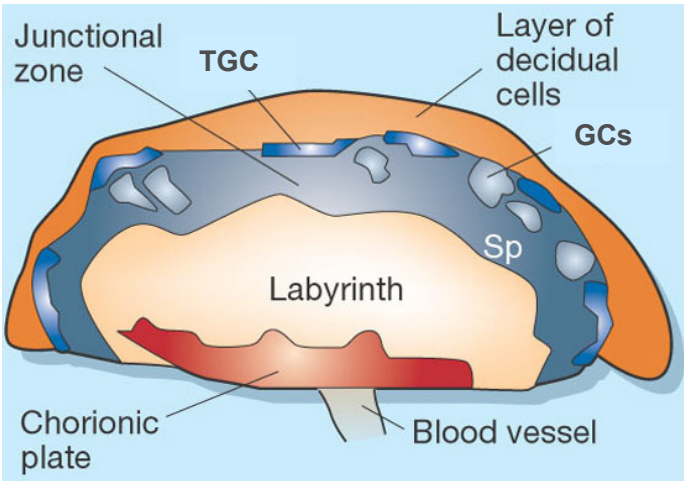


Figure 3. **Simplified figure of cross-section of the mouse placenta.** Modified from Tycko and Efstratiadis (Tycko and Efstratiadis 2002). Sp, spongiotrophoblast; GCs, glycogen cells; TGC, trophoblast giant cells.

2.1.3 Steroid metabolism in feto-placental unit

Steroid hormones progesterone and estrogen are essential to establishing and maintaining pregnancy in mammals (Cross et al. 1994, Strauss et al. 1996). Synthesis of steroid hormones begins in steroidogenic tissues upon conversion of cholesterol into pregnenolone, and the reaction is catalyzed by the cytochrome P450 (P450scc). The pregnenolone is further processed into active steroid hormones by many enzymes, such as hydroxysteroid (3-beta) dehydrogenase/isomerase (3βHSD), Cytochrome P45017α-hydroxylase/c17-20lyase (P450c17), and cytochrome P450 aromatase (Ben-Zimra et al. 2002, Pasqualini 2005). In the rodent, the progesterone production by the corpus luteum (CL) has been shown to be indispensable throughout pregnancy (Strauss et al. 1996, Pasqualini 2005) and the mature rodent placenta can only convert progesterone to androgens by the entzyme P450c17 (Warshaw et al. 1986) and androgen thereafter serves as substrate for estrogen synthesis in mouse CL (Warshaw et al. 1986, Ben-Zimra et al. 2002).

Even if the rodent placenta, as opposed to the human placenta, is known to produce very modest amounts of steroid hormones *de novo* in the second half of gestation, the role of local steroid hormone production in the rodent placenta is not clear. After implantation, expression of P450scc and 3β-HSD has been detected in decidual cells of the uterine wall (Schiff et al. 1993, Arensburg et al. 1999) and at the time of placentation at midpregnancy, the expression of steroidogenic genes including P450scc (Schiff et al. 1993), StAR, 3β-HSD, and P45017α are observed in the giant trophoblast cells, the steroidogenic cells of the rodent placenta (Ben-Zimra et al. 2002). Expression of these enzymes peaks at E9.5, after which there is a decline in expression between E10.5 and E14.5 (Arensburg et al. 1999). The expression of genes required for placental progesterone production has been associated with various theories related to effects of locally produced steroids (Salomon and Sherman 1975, Siiteri et al. 1977, Strauss et al. 1996) and earlier studies have shown that mouse giant trophoblast cells can produce progesterone

(Salomon and Sherman 1975). In the other hand, the mouse embryonic steroidogenesis appears not to be necessary, as P450scc and StAR knockout (KO) mice die postnatally for adrenal insufficiency (Caron et al. 1997, Hu et al. 2002). However, deficiency of P450c17 leads to early lethality prior to gastrulation (Bair and Mellon 2004). He cause of this early lethality is unknown, as DHEA, androstenedione, and 17-OH pregnenolone are not able to rescue the phenotype (Bair and Mellon 2004).

2.2. Gene targeting, advanced genetic manipulation of mouse genome

The most important advantage to use the mouse for biomedical research is the possibility to experimentally manipulate the mouse genome. The ability to introduce virtually any mutation into the mouse genome through the gene targeting in mouse embryonic stem (ES) cells provides a powerful tool for studying the gene function *in vivo*. Mouse ES cells are derived from the inner cell mass of blastocysts and are capable of unlimited and undifferentiated proliferation *in vitro*. Mouse ES cell lines were first established by culturing ICM (Martin 1981, Evans and Kaufman 1981) in the presence of the feeder cell layer made of mouse embryonic fibroblast (MEF) and leukemia inhibitory factors (LIF) (Williams et al. 1988). ES cells have a developmental potential to form derivatives of all three embryonic germ layers, endoderm, mesoderm, and ectoderm, even after extended culture (Thomson and Marshall 1998). Moreover, they are capable of generating germ-line chimeras after the blastocyst injections (Bradley et al. 1984).

Mammalian cells can mediate recombination between homologous DNA sequences. Homologous recombination allows the exchange of genetic information between two DNA molecules in a precise and specific manner (Kowalczykowski et al. 1994, Kowalczykowski and Eggleston 1994). Gene targeting is a technique utilizing homologous recombination between a modified exogenous DNA fragment and the genome of the mouse embryonic stem cells. (Capecchi 1989a, Capecchi 1989b, Koller and Smithies 1992). Thousands of mouse genes have been targeted in this fashion. Although the rat ES cells have been available for a while (Li et al. 2008, Buehr et al. 2008), the mouse is still the only species for which ES cells can be consistently and quickly transmitted through the germ line after genetic manipulation *in vitro* (Bradley et al. 1984). This ability to generate designated mutations in mouse genes has provided a comprehensive resource to understand the genetic basis of mammalian biology and disease progression (Glaser et al. 2005).

2.2.1. Knockout mouse models

Gene targeting, or homologous recombination, is the method of specifically manipulating a gene in the nucleus of an ES cell (Capecchi 1989b, Koller and Smithies 1992). Typically this is done by introducing an artificial piece of DNA that shares homologous sequence to the gene. This homologous sequence flanks the existing gene DNA sequence both upstream and downstream of the gene location on the chromosome. A homologous recombination results in exchange of the artificial DNA sequence for the corresponding area of genomic DNA as the DNA breaks and rejoins (Majzoub and Muglia 1996). Because the artificial DNA is inactive, the swap eliminates, or knocks out, the function of the existing gene. This kind of knockout is often referred as

constitutive knockout or traditional knockout, and the mice are often called loss of function models (Glaser et al. 2005).

In many cases, however, the complete deficiency of a gene leads to embryonic lethality, preventing the analysis of gene function in later developmental stages or in the adult. Alternatively, the absence of one gene during development may lead to up regulation of components in compensatory pathways, thereby complicating the final phenotype of the knockout mouse. This problem can be overcome by making conditional knockout mice, which allows a gene to be inactivated in a tissue or in temporal specific fashion (Nagy 2000). This can be achieved with site-specific DNA recombinase technology (SSR), such as the Cre-lox P (Rajewsky et al. 1996), which involves the insertion of specific nucleotide sequences called lox P either side of the targeted gene. The Cre enzyme recognizes the sequences flanked by the lox P sequences and causes DNA cleavage at these points, thereby deleting the gene. By expressing Cre recombinase under a tissue-specific promoter, the gene can be inactivated in a cell-type-specific fashion. The timing of Cre expression can also be controlled by using inducible Cre expression systems (Hayashi and McMahon 2002) or viral delivery systems such as adenovirus or lentivirus (Pfeifer et al. 2001), which makes it possible to inactivate a gene in a temporal-specific fashion. Today there are several SSR systems in use, Cre-loxP (Akagi et al. 1997), FLP/ FRT (Dymecki 1996), ϕC31-attB/P (Belteki et al. 2003) and Dre (Anastassiadis et al. 2009). These techniques have provided a valuable tool for the manipulation of mouse genome (Glaser et al. 2005, Schnütgen et al. 2006, García-Otín and Guillou 2006) and have made it possible to generate more sophisticated animal models for human diseases and drug development.

2.2.2. Novel methods for constructing gene targeting vectors

Apart from the low efficiency of homologous recombination a critical and rate-limiting step in generating a knockout mouse is the cloning of the gene targeting vector. Vector construction typically involves time-consuming cloning and mapping of genomic DNA fragments. The cloning of DNA molecules *in vivo* by homologous recombination has significantly improved the vector construction process (Zhang et al. 2000, Branda and Dymecki 2004, Court et al. 2002, Glaser et al. 2005). The time requirements and limitations of traditional *in vitro* cloning procedures have been significantly reduced through the development of the recombineering (Chartier et al. 1996, Muyrers et al. 2001, Copeland et al. 2001) and the Gateway cloning system (Walhout et al. 2000, Hartley et al. 2000). Variety of modifications, including sequence deletions, substitutions, and insertions, can be carried out with homologous recombination (Glaser et al. 2005).

Recombineering has been used to subclone or "rescue" genomic DNA fragments as large as 80 kb from a bacterial artificial chromosome (BAC) into a standard plasmid vector (Lee et al. 2001). Recombineering technique allows the cloning of DNA fragments without a need for conveniently positioned restriction sites or use of ligase, and it has been applied specially for cloning large DNA fragments, such as bacterial artificial chromosomes (BACs) (Muyrers et al. 2000c, Valenzuela et al. 2003, Yang and Seed 2003). As distinct from conventional cloning strategies, recombineering cloning can be carried out in a selected position on a given target DNA molecule. Manipulation of plasmid DNA by homologous recombination was first reported

in yeast (Baudin et al. 1993). In *E.coli* the first homologous recombination engineering was done with RecA-dependent recombineering by over-expressing RecA protein, and a variety of RecA dependent cloning approaches have been published since (Chartier et al. 1996, Degryse 1996, Jessen et al. 1998). However, the efficiency in all the RecA dependent strategies was low and unwanted non-homologous or rearranged recombination products were frequently detected (Muyrers et al. 2001). An alternative recombineering technique was developed where the recombination is not dependent on RecA, but instead the recombineering takes advantage of the phage-derived protein pairs, either RecE/RecT from the *Rac* phage or Redα/Redβ from the λ phage (Zhang et al. 1998, Muyrers, Zhang and Stewart 2000b). These protein pairs are functionally equivalent. RecE and Redα are 5´-3´ exonucleases and RecT and Redβ are single-stranded DNA annealing proteins (Kolodner et al. 1994, Muyrers et al. 2000a). A limitation of the original homologous recombination technique was due to the fact that bacterial RecBCD nuclease degrades linear DNA (Kuzminov and Stahl 1997, Amundsen et al. 2000). This was overcome by the discovery that Redα and Redβ were assisted by Redγ, which inhibits RecBCD nuclease activity making it possible to use the technique in *E. coli* and other commonly used bacterial strains (Poteete et al. 1988). The combination of these three enzymes (α, β and γ, or E, T and γ) in one vector was named Red/ET recombination (ET cloning) (Zhang et al. 1998, Muyrers et al. 2000b). As opposed to the RecA recombination, where the recombination occurs between to circular plasmids, the Red/ET recombination mediated by RecET or Redαβ allows recombination of a linear fragment to another linear or circular plasmid (Zhang et al. 2000).

A significant limitation of recombineering is that transfer of DNA fragments between different vectors is not as efficient as the Gateway system. The Gateway system uses elements of the site-specific recombination (SSR) system of bacteriophage lambda to enable inter-molecular transfer of DNA fragments between vectors. In this system the construct of interest is first cloned into an entry vector (pENTR) and is then transferred to a destination vector (pDEST) by site-specific recombination. This is done by using the Int and Xis recombination proteins that recognize the *att*L and *att*R sites in the entry and destination vectors. This system has been employed in large-scale cDNA projects, and compatible vectors have been developed for many different applications (Rozwadowski, Yang and Kagale 2008).

2.2.3. Gene trap mutagenesis

An alternative way to create mutagenic ES cells for generating knockout and other mutant mouse strains is the gene trapping (GT) strategy (Gossler et al. 1989, Wurst et al. 1995, Skarnes et al. 1995, Hardouin and Nagy 2000). Gene trapping approach can be used as a high-throughput method to generate mouse mutants in ES cells. The general term, gene trap, refers to the random integration of a reporter gene construct into the genome. An integration event brings the reporter gene under the transcriptional regulation of an endogenous gene. Gene trap vectors were originally developed in bacteria (Casadaban and Cohen 1979) and applied in *Drosophila* to identify novel developmental genes (Bier et al. 1989), but the gene trap effort has been most extensively used in mouse. Today there is more than 57 000 of ES cell lines, which currently represent approximately 50% of known mouse genes (Raymond and Soriano 2006). Four types of trap vectors are used in mouse, the enhancer (Gossler et al. 1989), promoter (Friedrich and

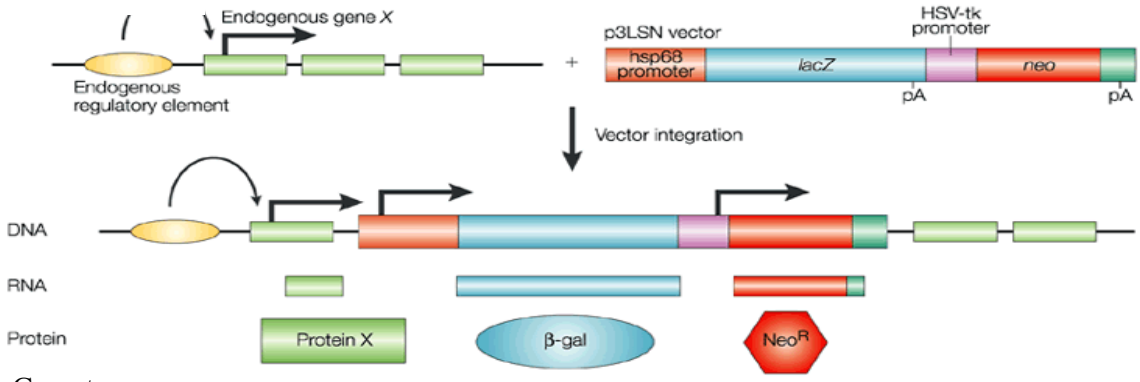
Soriano 1991, von Melchner et al. 1992), poly A (Niwa et al. 1993, Salminen et al. 1998) and gene trap vectors (Skarnes et al. 1992, Stanford et al. 2001) (Figure 6).

The enhancer trap vector contains a minimal promoter that requires that the vector is integrating near to a *cis*-acting enhancer element to produce expression of a reporter gene. Enhancer-traps were first used in bacteria to introduce reporter genes in *E.coli* genome (Casadaban and Cohen 1979), thereafter the method was adapted for use in the other model systems including the mouse (O'Kane and Gehring 1987, Hope 1991, Korn et al. 1992). Although enhancer trapping carried out in mouse ES cells showed reporter expression *in vivo,* the loss-of-function mutation rate from enhancer trapping has not been reported. The enhancer-trap vectors were initially used for finding novel genes in the genome and have not been widely used in the mouse for the production of mutations (Durick et al. 1999, Lee et al. 2007).

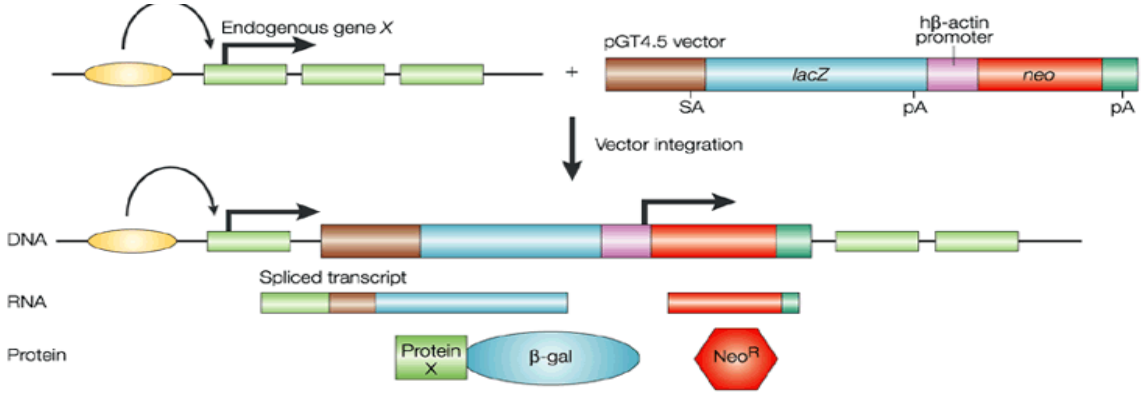
Promoter trap vectors consist of a promoterless reporter gene. The reporter expression and mutagenesis occur when the vector inserts into an exon and generates a fusion transcript that combines the upstream endogenous exonic sequences and the reporter gene (von Melchner and Ruley 1989). The frequency with which promoter-trap vectors insert into exons is remarkably low, therefore, promoter-trap vectors generally have a selectable marker, such as the neomycin resistance gene (*neo*), so that only ES cell clones that contain vector insertions can be selected. Promoter-trap approach, however, means that only insertions into those genes that are transcriptionally active in undifferentiated ES cells will be selected (Skarnes et al. 1992, Yamamura and Araki 2008). The gene trap vector has a splicing acceptor site upstream of a promoterless reporter gene and selectable marker expression cassette. The integration of gene trap vector into an intron results in premature termination of captured endogenous gene and forms a spliced fusion transcript between the reporter gene and the endogonous gene (Skarnes et al. 1992, Salminen et al. 1998).

Promoter and gene trap vectors can be used for those genes that are expressed in mouse ES cells but for the trap genes that are silenced. To overcome this problem the poly A traps were generated (Niwa et al. 1993). Poly A trap vector consists of constitutive promoter driving the expression of a selectable marker that lacks its own poly A addition signal sequence. The reporter gene is expressed only when the trap vector is able to use the poly A addition signal of the endogenous gene (Niwa et al. 1993, Salminen et al. 1998). The limitation in poly A trapping is that the vector integration favors the 3´-end of the genes due to nonsense-mediated mRNA decay of the fusion transcript, resulting in the deletion of only a limited C-terminal portion of the protein encoded by the trapped gene (Shigeoka et al. 2005). Development of improved poly A traps overcomes this problem by using either an internal ribosome entry site (IRES) sequence just downstream of a marker gene containing a termination codon (Shigeoka et al. 2005) or incorporating an *cis*-acting mRNA destabilizing AU-rich element (ARE). ARE ensures that the right integration is achieved only when splicing removes ARE sequence from the transcript (Tsakiridis et al. 2009).

A. Enhancer trap



B. Gene trap



C. Promoter trap

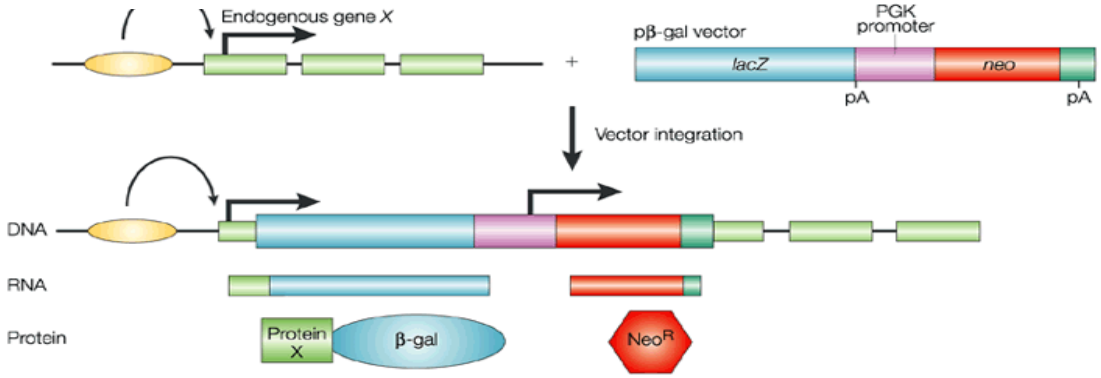


Figure 6. Simplified examples of enhancer, gene and promoter trap vectors, which all contain a *lacZ* reporter gene and a neomycin gene (*neo*) (Stanford et al. 2001).

2.3. Hydroxysteroid (17-beta) dehydrogenase enzymes

Hydroxysteroid (17-beta) dehydrogenase (HSD17B) enzymes catalyze the conversion between 17-keto and 17β-hydroxysteroids *in vitro*. Both androgens and estrogens are highly active in the 17β-hydroxy configuration whereas the affinity of the 17-keto derivatives for the corresponding receptors is markedly lower (Luu-The et al. 1995). HSD17B enzymes belong to two families: aldo-keto reductases and ketosteroid reductases (Peltoketo et al. 1999). To date, in these two enzyme families, 14 different enzymes with HSD17B activity have been characterized. The enzymes possess different substrate and cofactor specificities, different tissue distribution, localication in different subcellular compartments, and different preferences for the direction of the reaction (Labrie et al. 1997, Labrie et al. 2000, Luu-The 2001, Moeller and Adamski 2009). Several studies *in vivo* have shown that some of these enzymes may have key functions also in other metabolic pathways, apart from those attaching to steroid metabolism (Baes et al. 2000, He et al. 2002, Zhongyi et al. 2007).

2.3.1. Hydroxysteroid (17-beta) dehydrogenase 2

Hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2) enzyme has oxidative activity and it is capable of catalyzing *in vitro* the conversion of estradiol (E2), testosterone (T) and dihydrotestosterone (DHT) to their less active 17-keto forms, estrone (E1), androstenedione (A-dione) and 5α-androstanedione (5αA-dione), respectively. The enzyme also possesses 20α-HSD activity, thereby activating 20α-hydroxyprogesterone to progesterone (P) (Wu et al. 1993, Labrie et al. 1995). *Hsd17b2* is localized to endoplasmic reticulum and it is widely expressed in peripheral tissues both in humans and in rodents. Studies in human tissues have shown that the enzyme is expressed in various estrogen and androgen target tissues such as endometrium (Casey et al. 1994), placenta (Mustonen et al. 1998b) and prostate (Elo et al. 1996), thus indicating the role in the hormonal regulation of these tissues (Peltoketo et al. 1999). However, studies in mice have revealed that the enzyme is also expressed throughout the gastrointestinal tract (Mustonen et al. 1998a, Sano et al. 2001) and the liver (Mustonen et al. 1997b), both in fetal and adult mice. Phylogenic studies have suggested a close relationship with *Hsd17b2* and all-*trans*-retinol dehydrogeses (Baker 1998). In addition, *in vivo* studies have shown that RA-induced expression of *Hsd17b2* in a dose- and time-dependent manner in human endometrial epithelial cells (Cheng et al. 2008) and placental cells (Su et al. 2007), and by using an RA antagonist the RA-induced *Hsd17b2* expression can be eliminated (Cheng et al. 2008).

2.3.2. Hydroxysteroid (17-beta) dehydrogenase 7

The cDNA encoding for hydroxysteroid (17-beta) dehydrogenase 7 (HSD17B7) enzyme was first cloned from the rat *corpus luteum* and was identified as a prolactin receptor-associated protein (PRAP) in endoplasmic reticulum (Duan et al. 1996). The rodent (Nokelainen et al. 1998) and human (Törn et al. 2003) HSD17B7 enzymes catalyze the conversion of E1 to E2 *in vitro,* and *Hsd17b7* is considered to be important in pregnancy based on the high expression in mouse ovary during pregnancy (Nokelainen et al. 1998, Nokelainen et al. 2000). Shorter form of human HSD17B7 has also been described (Liu et al. 2005) and it has been shown to catalyze

conversion of estrone to estardiol in HEK293 cells. On the other hand, it has also been shown that the truncated HSD17B7 protein is inactive (Marijanovic et al. 2003). The human HSD17B7 enzyme has also been predicted to possess a 3-ketosteroid reductase activity by its amino acid sequence similarity with the yeast ERG27 protein (Breitling et al. 2001b, Breitling et al. 2001a, Marijanovic et al. 2003). Accordingly, the recombinant human and mouse HSD17B7 enzymes have been found to catalyze the reaction from zymosterone to zymosterol *in vitro* (Marijanovic et al. 2003), a reaction essential for cholesterol biosynthesis. The putative role of HSD17B7 in cholesterol biosynthesis was also supported by a similar expression pattern of the enzyme along with other cholesterogenic enzymes during mouse embryonic development (Laubner et al. 2003, Marijanovic et al. 2003).

2.3.3. Hydroxysteroid (17-beta) dehydrogenase 12

Mammalian hydroxysteroid (17-beta) dehydrogenase 12 (HSD17B12) was initially characterised as a 3-ketoacyl-CoA reductase (KAR), involved in the long-chain fatty acid synthesis in endoplasmic reticulum (Moon and Horton 2003). Both in humans and mice, the *Hsd17b12* shares around 40 % sequence similarity with *Hsd17b3,* and phylogenetic studies have suggested that *Hsd17b12* would be an ancestor of *Hsd17b3* (Mindnich et al. 2004). In humans, the highest level of *Hsd17b12* has been detected in the tissues involved in lipid metabolism, such as the liver, kidney and muscle, whereas in mouse, the expression has also been detected in the brown and white adipose tissue (Moon and Horton 2003, Sakurai et al. 2006, Blanchard and Luu-The 2007). In addition to its role in the fatty acid synthesis, human HSD17B12 has been shown to catalyze the reduction of E1 to E2 in HEK293 cells (Luu-The et al. 2006), and HSD17B12 has been suggested to be the major enzyme converting E1 to E2 in women after menopause (Luu-The et al. 2006). However, neither over-expression nor knocking down of *Hsd17b12* expression affects the E2 production in cultured breast cancer cells (Day et al. 2008). Recent studies from zebrafish have further supported the role of HSD17B12 in the fatty acid synthesis (Mindnich et al. 2004). It has also shown that *Hsd17b12* is involved in the production of monomethyl branched and long chain fatty acids, crucial for proper growth and development of *C. elegans* (Entchev et al. 2008). *Hsd17b12* is, at least partly, regulated by sterol regulatory element-binding proteins (SREBPs), similar to many of the genes involved in lipid metabolism and fatty acid and cholesterol biosynthesis (Nagasaki et al. 2009a). The siRNA-mediated knockdown of the *Hsd17b12* expression in cultured breast cancer cells results in significant inhibition of cell proliferation, which is fully recovered by supplementation of arachidonic acid (Nagasaki et al. 2009b).

2.3.4. Mouse models for hydroxysteroid (17-beta) dehydrogenases

Transgenic male mice over-expressing human HSD17B1 are normal and fertile (Saloniemi et al. 2007) but mascularization is observed in female mice. However, human HSD17B1 is predominantly converting E1 to E2 and is only slightly converting A-dione to T *in vitro* (Puranen et al. 1997). HSD17B1 expressing female mice have increased testosterone concentration, show changes in sexual characteristics and develop ovarian benign serous cystadenomas. The phenotype can be rescued with anti-estrogens or transplantation of wild type (WT) ovaries

(Saloniemi et al. 2007, Saloniemi et al. 2009). Over-expression of human HSD17B2 leads to growth retardation, delayed eye opening, and disrupted spermatogenesis (Zhongyi et al. 2007). The excess of the enzyme causes a decreased bone formation rate at pre-pubertal age associated with lower serum IGF-I, osteocalcin and testosterone (Shen et al. 2008). The phenotype seems not to be due to a reduced estrogen or androgen action but instead to an imbalanced retinoic acid signaling, since the testis phenotype can be rescued by supplying retinoic acid receptor agonist (Zhongyi et al. 2007).

The knockout mice for *Hsd17b4* [peroxisomal multifunctional enzyme type 2 (MFE-2)] survive but show growth retardation and die before 6 months of age. An accumulation of VLCFAs and branched fatty acids is observed. Female mice have reduced fertility while male mice are infertile due to defective spermatogenesis (Baes et al. 2000). Excessive accumulation of fatty acids in Sertoli cells is the most probable reason for the degeneration of the testis and total loss of spermatogenesis at the age of 4 month (Huyghe et al. 2006a). Knockout animals exhibit severe astrogliosis and microgliosis in the central nervous system within the grey matter of the brain and the spinal cord (Huyghe et al. 2006b). Knockout mice for *Hsd17b7* are embryo lethal. Embryos are reabsorbed between E10.5–11.5 of development due to severe brain malformations and heart failure (Present study, Shehu et al. 2008). Tissue-specific over-expression of human HSD17B10 in neuronal tissue seems to protect from cerebral infarction, ischemia (Du Yan et al. 2000) and neuronal stress induced by the neurotoxin MPTP (Tieu et al. 2004).

2.4. Overview of important metabolic pathways in mouse development

2.4.1. Vitamin A in development of mouse embryos

Overview of vitamin A metabolism

Retinoids are required during the normal embryogenesis and have an effect on many biological processes, such as cell differentiation, cell proliferation, and cell death (Chambon 1996, Yashiro et al. 2004, Blomhoff and Blomhoff 2006, Williams et al. 2009). Mammals are unable to synthesize vitamin A *de novo*, hence it must be obtained from dietary sources, most commonly derived from plant carotenoids or as retinyl esters from animal sources (Blomhoff and Blomhoff 2006). Retinoid acids (RA) are synthesized intracellularly from its precursor, vitamin A (or retinol). Upon the entry into the cell, retinol is reduced to retinaldehyde (retinal). Several enzymes have been identified that are capable of catalyzing this reaction, including cytosolic alcohol dehydrogenases (ADHs) of the medium-chain dehydrogenase/reductase (MDR) superfamily and microsomal retinol dehydrogenases (RDHs) of the short-chain dehydrogenase/reductase (SDR) superfamily (Duester et al. 2003). Retinal acts as a substrate for retinaldehyde dehydrogenases (ALDH) that catalyze the irreversible formation of RA (Niederreither et al. 1999b). RAs are the most biologically active form of vitamin A and are able to modulate gene expression by binding to nuclear RA receptors (RAR) or the retinoid X receptors (RXR). Both the all-trans and 9-cis forms of retinoic acid can bind to the retinoic acid receptor (RAR) family members (Allenby et al. 1993). The 9-cis form of retinoic acid (Heyman et al. 1992, Levin et al. 1992), as well as essential fatty acid (EFA), docosahexenoic acid (DHA) binds to RXR (de Urquiza et al. 2000). The RAR and RXR do not act alone, but as heterodimers

(Allenby et al. 1993), and the RXR can also heterodimerize with a variety of other related receptors such as the thyroid hormone receptors, vitamin D receptors, peroxisomal proliferator-activated receptor and several other orphan receptors (Chambon 1996, Rowe 1997). RA is further oxidized by the CYP26 proteins, which are cytochrome P450 enzymes that catalyze the conversion of RA to more polar metabolites including 4-oxo-RA, 4-OH-RA and 18-OH-RA (White et al. 1996, White et al. 2000, Tahayato et al. 2003) (Figure 4). These oxidation reactions are thought to inactivate RA, thus limiting expression of RA-responsive genes in tissues where the CYP26 proteins are expressed. In addition to RA, there are several other forms of bioactive retinoids (Blomhoff and Blomhoff 2006).

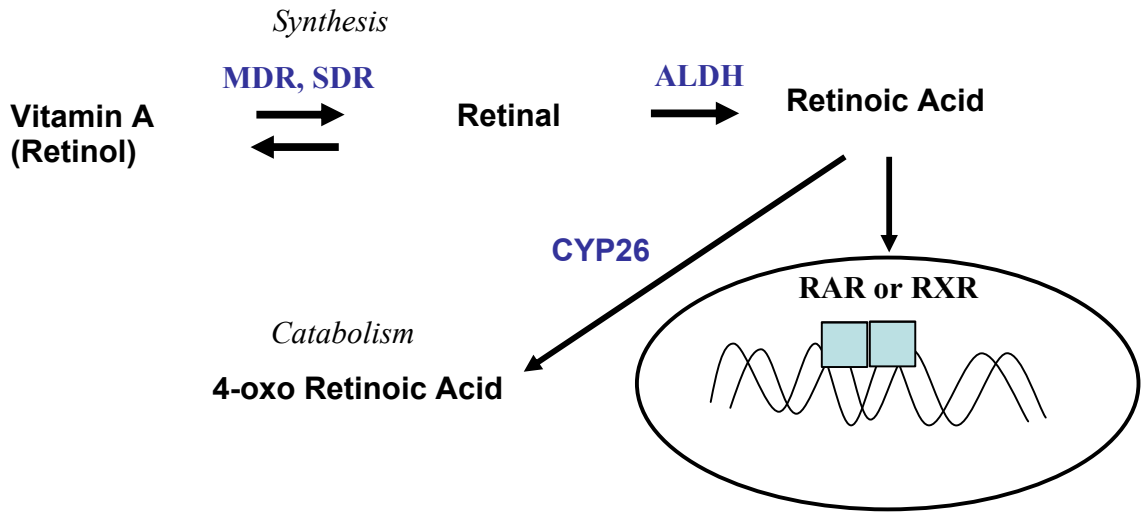


Figure 4. Diagram of retinoic acid pathway.
Abbreviations: MDR, medium-chain dehydrogenase/reductase (MDR); SDR, short-chain dehydrogenase/reductase; ALDH, aldehyde dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid X receptor; CYP26, Cytocrome P450 RA hydroxylase.

Retinoid-synthesizing enzymes in the mouse embryo

Members of alcohol dehydrogenases (ADH) are thought to be involved in the oxidation of retinol to retinal as well as its reduction back to retinol. The spatiotemporal expression patterns of *Adh2, 3* and *4* are detected during mouse embryogenesis (Vonesch et al. 1994, Ang and Duester 1997, Haselbeck and Duester 1998a, Haselbeck and Duester 1998b, Molotkov et al. 2002). Studies with mutant mice have shown that these enzymes are not essential for normal mouse development, as single null mutants of *Adh1, Adh3* and *Adh4* are developmentally normal and viable (Deltour et al. 1999).

Oxidation of retinol to retinal can also be catalyzed by the membrane bound short-chain dehydrogenase/reductase (SDR) family of microsomal enzymes (Duester et al. 2003). However, only retinol dehydrogenases 5 (RDH5) and 10 (RDH10) have been shown to have any function in retinoic metabolism *in vivo* (Duester 2000). *Rdh5* is expressed in mouse embryo already at the time of gastrulation and the expression can be detected through all the stages of development (Ulven et al. 2000). *Rdh5* deficient mice are viable but suffer a mild visual defect (Driessen et al. 2000). *Rdh10* is expressed in mouse embryos from midgestation (Sandell et al. 2007). N-ethyl-

N-nitrosourea (ENU)-induced RDH10 mutation results in lethality at E13.0 (Sandell et al. 2007). Importantly, the developmental anomalies observed in RDH10 knockout mouse can be rescued through RA supplementation of the maternal diet, demonstrating thus an essential role for RDH10 in retinol metabolism during the mouse development (Sandell et al. 2007).

Members of the aldehyde dehydrogenase (ALDH) family *Aldh1a1, Aldh1a2*, *Aldh1a3* and *Aldh1a4* (ALDH1, ALDH2, ALDH3 and ALDH4) are playing an important role in the retinoic acid generation. The expression of *Aldh1a2* begins during the gastrulation (Niederreither et al. 1997, Niederreither et al. 2002) and later on in development, *Aldh1a2* becomes quite widespread in the mesodermal tissues and is highly expressed in the heart (Niederreither et al. 1997, Moss et al. 1998). The expression of other *Aldh1a* genes appears later in mouse development (Mic et al. 2000, Suzuki et al. 2000, Niederreither et al. 2002, Lin et al. 2003). Mice deficient in *Alhd1a1* develop normally (Fan et al. 2003). On the other hand, *Aldh1a2* is inexpansible during embryogenesis, as *Aldh1a2* null mutants die *in utero* at midgestation under going axial rotation and exhibiting other malformation (Niederreither et al. 1999b). Embryonic development of *Aldh1a2* null mouse can be partly rescued by the maternal administration of large dose of RA, thus confirming that *Aldh1a2* plays a critical role in the synthesis of retinoic acid (Niederreither et al. 1999b). *Alhd1a3* null mutant mouse displays several malformations that result in a lethal respiratory distress syndrome at birth (Dupé et al. 2003). The targeted mutation of the *Alhd1a4* has not yet been reported.

Cytocrome P450 RA hydroxylases (CYP26) form a family of enzymes that has three known members in mammals (CYP26A1, CYP26B1and CYP26C1) that are responsible for the oxidization of retinol and RA into more polar metabolites (White et al. 2000, Abu-Abed et al. 2002, Taimi et al. 2004). All three *Cyp26* genes are expressed during mouse embryonic development in various tissues, with some spatial and temporal differences (MacLean et al. 2001, Abu-Abed et al. 2002, Tahayato et al. 2003). *Cyp26a1* null mutant mouse embryos show abnormal patterning of the hindbrain and posterior transformation of the cervical vertebrae and they die at midgestation (Sakai et al. 2001, Abu-Abed et al. 2002). Deletion of one copy of *Aldh1a2* in *Cyp26a1* deficient mice rescued many of the defects attributable to the lack of *Cyp26a1*. The rescue is the result of the reduction of RA levels to a range that is compatible with proper embryonic development (Niederreither et al. 2002). *Cyp26b1* knockout mouse embryos display craniofacial defects and malformations in limbs. Homozygous mutants die immediately after birth of respiratory distress (Yashiro et al. 2004), whereas *Cyp26c1* knockout mice do not have any obvious anatomic abnormalities during the embryogenesis (Uehara et al. 2007).

Cellular retinoid binding proteins are present in the cytoplasm of many cells (Ulven et al. 2000, Blomhoff and Blomhoff 2006). Four cellular retinol binding RBP proteins (CRBP1, CRBP2, CRBP3 and CRBP4) and two cellular retinoic acid binding CRABP proteins (CRABP1 and CRABP2) are expressed in different levels in cell type-specific and tissue-specific patterns during the mouse development (Ruberte et al. 1992, Lyn and Giguère 1994, Sapin et al. 1997, Ulven et al. 2000, Quadro et al. 2004). These proteins insure the solubility on otherwise insoluble retinoids, protect them from degradation, protect membranes from accumulation of retinoids and escort retinoids to enzymes that metabolize them (Napoli 1999). *Crbp1, Crbp2* and *Crbp4* null mutant mice appear healthy and fertile (Ghyselinck et al. 1999, Quadro et al. 2003). Mice deficient in *Crabp1* are normal (Gorry et al. 1994) and *Crabp2* null mutant mice do not exhibit any obvious phenotypic defects (Lampron et al. 1995). Moreover, *Crabp1 /Crabp2*

double null mutant mice are also normal. Thus, all of these binding proteins appear to be dispensable for mouse development (Lampron et al. 1995, Romand et al. 2000).

The retinoid receptors

In mouse, three *RAR* and three *RXR* genes have been identified, *RARα, RARβ, RARδ, RXRα, RXRβ* and *RXRδ* (Dollé et al. 1989, Johansson et al. 1997, Ghyselinck et al. 1998). Furthermore, each receptor has several isoforms as a result of differential splicing and the use of alternate promoters (Mark et al. 2006). RAR/RXRs are involved in many stages of development, among antero-posterior pattering of the somatic mesoderm and hindbrain neuroectoderm, in cardiomyocyte differentiation and in control of apoptosis (Lohnes et al. 1994, Mendelsohn et al. 1994, Moss et al. 1998, Mark et al. 2009).

The *RARα* is considered to be ubiquitously expressed in mouse embryo, whereas *RARβ* and *RARδ* are expressed in spatially and temporally restricted patterns (Dollé et al. 1989, Dollé et al. 1990, Ruberte et al. 1990, Lohnes et al. 1994, Mark et al. 2006) and there are also differential expression patterns of individual isoforms (Chambon 1996). Mice deficient in individual *RARs* and all the *RARβ* isoforms are normal (Luo et al. 1995), but disruption of all isoforms of *RARα* or *RARδ* resulted in early postnatal lethality (Lufkin et al. 1993, Lohnes et al. 1993). Compound *RAR* mutant mice show severely disrupted development, and almost all the abnormalities of the vitamin A deficiency syndrome (Lohnes et al. 1994, Mendelsohn et al. 1994, Grondona et al. 1996, Mark et al. 2009). The null mutant mice of *RXRβ* or *RXRδ* are developmentally normal (Lohnes et al. 1993, Krezel et al. 1996). However, the *RXRα* null mutant mice display cardiac malformations and die of cardiac failure around E15 (Kastner et al. 1994, Kastner et al. 1997). All the compound mutants with *RXRα* have the same phenotype, suggesting a vital role for this receptor in development (Kastner et al. 1994, Kastner et al. 1997, Clagett-Dame and DeLuca 2002).

2.4.2. Cholesterol in development of mouse embryos

Overview of cholesterol biosynthesis

Cholesterol is a key component of cell membranes and is the precursor for the synthesis of all known steroid hormones and bile acids (Simons and Ehehalt 2002). In mammalian cells, cholesterol can be synthesized from acetate precursors or taken up from the diet (Bloch 1965). The 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), which catalyzes the conversion of HMG-CoA to mevalonate, is the rate-limiting enzyme in the mevalonate pathway and the formation of squalene is the first enzymatic step which is unique to the biosynthesis of cholesterol (Goldstein and Brown 1990) (Figure 5). The mevalonate pathway produces isoprenoids that are essential for diverse cellular functions, example cholesterol synthesis (Nwokoro et al. 2001).

Mouse models of enzymes involved in cholesterol biosynthesis

The homozygous embryos of *Hmgcr* knockout are found only at the blastocyst stage (E3.5), indicating that HMG-CoA reductase is crucial for early development of the mouse embryo

(Ohashi et al. 2003), and the lethal phenotype is not completely rescued by supplementing the pregnant female mice with mevalonate (Ohashi et al. 2003). Squalene synthase (SS) is the first committing enzyme for the cholesterol biosynthetic pathway (Goldstein and Brown 1990) and it catalyzes the reductive condensation of two molecules of farnesyl diphosphate to form squalene. The homozygous mice for the disrupted *SS* die around midgestation and exhibit severe growth retardation and defects in neural tube closure (Tozawa et al. 1999). The lethal phenotype is not rescued by supplementing either squalene or cholesterol during the pregnancy (Tozawa et al. 1999). When squalene synthase is eliminated conditionally (*fdft1*$^{flox/flox}$) in myelinating glia, the homozygous animals show severe hypomyelination and some of the animals die before weaning (Fünfschilling et al. 2007). When squalene synthase is deleted embryonically in nestin-positive neuronal and glial precursors, no viable pups are born (Tronche et al. 1999).

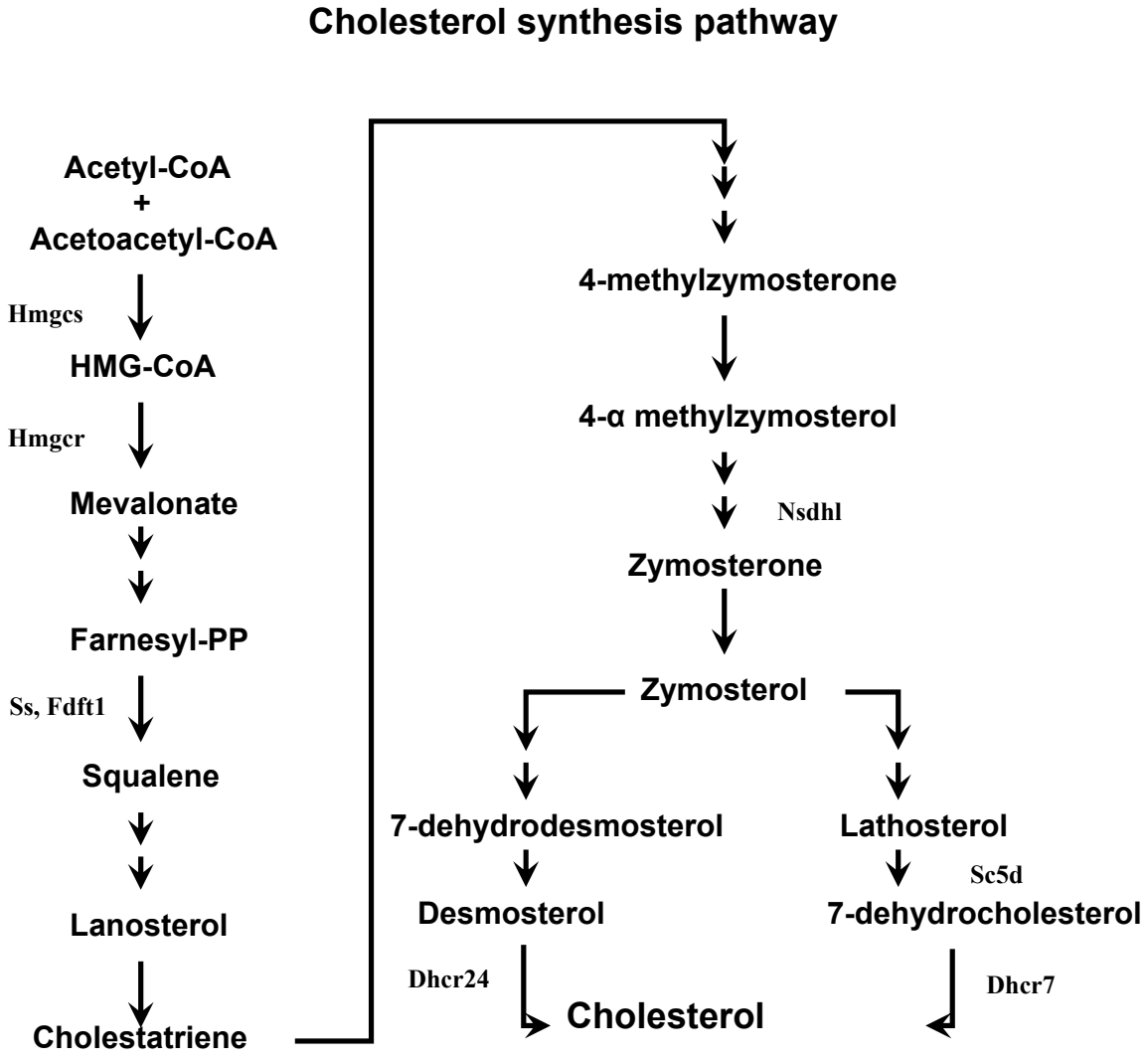


Figure 5. Simplified overview of cholesterol biosynthesis.
Abbreviations: Hmgcs, 3-hydroxy-3-methylglutaryl-CoA synthase ; Hmgcr, 3-hydroxy-3-methylglutaryl-CoA reductase; Ss and Fdft1, squalene synthase; Nsdhl, NAD(P) dependent steroid dehydrogenase-like enzyme; Sc5d, lathosterol 5-desaturase; Dhcr24, 24-dehydrocholesterol reductase; Dhcr7, 3ß-hydroxysterol delta7-reductase.

Nsdhl [NAD(P) dependent steroid dehydrogenase-like] encodes a 3ß-hydroxysterol dehydrogenase enzyme that functions as a sterol dehydrogenase involved in the removal of C-4 methyl groups from the cholesterol precursor lanosterol (Levin et al. 1996). Several mutations of mouse *Nsdhl* gene have been identified as responsible for the X-linked dominant, male lethal mouse mutations bare patches (*Bpa*) and striated (*Str*) (Phillips and Kaufman 1974, Liu et al. 1999). Male embryos for mild (*Str*) and moderate (*Bpa*) *Nsdhl* alleles die in midgestation, between E10.5-13.5 due to diminished labyrinth layer of placenta (Happle et al. 1983, Nwokoro et al. 2001, Caldas et al. 2005). Another X-linked mouse mutation Tattered *(Td)* is caused by deficiency of Δ8-Δ7 sterol isomerase (emopamil binding protein, *Ebp*) gene in mouse (Derry et al. 1999). Affected heterozygous male embryos die prenatally between E12.5 and birth, depending on the genetic background. Heterozygous male *Td* embryos are growth retarded and edematous, have a cleft palate and craniofacial anomalies, lack intestines and have limb anomalies (Derry et al. 1999).

After formation of desmosterol, two divergent pathways can be used for cholesterol synthesis, the first is from zymosterol through lathosterol to 7-dehydro-cholesterol and then to cholesterol, and the second from zymosterol to desmosterol and then to cholesterol (Goldstein and Brown 1990). Lathosterol 5-desaturase (*Sc5d*) catalyzes the conversion of lathosterol to 7-dehydrocholesterol in the cholesterol synthesis. Homozygous lathosterol 5-desaturase deficient mice die during or immediately after birth (Krakowiak et al. 2003). Mutant mice have elevated lathosterol, decreased cholesterol levels, craniofacial defects including cleft palate and micrognathia, and limb patterning defects. Many of the malformations found in *Sc5d* homozygous mice are consistent with impaired hedgehog signaling, and appear to be a result of decreased cholesterol rather than increased lathosterol (Krakowiak et al. 2003). 3ß-hydroxysterol Δ7-reductase (*Dhcr7*) converts 7-dehydrocholesterol to cholesterol (Wassif et al. 1998, Moebius et al. 1998). Two mouse models for *Dhcr7* have been generated (Wassif et al. 2001, Fitzky et al. 2001). In both of the mutations the homozygous *Dhcr7* mice show several abnormalities, decreased cholesterol levels, accumulation of 7-dehydrocholesterol and they die within hours of birth as a consequence of respiratory failure and failure to suckle (Wassif et al. 2001, Fitzky et al. 2001). The 24-dehydrocholesterol reductase (*Dhcr24*) is an enzyme catalyzing the last step of cholesterol biosynthesis, the conversion of desmosterol to cholesterol. Two knockout mouse models have been developed for *Dhcr24* (Wechsler et al. 2003, Mirza et al. 2006). Mice deficient for the *Dhcr24* with the milder phenotype are viable, have smaller stores of subcutaneous and mesenteric fat, and both males and females are infertile (Wechsler et al. 2003) whereas the other *Dhcr24* deficient mouse model in an another genetic background is showing early postnatal lethality (Mirza et al. 2006).

2.4.3. Role of fatty acids in development of mouse embryos

Overview of fatty acid biosynthesis

Fatty acid biosynthesis is essential to maintaining energy homeostasis in animals and it is characteristic to almost all living organisms. There is only some parasitic organisms, such as the human pathogen *Mycoplasma genitalium*, that are not capable of *de novo* fatty acid synthesis (Razin 1997). During the time of caloric excess, glucose is converted to pyruvate, which is

converted to citrate in the mitochondria and transported to the cytosol where ATP citrate lyase uses citrate to produce acetyl-CoA. Acetyl-CoA is thereafter carboxylated by acetyl-CoA carboxylases (ACC) to form malonyl-CoA. The fatty acid synthase (FAS) uses malonyl-CoA, acetyl-CoA and NADPH to elongate fatty acids in 2-carbon additions in the cytosol (Aprahamian et al. 1982, Wakil 1989, Chirala and Wakil 2004). In mammals, *de novo* fatty acid synthesis up to palmitic acid (16:0) is carried out in the cytosol by FAS and further FA elongation activity making long-chain and very-long-chain fatty acids (VLCFA) and unsaturation of fatty acids exist in the endoplasmic reticulum (ER) and peroxisomes (Ghesquier et al. 1987, Horie, Suzuki and Suga 1989). Elongation of VLCFAs is catalyzed by several different enzymes. In the ER, a family of enzymes, elongation of very-long-chain fatty acids (ELOVL), catalyses the first rate-limiting condensation step of the VLCFA synthesis reaction (Leonard et al. 2004, Jakobsson et al. 2006). Elongases interact with 3-ketoacyl-CoA reductase, a dehydratase, and *trans*-2,3-enoyl-CoA reductase to elongate fatty acids (Prasad et al. 1986, Moon and Horton 2003, Leonard et al. 2004).

Mammals lack the enzymes to introduce in fatty acids the double bonds at carbon atoms beyond C9. Hence, all the fatty acids containing a double bond at positions beyond C9 have to be supplied in the diet as essential fatty acids (EFA) (Gill and Valivety 1997). The n-3 and n-6 polyunsaturated fatty acids (PUFAs) must be obtained from dietary sources, predominantly in the form of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) (Innis et al. 1999, Sprecher 2000). These 18-carbon PUFAs are further processed to specific very-long-chain PUFAs (VLC-PUFAs) through a series of metabolic steps involving desaturation and subsequent chain elongation (Cook et al. 1991). The predominant n-3 PUFA end-product is docosahexaenoic acid (DHA, 22:6n-3), which is essential for visual and neurological development (Yehuda et al. 2005, Innis 2007a). DHA is particularly concentrated in the synaptic membranes and the microsomes of grey matter, as well as in the membranes of the rod outer segments of the retina (Galli and Spagnuolo 1976, Crawford 1993). Arachidonic acid (AA, 20:4n-6) is the predominant end product of n-6 PUFAs generated from linoleic acid in most of the tissues under ordinary circumstances (Sprecher 2000). AA gives rise to a whole group of 20-carbon, biologically important substances known as eicosanoids, including prostaglandins, leukotrienes and thromboxanes, which are important for the regulation of many metabolic and physiological processes, such as lipolysis, blood pressure, platelet aggregation, renal electrolyte regulation, blastocyte implantation, and activation of immune cells (Chatzipanteli et al. 1996, Wainwright et al. 1997, Tapiero et al. 2002). AA is also known to be responsible for fetal cognitive development, and diminished levels of AA can lead to impaired fetal growth, as well as impaired nerve transmission in the developing infant (Leaf et al. 1992, Crawford 1993, Birch et al. 1998, Innis et al. 1999, Birch et al. 2000, Innis 2007b).

Mouse models of fatty acid synthesis

Acetyl-CoA carboxylases 1 and 2 (ACC1 and ACC2) catalyze the synthesis of malonyl CoA, an intermediate metabolite that plays a key role in the regulation of fatty acid metabolism (Munday and Hemingway 1999). ACC1 is essential for embryonic development. *Acc* deficient embryos are undeveloped at E7.5 and they die by E8.5. This indicates that the *de novo* fatty acid synthesis is essential for embryonic development (Abu-Elheiga et al. 2005). The adipose-specific ACC1 knockout (FACC1KO) mice are growth-retarded *in utero* and also present with retarded postnatal

growth under normal diet. However, on a high fat/high carbohydrate diet FACC1KO mice become obese (Mao et al. 2009). In the liver-specific ACC1 knockout (LACC1KO) mice, the liver development is not affected, and the mice have no obvious health problems (Mao et al. 2006). The *Acc2* knockout mice are viable but smaller than the controls and have increased rates of fatty acid oxidation (Abu-Elheiga et al. 2001).

Mammalian cytosolic FAS is a complex enzyme that carries seven functional domains on a single polypeptide (Wakil 1989). FAS is widely expressed during the mouse development, and the expression is most intense in the regions known to be undergoing tissue–tissue interactions, tissue outgrowth, and progressive modeling/remodeling (Chirala et al. 2003). FAS is indispensable for normal mouse development as deletion of *Fasn* gene during the development leads to embryonic lethality (Chirala et al. 2003). The homozygous *Fasn* mice die before implantation around E4.5, and addtionally that, part of the heterozygous mutant mice also die at various stages of the development (Chirala et al. 2003).

Seven individual fatty acid elongase (*Elovl*) subtypes (*Elovl-1* to *Elovl-7*) are present in the mouse and most of them are ubiquitously expressed in mice (Jakobsson et al. 2006). Disruption of the *Elovl3* gene by homologous recombination in mouse, displayed a sparse hair coat, hyperplastic pilosebaceous system, and the hair lipid content was disturbed with exceptionally high levels of eicosanoic acid (Westerberg et al. 2004). Mice lacking a functional copy of *Elovl4* have reduced VLCFA levels that are affecting the skin ability to hold water, and ultimately results in dehydration and death shortly after birth (Raz-Prag et al. 2006, Vasireddy et al. 2006, Cameron et al. 2007). Null mutation of *Elovl5* mice showed the hepatic steatosis as accumulation of the C18 substrates and the decrease levels of the downstream products, arachidonic acid and docosahexaenoic acid (Moon et al. 2009). Mice deficient in *Elovl6* are obese and develop hepatosteatosis when being in a high-fat diet or mated to leptin-deficient ob/ob mice (Matsuzaka et al. 2007).

3 AIMS OF THE PRESENT STUDY

The present investigation was initiated to broaden our understanding of the role of the HSD17B enzymes *in vivo*. The basic question for the HSD17B enzymes is that in which metabolic pathways the enzymes play a physiological or pathopysiological role. To gain more information to this essential question we generated knockout mouse models for HSD17B 2, 7 and 12.

The specific aims of the study were:

1. The generation of knockout mouse models for hydroxysteroid (17beta) dehydrogenases 2, 7 and 12.

2. To study the role of HSD17B enzymes during the mouse embryonic development.

3. To identify novel metabolic pathways for hydroxysteroid (17beta) dehydrogenases 2, 7 and 12.

4 MATERIALS AND METHODS

4.1 Production of gene modified mouse lines

4.1.1. Cloning of targeting vectors for *Hsd17b2* and *Hsd17b7* (I, III)

In Red/ET recombineering, target DNA molecules are accurately altered by homologous recombination inside the *E.coli* that are expressing phage-derived protein pairs, either RecE/RecT from the Rac prophage, or Redα/Redβ from λ phage. RecE and Redα are 5´->3´ exonucleases, and RecT and Redβ are DNA annealing proteins (Zhang et al. 1998, Muyrers et al. 2001). A functional interaction between RecE and RecT or between Redα and Redβ is also required in order to catalyze the homologous recombination reaction. Schematic picture of ET cloning used to generate the targeting vectors (pACYC177-HSD17B2-NEO and pACYC177-HSD17B7-NEO) is presented in Figure 7. Recombinogenic *E.coli* bacteria (indicated as big ovals in figure 7) were used as a host for ET recombination steps.

A BAC clone, containing the mouse *Hsd17b2* gene was isolated by screening the mouse genomic 129/SvJ library (Genome Systems, MO, USA), by using a fragment of mouse *Hsd17b2* cDNA (GenBank accession NM_008290) as a probe. A 6.5 kb fragment of the BAC clone containing the 130 bp long exon 4 of the *Hsd17b2* gene and the flanking regions was subcloned into the pACYA177 plasmid (New England Biolabs, Inc, Ipswich, MA, USA) by ET cloning (GeneBridges, Dresden, Germany) (Zhang et al. 1998, Muyrers et al. 1999, Zhang et al. 2000). The linear targeting molecule used in subcloning was generated by polymerase chain reaction (PCR) using the pACYA177 plasmid as a template. The amplicon contained a replication origin (*ori*) and a selectable marker (*Amp*). The primers (A) 5´-**CAG AAA ATG GAA GCA GTA AAG TTA AAC TCT CCT CCA GAG ACT TCA GGA GA***G GCA GAC CTC AGC GCT* AG-3´ and (B) 5´-**TTT CAT TGT AAT ATA TTA TAT CTA GAA GTT TTG GCA ATA TTT TTA CAA T***GT GAA GAC GAA AGG GCC TC*-3´ included homology arms to the intron 3 (primer A) and 4 (primer B) of the *Hsd17b2* gene (indicated as bold). These homology regions were chosen to define the boundaries of the DNA region of interest that is present in the intact circular BAC clone. The linear PCR amplicon was electroporated into ET-competent host cells, containing the plasmid encoding recombination enzymes (E), to create recombinant plasmid pACYC177-HSD17B2 (figure 7).

The resulting plasmid pACYA177-HSD17B2 was targeted again with the ET cloning technique. Exon 4, containing the active center of the enzyme, was disrupted by inserting a neomycin resistance (*neo*) gene that was under the control of the mouse *phosphoglycerol kinase 1* (*PGK1*) promoter for eukaryotic expression and under the *tn5* promoter for bacterial expression. The neomycin dual transcription unit used was amplified from pPGK-loxP-neo plasmid (GeneBridges, Dresden, Germany) using the primers (C) 5´-**AAT CTC CCA TTC TTT CCA TAG GCA CGG TTC CAC TTC AAA TGA CAT CAG CT***G AAG CAG GGA TTC TGC AAA C*-3´ and (D) 5´-**CTC CTG TCT GAT GAT TGT TGA GAA CAT GGT TAG AGC TGC CTT TGT GGC TG***C GCG GAT TTG TCC TAC TCA GG*-3´ where homology arms to the target gene are indicated in bold, and PCR primers for pPGK-loxP- NEO are shown in italics. The linear targeting molecule was electroporated into ET-competent host cells with plasmid pACYC177-HSD17B2 to create the final targeting plasmid pACYC177-HSD17B2-

NEO (figure 7). The final targeting vector contained 1.9 and 4.6 kb long homology regions upstream and downstream of the exon 4 of mouse *Hsd17b2*.

The same cloning system was used to clone the targeting vector for *Hsd17b7*. Primers used for the subcloning step were (A) 5′- **CCG GTG ATC AAA ACC ACC TTC CGC ATC TTC CAC GCC CTG CAC CGA AGT AG***G GCA GAC CTC AGC GCT AG*-3′ and (B) 5′- **GCG GAA GGA ATT TTG ACC CAG AAT GAC TCG GTC ACT GCC GAC GGG TTG CA***T GAA GAC GAA AGG GCC TC-3′*. Primers for adding the *neomycin* resistant gene in the second round of ET cloning were (C) 5′-**ACC TGT GTT TGG CGT GTA GGA ACC TGA GCA AAG CAA GAG CTG TTC GAG AT***G AAG CAG GGA TTC TGC AAA C*-3′ and (D) 5′-**TCC ATC TGC ACG ATG CTG ACT TCG GCG GAG GGG TGA GAG GCC AGC AGG GT***G GCG GAT TTG TCC TAC TCA GG*-3′. The final targeting vector (pACYC177-HSD17B7-NEO) contained 1.7 and 5.3 kb long homology regions upstream and downstream of the exon 2 of mouse *Hsd17b7*, respectively.

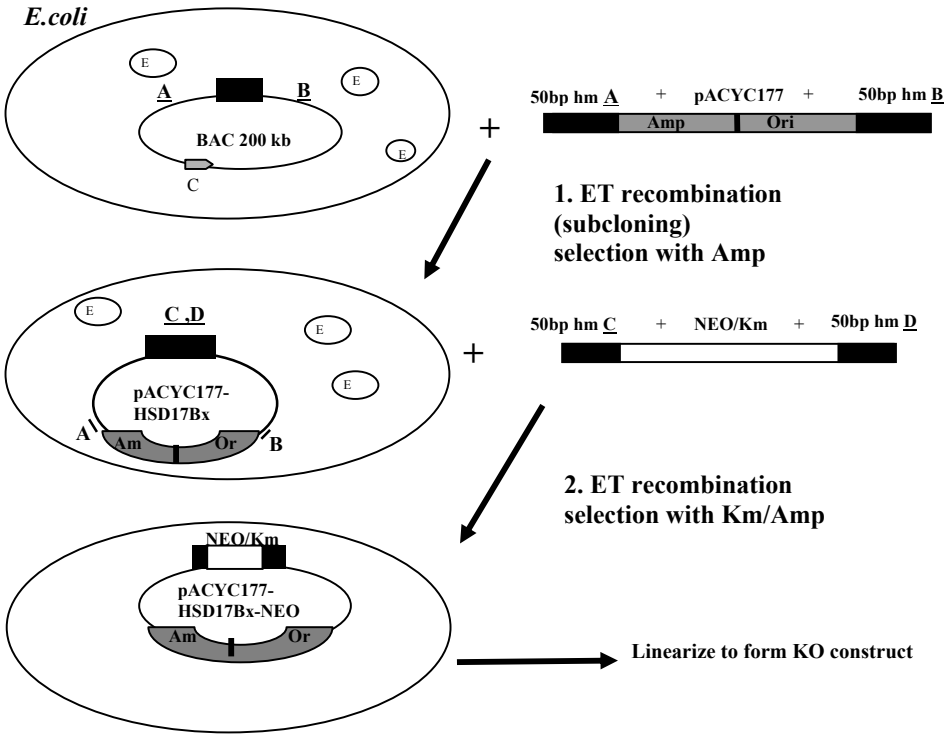


Figure 7. Simplified depiction of the ET cloning method

Recombinogenic *E.coli* bacteria (indicated as big ovals) were used as a host for ET recombination steps. Linear targeting molecules used in the cloning were generated by polymerase chain reaction (PCR). In first ET-cloning step the amplicon contained a replication origin (ori), a selectable marker (Amp) and homology regions A and B that are chosen to define the boundaries of the DNA region of interest present in intact circular BAC clone. The PCR amplicon was electroporated into an ET-competent host cells, containing the plasmid encoding recombination enzymes (E), to create recombinant plasmid pACYC177-HSD17Bx (*i.e.* HSD17B2 or HSD17B7). In the second ET-cloning step, the PCR amplicon contained a neomycin resistance cassette and the homology regions C and D. The second PCR amplicon was electroporated into ET-competent host cells with plasmid pACYC177-HSD17Bx to create the final targeting plasmid pACYC177-HSD17Bx-NEO (*i.e.* HSD17B2 or HSD17B7).

4.1.2. Establishment of the of *Hsd17b12* deficient mice (II)

Mouse lines were generated from the gene trap clone AO3OEO6 (German Gene Trap Consortium; Munich, Germany) and clone DC0186 (Sanger Institute, Hinxton, England). In order to locate the exact integration site of the gene trap, overlapping PCR amplicons were designed to cover intron 1 (clone DC0186) and 3 (clone AO3OEO6) of the *Hsd17b12* gene. Primers used for the PCR reaction are indicated in table 1. Purification and direct sequencing revealed the exact integration site of the gene trap.

4.1.3. ES cell culture and targeting (I, III)

The linearized targeting vector for *Hsd17b2* or *Hsd17b7* was introduced into AB 2.2 ES cells (Lexicon Genetics, The Woodlands, TX, USA) by electroporation (one pulse of 230V, 500 μF; Gene Pulser, Bio-Rad, CA, USA). Cells were subsequently cultured for 8 days in the presence of a selective antibiotic, G418, (0.35 mg/ml; Gibco, Invitrogen, Carlsbad, CA, USA), after which 180-250 clones were manually picked, expanded, and stored frozen at -70^{o}C. Homologous recombination was screened by PCR and Southern blot analysis.

4.1.4. Animals (I-III)

The ES cells were injected into C57BL/N6 mouse blastocysts to generate chimeric mice. Breeding of the chimeras with C57BL/N6 mice produced heterozygous animals carrying the targeted allele. All the studies were carried out in mixed (129:C57BL/6N, 1:1) genetic background. C57BL/6N mice used as blastocyst donors were obtained from Charles River Laboratories (Wilmington, MA, USA). The mice were fed with a soya free diet, and they were maintained in a specific pathogen-free stage at Central Animal Laboratory at the University of Turku. All studies carried out with the mice were approved by The Finnish ethical committee for experimental animals, complying with international guidelines on the care and use of laboratory animals.

4.1.5. Genomic PCR and Southern blot analysis (I, III)

Southern blot analyses were performed on genomic DNA to confirm homologous recombination in PCR-positive ES clones, as well as in the tissues of the HSD17B2KO and HSD17B7KO mice. Genomic DNA was digested, size-fractioned in 0.8% agarose gel and transferred onto Hybond-N+ membrane (GE Healthcare, Buckinghamshire, UK). The membranes were hybridized overnight at 42°C in UltraHyb buffer according to manufacturer's instructions (Ambion, Austin, TX, USA) with a 400 bp-long PCR fragment of the *Hsd17b2* or a 600 bp-long PCR fragment of the *Hsd17b7* gene, labeled with [α-^{32}P] dCTP (random-primed method, Promega, Madison, WI, USA). The signal was detected with Fuji Bas 5000 PhosphoImager (Fuji, Film Ltd., Tokyo, Japan).

4.1.6. DNA extraction and genotyping (I, II, III)

Genotyping of the mice was carried out with DNA extracted from yolk sacs of the embryos or ear marks of 2-week-old mice. PCR conditions and primers for genotyping are shown in Table 1.

4.2 Analysis of the phenotypes

4.2.1. Morphological, histological and immunohistochemical analyses (I, II, III)

Pregnant female mice were euthanized by cervical dislocation, and blastocysts at E3.5 (plug day = E0.5), and embryos at E6.5-E11.5, E15.5 and E17.5 together with the yolk sack were dissected free from the uterine tissue, placed in ice-cold PBS, and macroscopic abnormalities were evaluated. For histological analysis, the embryos, the placentas and various organs from newborn mice were weighed, fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 24-48 h at 4°C, and embedded in paraffin. The embedded tissues and whole embryos were cut in 4 or 5 µm thick sections, and stained with Harri´s hematoxylin and eosin (BDH Ltd., Poole, UK). For bone and cartilage analyses, newborn mice were euthanized, skinned, eviscerated and fixed with 100 % EtOH for 24 h. The cartilages were stained with 0.3% Alcian Blue and the bones were counterstained in 0.1% Alizarin Red solution. The gender of the newborns was determined by the presence of the ovaries or the testes.

Immunohistochemical staining was performed to examine the GATA-4 expression in cardiomyocytes in 5 µm thick sections. Novolink™ Polymer Detection System Kit (Novocastra™, Benton Lane, UK) was used with a rabbit polyclonal anti-GATA-4 IgG (dilution 1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions.

Apoptosis in E17.5 placentas was studied using ApopTag Peroxidase *In situ* Oligo Ligation (ISOL) Apoptosis Detection Kit (Chemicon International, Inc, Temecula, CA, USA) according to the manufacturer's instructions. The activation of caspase 3 from E8.5-10.5 embryos was studied in order to analyze the rate of apoptosis in embryos. After deparaffinization and rehydration, the sections were boiled in 0.01 M sodium citrate (pH 6.0) for 15 min and cooled slowly to room temperature. The sections were then exposed to 3% hydrogen peroxide for 20 minutes and incubated overnight with cleaved caspase 3 (Asp175) antibody (Cell Signaling Technology, Beverly, MA, USA). The sections were afterwards incubated with labelled polymer anti-rabbit (Dako EnVision+ System-HRP; Dako, CA, USA), and the color reaction was performed with DAB substrate (Dako Liquid DAB+ Substrate Chromogen system; Dako). The sections were counterstained with hematoxylin and dehydrated in ethanol and xylene before mounting with Pertex mounting solution (Histolab, Gothenburg, Sweden).

4.2.10. Hormone measurements (I)

Placentas were homogenized in 1 ml PBS for the analysis of the intra-tissular estradiol and testosterone concentrations. The steroids were extracted from the homogenates or from amniotic fluid by using diethyl ether, the organic phase was evaporated into dryness, and the steroids were

solubilized in PBS. Estradiol and progesterone concentrations were measured by Delfia kits following the manufacturer's protocol (PerkinElmer wallac, Turku, Finland). Measurements of testosterone concentrations were performed by a radioimmunoassay, as described previously (Huhtaniemi, Nikula and Rannikko 1985).

4.2.11. Steroid treatments (I)

On pregnancy day 7.5, HE and WT female mice (mated with HE and WT males, respectively) were implanted with pellets containing either an antiestrogen, ICI 182,780 (1.5 mg/12 d release, Innovative Research of America, Sarasota, FL, USA) or progesterone (5.0 mg / 21 d release, Innovative Research of America). Control mice were implanted with placebo pellet. The females were euthanized on pregnancy day 17.5, and living embryos of various genotypes were determined by the presence of a beating heart. Amniotic fluids and placentas were collected for further analyses.

4.2.3. β-Galactosidase staining (II)

E7.5-E9.5 embryos were studied to visualize the expression of the fusion gene resulting of the gene-trap insertion. The embryo**s** were dissected, washed in PBS and fixed in PBS (pH7.3) containing 0.2% gluteraldehyde, 2 mM $MgCl_2$ and 5 mM EGTA for 30 min. Thereafter, they were washed overnight in PBS containing 0.02% NP-40, 2 mM $MgCl_2$ and 0.01% Na-deoxycholate, and incubated overnight at 37^{o}C with β-galactosidase substrate (X-Gal, 1 mg/ml; Fermentas, Glen Burnie, MD, USA) in washing buffer, supplemented with 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide. The stained embryos were further fixed in 4% paraformaldehyde at RT for 20 minutes, washed with PBS, and photographed.

4.2.7. *In vitro* blastocyst culture (II)

Heterozygous HSD17B12HE males and females were mated and E3.5 embryos were collected by flushing the uterus of the pregnant females. Blastocysts were individually cultured in ES cell medium without LIF on gelatin or feeder cells (Gibco, Invirogen) in 5% CO_2 at 37°C. The growth and expansion of the ICM was followed for 7 days, after which the cultured cells were genotyped.

4.2.9. Fatty acid measurements (II)

ES cells where isolated from E3.5 blastocysts and cultured *in vitro* (Bryja et al. 2006). Lipids were extracted by modified Folch method (Folch 1957). Briefly, cells of a confluent 10-cm cell culture dish were pelleted and mixed with an internal standard (1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine in chloroform). A 200 µl aliquot of 0.88% potassium chloride and 1.5 ml of methanol was added. The samples were mixed, and 3 ml of chloroform was added. Thereafter, nitrogen was added to the tubes to prevent lipid oxidation, and the tubes were capped. The

sample tubes were mixed by vortexing for 1 min, followed by shaking for 1 hr. After mixing, 915 µl of 0.88% potassium chloride was added and the tubes were carefully mixed and centrifuged at 1300 x g for 5 min. The lower phase was collected to a tared glass vial, evaporated to dryness, and the vial was weighed. The extracted lipids were dissolved in chloroform and stored at -80 °C until the preparation of fatty acid methyl esters. Fatty acid methyl esters (FAMEs) were prepared from the lipid extracts at 92°C by boron trifluoride-catalyzed transesterification (Morrison 1964, Agren et al. 1992) and then the solvent was evaporated under nitrogen. The esters were dissolved in hexane and the esterified lipids were analyzed by gas chromatography with flame ionization detection (GC-FID) (PerkinElmer AutoSystem, Shelton, CT, USA) using a DB-23 column (60 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). The linoleic acid (LA) and arachidonic acid (AA) were identified with help of FAME mixtures 68D and 37 obtained from Nu-Check Prep Inc. (Elysian, MN, USA) and Supelco (Bellefonte, PA, USA), respectively.

4.2.4. *In situ* hybridization (I, III)

In situ hybridization was performed on 4 µm paraffin-embedded tissue sections with 1 µg/ml antisense and sense digoxigenin-labeled riboprobes generated from a linearized plasmid for *Tpbpa* and *PL-1* cDNA and transcribed with T7 and SP6 RNA polymerases (Roche Molecular Biochemicals, Basel, Switzerland). Tissue sections were deparaffined and incubated with proteinase K (2 mg/ml) for 10 min, then fixed in 0.4% paraformaldehyde in PBS for 20 min, and finally treated with 2 mg/ml glycine for 15 min. Prehybridization was carried out in 1× SSC in 50% deionized formamide for 1 h. Hybridization was performed at 55 C overnight with a DIG-labeled RNA probe in 20 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, 0.3 M NaCl, 1 mg *Escherichia coli* tRNA, 50% deionized formamide, 1× Denhardt's reagent, and 10% dextran sulfate. Tissue sections were washed with 4× SSC in 50% deionized formamide at RT for 5min, 2× SSC, and 1× SSC at 37 C for 2x15 min each, and 0.5× SSC at 37 C for 10 min. Slides were incubated in 0.1 M Tris–HCl, pH 7.4, at room temperature for 1h followed by 1h incubation with 5 mg/ml blocking reagent (Roche Molecular Biochemicals) at RT and an o/n incubation with 1:500 alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals) at room temperature. Slides were washed and stained using nitro blue tetraxolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) stock solution (Roche Molecular Biochemicals), and counterstained with 0.5% nuclear Fast Red.

To study the mRNA expression of *Shh* and *Ptch1* by whole mount *in situ* hybridization, E8.5 embryos were dissected as described above and fixed in 4% paraformaldehyde (PFA) overnight at 4ºC. After fixation, the embryos were washed in PBT and dehydrated with methanol. Wholemount *in situ* hybridization with *Shh* [gift from Dr. Thomas Edlund, Umeå Centre for Molecular Medicine (UCMM), Umeå, Sweden] and *Ptchl* (Kim et al. 1998) probes was performed with InSituPro automate (Intavis, Cologne, Germany).

4.2.5. RNA analyses (I, II, III)

For analyzing the mRNA expression of *PL-1, Esx1* from E17.5 placentas or *Hmgcr*, *Cyp51*, *Nsdhl*, *Hsd17b7*, *Dhcr24*, *Dhcr7*, *Shh* and *Ptch1, Smo, Tnn2,Ankrd1* and *Lefty2* from E9.5 whole embryos, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA samples were DNAse-treated (DNase I Amplification Grade Kit, Invitrogen, Paisley, UK), and used for either standard RT-PCR or quantitative RT-PCR analysis. Quantitative RT-PCR was performed utilizing DyNAmo™ HS qRT-PCR kit for two-step SYBR® Green qRT-PCR (Finnzymes Oy, Espoo, Finland). The results were normalized to mouse *β-actin* and *Ppia* expression in the samples. Primer sequences for PCR, and annealing temperatures used are listed in Table 1.

For Northern blot analysis, total RNA (10 μg/lane) from liver and kidney was resolved on an agarose gel, and the RNA was transferred onto Hybond-N+ membrane (GE Healthcare). The membranes were hybridized overnight at 42°C in UltraHyb buffer according to manufacturer's instructions (Ambion) with an [α-^{32}P] CTP-labelled 734 bp-long cDNA fragment of mouse *Hsd17b2*. The nonspecific signal was removed by a series of washes with 2× SSC and 0.5% SDS. Hybridization results were visualized by autoradiography using a Fuji Bas 5000 PhosphoImager (Fuji).

4.2.6. Tetraploid aggregation (III)

To generate tetraploid-diploid aggregates, two-cell-stage embryos were obtained from the oviducts of female mice expressing green fluorescent protein (GFP) under the chicken *beta-actin* promoter (Hadjantonakis et al. 1998) at E1.5, placed in 0.3 M mannitol (Sigma Aldrich, St. Louis, MO, USA) and aligned in a 5 V field of alternating current. Embryos were then fused with two 36 μs of 160 V direct current pulses using a 1 mm electrode and an electrofusion apparatus (BLS, Budabest, Hungary) resulting in tetraploid embryos. The fused embryos were cultured overnight in order to monitor the viability and normal cleavage of the embryos. Superovulated heterozygous HSD17B7KO females were mated with heterozygous males, and diploid E2.5 embryos at six- to eight-cell stage were isolated from the oviducts of the pregnant females. Thereafter, the zona pellucida of both the diploid and tetraploid embryos was removed and two tetraploid embryos were aggregated with one diploid embryo (Nagy et al. 1993, Tanaka et al. 2001). After 24-26 h incubation, successfully aggregated embryos formed blastocysts which were transferred to the uterine horns of day 0.5 pseudopregnant NMRI female recipients. Chimeric embryos were dissected and photographed at E10.5-11.5.

4.2.8. Sterol measurements (III)

Embryos at the age of E10.5 were weighed and homogenized in chloroform-methanol with Ultra Turrax after addition of internal standards (5-alfa cholestane and epicoprostanol). Sterols were then saponified, extracted and subjected to Gas-Liquid Chromatography (GLC) analysis as trimethylsilyl-derivatives by using a 50 m long Ultra 2 capillary column (Agilent Technologies, Wilmington, DE, USA) as described previously (Miettinen 1970, Miettinen 1988, Miettinen et al. 1990). The GLC procedure measures the concentrations of squalene, cholesterol, cholestanol,

cholestenol, desmosterol, lathosterol, campesterol, lanosterol, sitosterol, sitostanol and avenasterol in the increasing order of retention time. The coefficients of variations (CVs) for the different sterols were between 1.8 and 6.0%.

4.2.2. Whole mount immunofluorescence (III)

E9.5 embryos were dissected in Dulbecco's PBS and fixed for 1 hour in 4% PFA. All washes and antibody incubations were performed at 4ºC with agitation. Embryos were first washed with phosphate buffer + 0.1% tween (PBT) and incubated with a blocking solution (PBT + 1 % heat inactivated fetal calf serum + 10% goat serum) for 2 hours. The primary and secondary antibodies were incubated overnight in blocking solution, and antibody washes were performed with PBT + 1% fetal calf serum (FCS). Monoclonal rat anti-mouse PECAM-1 (MEC 13.3; Pharmingen, CA, USA) was incubated at 1:100 and secondary antibody goat anti-rat Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was incubated at 1:400 dilution.

4.2.12. Statistical analyses (I, II, III)

Statistical analyses were performed using the SigmaStat program (SYSTAT Software Inc., San Jose CA, USA). The results for body and organ weights were analyzed by Student's *t*-test or Mann-Whitney Rank Sum test with a limit of significance set at $P < 0.05$. One way ANOVA was used to analyze the data from antiestrogen and progesterone treatment study.

TABLE 1. Primers used

Gene	Primer sequence	Lenght	Tm
Genotyping primers			
HSD17B2KO			
Wt1	5′-GGCACGGTTCCACTTCAAAT-3′	173 bp	59
Wt2	5′-CGGGTTTGATGGCACAACTT-3′		
KO	5′-GACCCATGGCGATGCCTGCTTG-3′	420 bp	60
HSD17B7KO			
Wt1	5′-GCATTGGGCTAGCCCTTTG-3′		
Wt2	5′-GCTTGACTTCCTCTGCACC-3′		
KO′	5′-GCAGGGCTTCCCAACCTTAC-3′		
HSD17B12KO			
Wt1	5′-AATCGACAAATGGGACCTAATG-3′	1100 bp	60
Wt2	5′-AGCCAATTACAGAGAGCAGAGG-3′		
KO	5′-CGTGTCCTACAACACACAATACAACC-3′	420 bp	60
Primers and annealing temperatures used for localisation of HSD17B12 genetrap			
Local1_S	5′-ACAACAACAACGAAAACCACAG-3′		60
Local2_S	5′-TTCCAGAAGAATAGAGGGACCA-3′		
Local3_S	5′-CCTGACATTCACCATTCACTCA-3′		
Local4_S	5′-AAGGCGATTTTGTTGTTGTTTT-3		
Local5_S	5′-AGTGGAGTTCCCTTCAAGACTG-3′		
Local6_S	5′-CACTCTACCCCTTTCCTCATTG-3′		
Local7_S	5′-GGGGAGGTAGGAACCACTTAAC-3		
Local8_S	5′-TGGCTTCAGGCCTAGTTAAGAG-3′		
Local9_S	5′-TAGCTGACTGTGAGCATCCACT-3′		
Local10_S	5′-AAACCATCCAGTGGAAGAAAGA-5′		
Local11_S	5′-GCCTGCTGGGTTACAGTCTATC-3′		
Placenta layer spesific genes			
Tpbpa,	5′-GAGACATGACTCCTACAATCTTCC-3′		
	5′-TTGCCTAACTTCATACTGCTGTCC-3′	407 bp	60
Pl-1	5′-TCCGCAGGAATGCAATTGTTGCTG-3′		
	5′-GGGAAAGCATTACAAGTCTGGTTC-3′	711 bp	60
Esx1,	5′-TTGCTAGAGTGGAGCTTGCC-3′		
	5′-GCCTAAATGGTGGAGGCATTC-3′	279 bp	60
bAkt1	5’-CGTGGGCCGCCCTAGGCACCA-3’		
	5’-TTGGCCTTAGGGTTCAGGGGG-3’	243bp	54
Other genes used			
Hmgcr	5’-TGGAGATCATGTGCTGCTTC-3’		
	5’-GCGACTATGAGCGTGAACAA-3’	248bp	60
Cyp51	5’-ATTGCCCGTATTCTGTGCTC-3’		
	5’-TGGCCTCAAATTCTCAATCC-3’	174bp	60
Nsdhl	5’-GATGCCAACGACCCTAAGAA-3’		
	5’-AACCACATTCTCCACGAAGG-3’	160bp	60
Hsd17b7	5’-TGGCAGAAGACGATGACCTC-3’		
	5’-GGCAGGATTCCAGCATTCAG-3’	217bp	60
Dhcr24	5’-CATCTTCCGCTACCTCTTCG-3’		
	5’-CTCTGCTTCATCTCCCTTGG-3’	259bp	60
Dhcr7	5’-CGCTCCCAAAGTCAAGAGTC-3’		
	5’-GTGTCTTGGCCCAAATGTCT-3’	233bp	60
Shh	5’-GGCAGATATGAAGGGAAGAT-3’		
	5’-ACTGCTCGACCCTCATAGTG-3’	260bp	57
Ptch1	5’-GGCTGAGAGCGAAGTTTCAG-3’		
	5’-GCCTCTGTGGTCAGAACATT-3’	289bp	60
Ppia	5’-CATCCTAAAGCATACAGGTCCTG-3’		
	5’-TCCATGGCTTCCAGAATGTT-3’	165bp	63
Hsd17b12	5’-TTTGTTATGGGCAGATTCAGTG-3’		
	5’-TTGGCCCACTTACCTCTACATT-3’	261bp	60

5 RESULTS

5.1. Generation of knockout mice (I, II, III)

To investigate the function of *Hsd17b2* and *Hsd17b7* genes *in vivo*, we generated targeted mutations for the genes by homologous recombination using embryonic stem (ES) cell technology. The genetic manipulation of *Hsd17b2* (HSD17B2KO) and *Hsd17b7* (HSD17B7KO) was designed to result in lack of active enzyme expression. The structures of genes were disrupted by replacing part of exon 4 in *Hsd17b2* and a part of exon 2 in *Hsd17b7* with a PGK-*neo* cassette. PCR and Southern blot analysis was used to detect the appropriate targeting events.

In generation of mice lacking the functional *Hsd17b12* gene (HSD17B12KO), an embryonic stem (ES) cell line (AO3OEO6) containing a gene trap vector inserted in the *Hsd17b12* gene on mouse chromosome 2 was used. The gene trap vector AO3OEO6 was integrated into the intron between exons 3 and 4. The insertion, thus, caused the expression of a fusion protein including the first three exons of *Hsd17b12* fused with the lacZ reporter gene. Another ES cell line (DC0186) was used to confirm that the disruption of HSD17B12 caused the phenotype observed. The gene trap vector DC0186 was integrated into the intron between exons 1 and 2. The insertion, thus, caused the expression of a fusion protein including the first exon of *Hsd17b12* fused with the lacZ reporter gene. Same results where observed with the two different gene traps used.

5.2. Mouse embryonic development

5.2.1. Expression of *Hsd17b12* in early mouse development (III)

To investigate the expression of *Hsd17b12* gene in mouse embryos, the fusion gene product produced by the mutated allele was followed by ß-galactosidase staining. Low level of *Hsd17b12* expression was detected as early as at E7.5 in the embryonic ectoderm and in the yolk sac. At E8.5, the expression was observed in the open neural folds at both the anterior and posterior regions of the body, with the highest level of expression in the fore-, mid- and hindbrain, and in the tail bud. From E8.5, the expression was also observed in the yolk sac and in the ectoplacental cone. From E9.5 to E14.5, *Hsd17b12* expression was specially noted along the rostrocaudal axis, with the strongest staining detected along the neural tube (Figure 8).

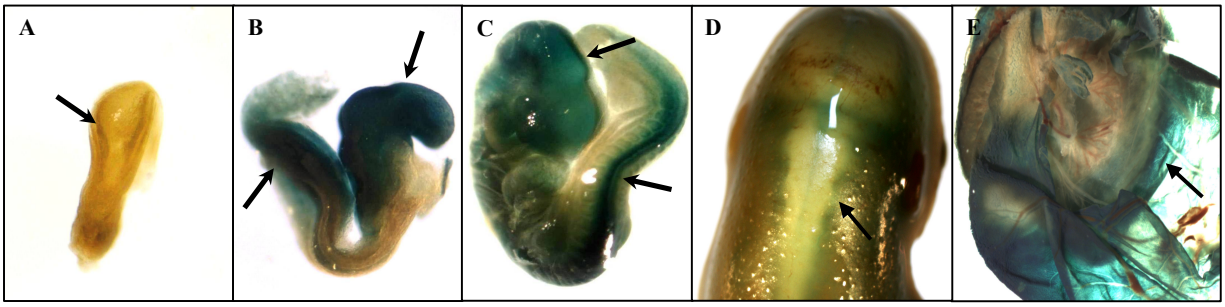


Figure 8. **The expression of the *Hsd17b12* during embryonic development.** **(A)** At E7.5, expression of *Hsd17b12* was detected in embryonic ectoderm (arrow). **(B)** At E8.5, the expression was observed in the open neural folds at both the anterior and posterior regions of the body (Black arrows). **(C)** At E9.5 and **(D)** at E14.5, the strongest expression was observed in the dorsal neural tube (black arrows). **(E)** At E8.5, and thereafter, the *Hsd17b12* expression is seen in the yolk sac.

5.2.2. *Hsd17b2, 7* and *12* deficiency in mouse embryo (I, II, III)

After genotyping 178 mice from heterozygous intercrosses, the number of HSD17B2KO mice identified was lower (6%, 11 out of 178) than expected (25%). The result indicated that the loss of *Hsd17b2* produced a recessive phenotype, resulting into lethality in close to 76% of the HSD17B2KO mice during embryonic development or during the first postnatal days. At the blastocyst stage (n=35) the percentages of homozygous (HO), heterozygous (HE) and wild WT genotypes were 26%, 48% and 26%, respectively. However, the percentage of live HSD17B2KO embryos reduced to 14% at E11.5, to 10% at E15.5, and to 6% at E17.5. The results indicated a loss of HSD17B2KO embryos at E9-12 onwards, and thus, markedly reduced number of HSD17B2KO embryos survived until birth. There was no evidence for gender influence in survival of the HSD17B2KO mice.

No viable HSD17B7KO or HSD17B12KO pups were identified among the mice genotyped at the age of 2 weeks. This indicated that the HSD17B7KO and HSD17B12KO mice died *in utero*. At E7.5 to E8.5, both knockout embryos were detected with the expected Mendelian frequency but reabsorptions and empty conceptuses were observed from this stage onward, and no live homozygous embryos were found beyond E9.5 for HSD17B12KO and beyond E10.5 for HSD17B7KO. All the HSD17B7KO and HSD17B12KO mutants were developmentally retarded.

At E9.5, the forebrain of the HSD17B7KO mice was smaller, and it did not develop further. The developmental malformation observed in the KO brain resulted in a defect in the development of hemispheres of the front brain. Staining for the caspase 3 indicated increased apoptosis in the neural tube of the HSD17B7KO mice at E9.5 as compared with the WT brain, and the amount of apoptosis was further increased in KO embryos at E10.5. In addition to the neural tube, apoptosis was observed in the dorsal root ganglia and trigeminal nerve. By E11.5, all the homozygous mutant embryos were necrotic with undifferentiated front brain and visible branchial arches, typically observable only at earlier developmental stages (Figure 9).

In addition to the brain defects in the HSD17B7KO embryos, the cardiac failure was observed as a pericardial effusion visible as a large transparent liquid-filled sac surrounding the developing heart (figure 9). The early stages of the heart development appeared to be normal, and the looping of the heart was normal. Accordingly, the mRNA expression of the genes related to heart development, *Tnnt2*, *Ankrd1* and *Lefty2*, was unaltered at E9.5. However, at E10.5, the heart was dilated and the thickness of the cardiac muscle in the HSD17B7KO embryos was markedly reduced as compared with that observed in the WT mice. Histological studies together with immunohistochemical staining for GATA-4 revealed that HSD17B7KO embryos had a reduced number of myocardial cells and a thin pericardium. The septum of the atrium was also defected in the KO mice. Surprisingly, immunohistochemical analysis with caspase 3 antibody showed no apoptosis in the hearts of the HSD17B7KO embryos, suggesting a proliferation defect in the cardiomyocytes.

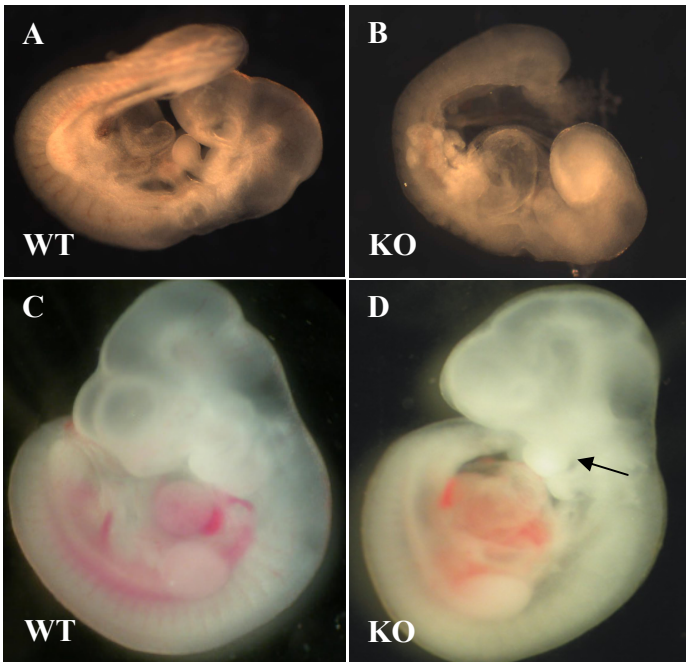


Figure 9. **Macroscopic phenotype of the HSD17B7KO embryos**
(A) The WT embryo at E9.5. **(B)** At E9.5 the HSD17B7KO embryos showed undifferentiated front brain. **(C)** The The WT embryo at E10.5. **(D)** Undifferentiated branchial arches (black arrow shows visible branchial arch in picture) and pericardial effusion was evident in HSD17B7KO embryos at E10.5.

At E7.5, HSD17B12KO embryos were smaller in size and had a poorly defined boundary between the embryonic and extraembryonic regions. In contrast, the WT embryos had a proper ectoplacental cone, primitive streak, and headfold. Furthermore, 90% of the KO conceptuses exhibited an enhanced accumulation of blood sinus at the antimesometrial pole. At E8.5, the WT embryos exhibited a normal yolk sac structure, while the KO embryos almost completely lacked the extraembryonic structures. At E8.5, most of the KO embryos were developmentally retarded. KO embryos had developed a headfold and a primordial neural tube but lacked somites. Also at E9.5, the HSD17B12KO embryos could be distinguished from their WT littermates by an abnormal yolk sac structure showing a condensed mass of cells without detectable blood vessels (Figure 10).

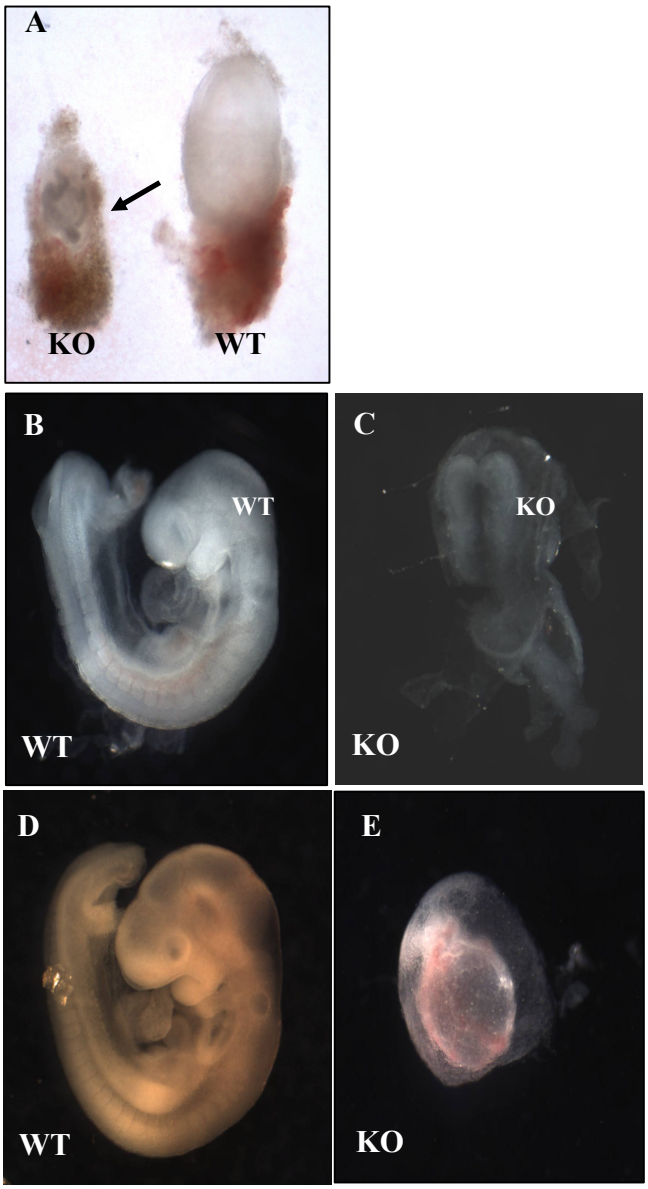


Figure 10. Morphology of HSD17B12KO embryos

(A) At E7.5, the KO embryos were smaller than their WT littermates, and they had poorly defined boundary between the embryonic and extra embryonic region (arrow). (**B**) A WT embryo at E8.5. **(C)** At E8.5 the KO embryos were notably smaller and developmentally retarded as compared with the WT embryos. **(D)** WT embryo at E9.5. **(E)** KO embryos at E9.5 were encased in spherical structure formed by the yolk sac and the amnion, and completely void of normal morphology.

Reduced size for the HSD17B12KO embryos as compared with the WT embryos was also observed in histological analysis at the age of E6.5. However, the egg cylinder structure of the HSD17B12KO embryos was normal. The WT embryos had a well-defined anterior-posterior axis with a proper formation of the epiblast, and typical structures for the mesodermal layer, ectoplacental cone, chorion and amnion at E7.5. Analysis of the KO embryos at this age revealed that the embryos had initiated gastrulation, indicated by a proper formation of the primitive streak. However, the embryonic, and particularly the extraembryonic, ectoderm of the HSD17B12KO embryos presented with an increased mass of cells, and the embryonic cavities were not properly formed. In addition, the chorion was undetectable and also the allantois showed an increased mass of cells. At E8.5, the development of the KO embryos was severely disrupted. The WT littermates were developed with proper headfolds, neural tube and

heart, whereas in KO embryos, the embryonic portion void of all the normal morphological structures and also the extra embryonic structures in the KO mice were partly disorganized (Figure11).

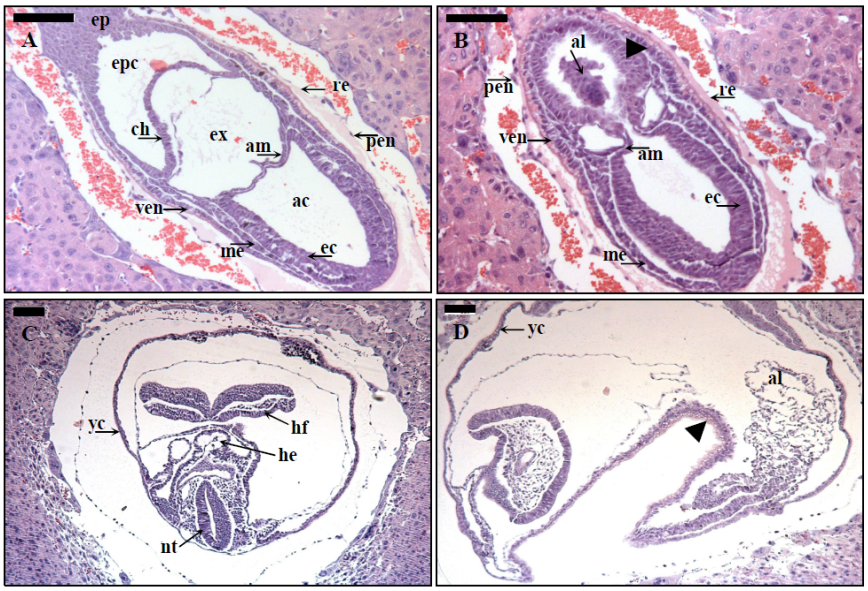


Figure 11. Histological analysis of HSD17B12KO embryos. (A) HE staining of a WT embryo at E7.5 **(B)**. HSD17B12KO embryos at E7.5. Abnormal formations of embryonic cavities were observed in the KO embryos at E7.5. Only the amniotic cavity was detectable, and there was increased cell mass in the ectoplacental region (arrow head in panel B). **(C)** WT embryo at E8.5 **(D)** At E8.5, the KO embryos were more severely affected, and they had no structures typical to those observed in the WT embryos. Abbreviations: ac, amniotic cavity; am, amnion; ex, exocoelomic cavity; ch, chorion; epc, ectoplacental cavity; ep, ectoplacental cone; al, allantois; ec, ectoderm; me, mesoderm; en, endoderm; ven, visceral endoderm; pen, parietal endoderm; re, Reichert's membrane; yc, yolk sac; nt, neural tube; hf, head folds; he, heart. Bars 100µm

5.2.3. *Hsd17b2, 7* and *12* deficiency in mouse extraembryonic tissues (I, II, III)

At the age of E12.5, the HSD17B2KO and WT placentas were identical in their histological appearance. However, at late gestation (E17.5) the placentas of HSD17B2KO were smaller in size as compared with the HE or WT mice placentas (WT, 77.4 ±8.12 mg; HE, 78.2 ± 10.40 mg; HO, 66.9 ± 7.11 mg, $p<0,05$) and the HSD17B2KO placentas showed variable structural abnormalities in all tree major layers: the decidua, the junctional and the labyrinth. Liquid-filled cysts were observed in the junctional region and decidua layer. Volume of the junctional layer, which includes spongiotrophoblast cells, glycogen cells and trophoblast giant cells, was increased and the labyrinthine layer was disorganized. Extensive trophoblast aggregates were present in the labyrinthine region, and the border between the junctional and labyrinth layer was disrupted (Figure 12).

In situ hybridization revealed the increased expression of a spongiotrophoblast-specific marker, *Tpbpa* (Lescisin, Varmuza and Rossant 1988), that indicated an expansion of the junctional layer in the KO mice. The expression of *PL-1*, a, marker gene for trophoblast giant cells was reduced. Furthermore, the expression of placental labyrinth marker gene *Esx1* was decreased in the HSD17B2KO placenta as compared with the WT. These results confirmed that both the junctional and labyrinth layers were affected by the absence of functional HSD17B2.

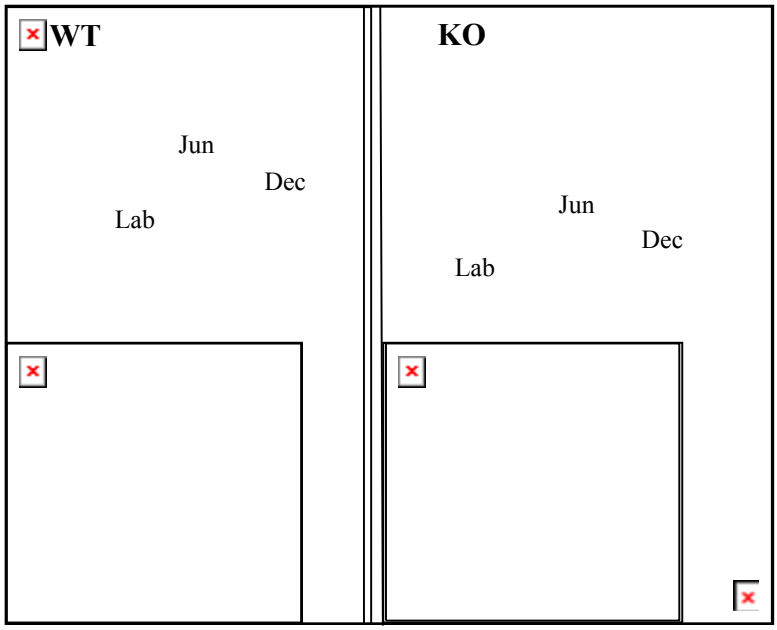


Figure 12. HSD17B2KO mice placentas at the age of E17.5.
Hematoxylin and eosin-stained sections of placentas of the wild-type and HSD17B2KO littermates. The normal appearance of the labyrinthine zone (Lab), junction zone (Jun) and decidua (Dec) observed in wild-type placenta was disrupted in HSD17B2KO placenta. The junctiol region which includes spongiotrophoblast cells, glycogen cells and trophoblast giant cells, was increased and unorganized.

The yolk sac of the HSD17B7KO mice lacked the proper vascularization (Figure 13). Although mesodermal, endodermal and hematopoietic cells were all detected by the histological analysis of the visceral yolk sac, no organized vessels were observed in the KO yolk sac. In addition to extraembryonic structures, the vascularization of the embryo was studied further with immunohistochemical detection of PECAM-1 expression. The staining indicated that the complexity of the vasculature was reduced in the HSD17B7KO embryos, particularly in the head capillary plexus and branchial arches. Based on the pattern of PECAM-1 staining, the lack of proper vasculature is likely to be due to a developmental delay in the KO embryos, rather than a defect in the vascular structure *per se*.

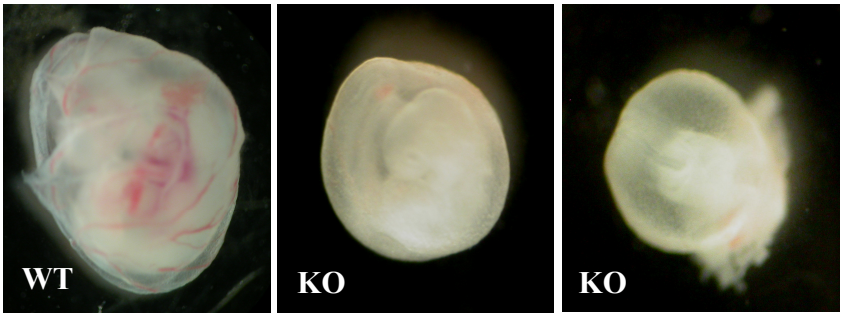


Figure 13. Vascular failure in the HSD17B7KO yolk sac.
As compared to wild type littermates, HSD17B7KO mice exhibited severely disrupted vascular networks in the yolk sac.

As a general tool, tetraploid aggregation can be used to rescue embryonic lethality caused by defects in extraembryonic tissues like the placenta, trophoblast or yolk sac. Tetraploid chimeric HSD17B7KO embryos were generated by aggregating WT tetraploid morulae with diploid morula stage embryos obtained from HSD17B7KO heterozygous matings. This approach takes advantage of the developmental bias of cells from tetraploid embryos to contribute preferentially to extraembryonic structures, whereas diploid embryos generate mostly embryonic tissues (Nagy et al. 1993). The tetraploid aggregation studies did not rescue the defects of the HSD17B7KO embryos, and the *Hsd17b7* mutant embryos presented with similar neural and cardiovascular defects than those obtained from heterozygous matings. This indicates that the phenotype observed in the HSD17B7KO embryos was not due to the defects in extraembryonic tissue.

5.3. Structural abnormalities of newborn HSD17B2KO mice (I)

Newborn HSD17B2KO mice were distinguishably smaller in size than their littermates. The mean body weight of KO newborns was 23% smaller as compared with their WT or HE littermates (WT, 1.3 ± 0.14 g, n=34; KO, 1.0 ± 0.14 g, n= 9; $p < 0.002$). A significant reduction in organ weight was found only for the kidney (WT, 6.9 mg ± 0.99; KO, 4.6 ± 0.29 mg; $p < 0.001$) and brain (WT, 90.9 ± 5.48 mg; KO, 71.1 ± 8.56 mg; $p < 0.001$), However, when compared to body weight, none of the organ weights in the KO mice were statistically different when compared to WT littermates. Although the HSD17B2KO mice were significantly smaller in size, the bone and cartilage structure showed no obvious morphological abnormalities.

In histological analysis, the newborn HSD17B2KO mice showed no obvious abnormalities in tissues other than kidneys and brain. The brains of HSD17B2KO mice were histologically abnormal, with enlarged lateral ventricle at one side, and the thalamus of some HSD17B2KO mice was also enlarged. In the developing cerebral cortex, an altered layering of cells was detected in HSD17B2KO mice together with abnormal laminar organization and increased cellular density. Hyperplasia and disrupted cellular strand was evident in most cases in the occipital and parietal cortical areas. The number of small cells in all the cortical layers were increased, however, the increased cortical cell mass was not due to decrease by apoptosis, as there was no difference in number of the apoptotic cells between WT and HSD17B2KO brains.

In the HSD17B2KO mice born alive, the enlargement of ventricles progressively increased during the postnatal age resulting in hydrocephalus with markedly enlarged bilateral ventricles covered with only a thin cortical mantel.

The newborn HSD17B2KO mice frequently had only one kidney, indicating unilateral degeneration of the whole kidney while the ureter was still detected. The remnant structures of the degenerated kidney were in most cases liquid-filled. The remaining kidney was pale in color. The weight of the remaining kidney of the newborn HSD17B2KO mice was also significantly reduced as compared with the WT littermates ($p<0.001$). In 1-day-old HSD17B2KO mice the developing cortical layer, where the new tubules form, was normal while the tubules in the kidney medulla and the renal pelvis were enlarged. The stromal cells at the developing medullary region had differentiated normally but were disorganized (Figure 14).

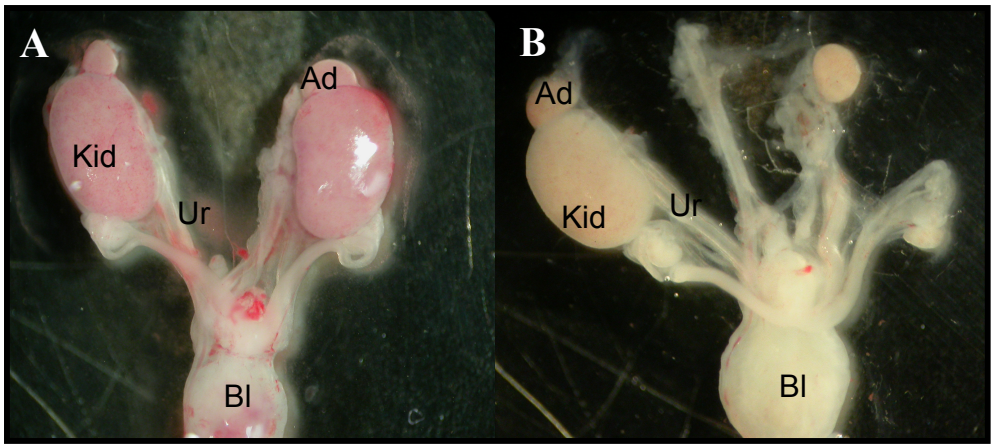


Figure 14. Kidney morphology of HSD17B2KO mice. **(A)** Urogenital block of WT mice at age of 1day demonstrating the kidneys (Kid), ureters (Ur), bladder (Bl) and the adrenals (Ad). **(B)** Urogenital block of HSD17B2KO mice at the age of 1 day revealing unilateral renal degeneration.

5. 4. *Hsd17b2, 7* and *12* in metabolic pathways

5.4.1. Rescue experiments of HSD17B2KO mice (I)

The data *in vitro* have suggested a role for HSD17B2 in the inactivation of sex steroids and in the conversion of 20α-dihydroprogesterone to progesterone. Intraplacental level of progesterone in HSD17B2KO mouse placenta at E17.5 was slightly elevated compared with the WT mice. Placental estradiol levels in both the KO and WT placentas were under the detection limit (0.05 nM) of the assay used. Additionally, we treated the pregnant mice with ICI 182,780, a potent antiestrogen, or with progesterone from E7.5 to E17.5. However, no knockout embryos with a rescued phenotype were observed, and the placentas from the rescue experiments appeared to be identical in morphology to those of untreated or placebo-treated mice at E17.5. Thus, the cause

of embryonic death does not appear to be neither due to the excess of estradiol nor lack of progesterone.

5.4.2. Cholesterol biosynthesis and Shh signaling in HSD17B7KO mice (II)

To study the role of hydroxysteroid (17-beta) dehydrogenase 7 in cholesterol biosynthesis, the concentration of cholesterol and its biosynthesis intermediates were measured in the whole embryos at the time of death, E10.5. The cholesterol biosynthesis intermediates before zymosterone (lanosterol and squalene) were accumulated in the HSD17B7KO embryos, while the amount of the demetylated intermediates after zymosterol in cholesterol biosynthesis (cholestenol, lathosterol and desmosterol) were markedly reduced. However, the level of total cholesterol was identical in the WT, HE and HSD17B7KO embryos at E10.5 (Figure 15). This indicates that the maternal supply is the major source of fetal cholesterol until E10.5, and that the KO mice were not devoid of maternal cholesterol. There was no difference in the sterol profiles between the WT and heterozygous mutant mice, indicating the capability of one intact allele to compensate for the missing allele.

Except for *Hsd17b7***,** there was no difference between the WT and KO embryos in the mRNA expression for the cholesterol biosynthetic enzymes at E9.5, suggesting that the lack of HSD17B7 was the only blockage in cholesterol synthesis in the KO mice. Thus, the embryonic lethality in the HSD17B7KO mice suggests the need of the *de novo* zymosterol biosynthesis in mice at E9.5-10.5 onwards, in addition to the presence of normal maternal contribution of cholesterol.

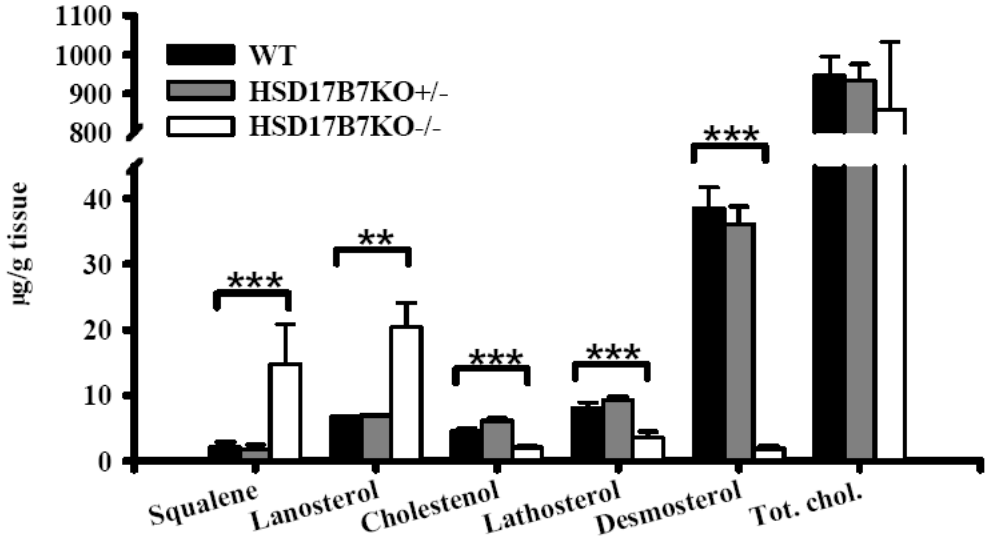


Figure 15. **Cholesterol biosynthesis in the HSD17B7KO mice.** Sterol analysis was done from the wild type (WT), heterozygous (HSD17B7KO+/-) and homozygous (HSD17B7KO-/-) knockout mice at E10.5. Whole-embryo sterol profile showed accumulation of cholesterol intermediates, lanosterol and squalene, and reduced amount of cholestenol, lathosterol and desmosterol in HSD17 577 B7KO-/- mice. The total concentration of cholesterol was unaltered at the time of death, E10.5. Bars represent the mean and the standard error of the mean. ***p<0.001 and **p<0.01.

In line with the normal level of total cholesterol, the expression patterns of the cholesterol modified sonic hedgehog (*Shh)* and its target gene *Ptch1*, were not altered in the HSD17B7KO mice as analyzed by *in situ* hybridization at E8.5 or by qRT-PCR at E9.5 embryos.

5.4.3. Proliferation experiments of HSD17B12KO mice (III)

To further investigate the cell proliferation in the HSD17B12KO embryos, the growth of the inner cell mass (ICM) was examined *in vitro*. Blastocysts from heterozygous matings were collected (E3.5) and cultured individually *in vitro*. The KO blastocysts showed a normal appearance, indicating that the lack of functional HSD17B12 does not affect the preimplantation stage of development. However, after 7 days in culture, the ICM did not grow in 90% of the mutant blastocysts, while only 20% of the WT and heterozygoust blastocysts failed to grow. However, trophoblast giant cells developed normally in the cultured KO embryos. In line with this, we could not isolate homozygous ES cells from blastocysts. From 22 isolated ES cell lines, 14 were heterozygous for the gene trap insertion (HSD17B12HE) and 8 were WT cells. The amount of arachidonic acid was significantly decreased in HSD17B12HE ES cells, whereas the amount of linoleic acid (LA), which is obtained from mother through the placenta, was the same as in the WT ES cells. This was in accordance with the 30-40% reduced amount of *Hsd17b12* mRNA in HSD17B12HE ES cells as compared with the WT ES cells (Figure 16).

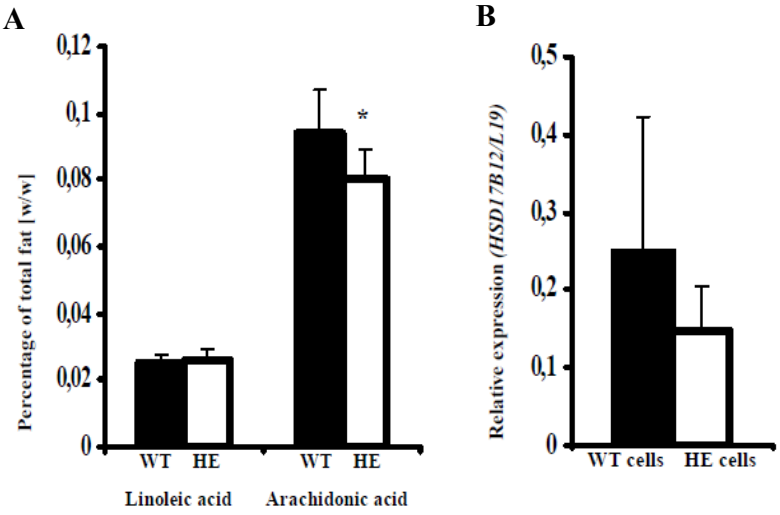


Figure 16. Imbalanced fatty acid synthesis and *Hsd17b12* mRNA expression in ES cells)
(A) The percentage of total amount of linoleic acid was unchanged, whereas the amount of arachidonic acid was significantly decreased (p= 0.013) in HSD17B12HE ES cells as compared with the WT cells. **(B)** RT-PCR analysis indicated a tendency for a reduced *Hsd17b12* mRNA expression in the heterozygous HSD17B12HE ES cells as compared with the WT cells.

6 DISCUSSION

6.1. Genetic manipulations

Sequencing the complete human genome (Istrail et al. 2004) resulted in the discovery of a large number of previously unknown genes, and for the majority of these genes the function *in vivo* is still unknown. Over the next years, one of the most important aims of biologic research will be revealing the function of these genes. Manipulating the genome of model organisms allows us to study gene function *in vivo*, and the data obtained from orthologous gene studies can be applied to the human with some appropriate criticism.

The ability to generate fertile mice from cultured cells has led to the development of a wide range of sophisticated genome engineering technologies, most notably gene targeting, gene trapping and conditional mutagenesis (Gossler et al. 1989, Capecchi 1989b, Glaser et al. 2005). Gene targeting by homologous recombination, although providing the desired specified mutation, has been labor-intensive and it has depended upon time-consuming methodologies to produce gene modifications on a gene by gene basis. One of the difficult steps in gene targeting has been the cloning of targeting vectors that are used to replace the native gene in mouse ES cells by homologous recombination (Capecchi 1989a). Most cloning methods are applying restriction endonuclease cleavage, followed by DNA ligation. These *in vitro* cleavage and ligation reactions have been the basis for creating DNA recombinants and for cloning of DNA segments in plasmid vectors. Those limitations of traditional cloning procedures are now avoided through the use of the new cloning methods (Chartier et al. 1996, Muyrers et al. 2001, Copeland et al. 2001, Schnütgen et al. 2006). In this study, the recombineering cloning (Zhang et al. 1998, Muyrers et al. 2000b) was used to generate the targeting vectors for *Hsd17b2* and *7*. In the present study, the recombineering was used to subclone the DNA fragments of *Hsd17b2* and *7* from bacterial artificial chromosomes into standard plasmid vector and for disruption of important exons. Recombineering cloning markedly improved the cloning efficiency as the targeting vectors used in this study were created with only two rounds of transformation and selection in *E.coli*. However, although the recombineering enhanced the cloning efficiency, it did not improve the procedure of identifying the rare ES cell clones in which the targeting vector had correctly altered the native gene.

An alternative method to generate gene-modified mice utilizes the gene trap libraries in ES cells. The gene trap method avoids the challenging tasks of making targeting vectors and screening the ES cell colonies and it allows the simultaneous mutation and identification of genes in the mouse (Gossler et al. 1989, Wurst et al. 1995, Skarnes et al. 1995, Hardouin and Nagy 2000). In this study, the gene trap ES cell lines were used to generate the conventional knockout mouse for *Hsd17b*12. The gene trap vectors contained a promoterless selectable marker gene that formed a fusion transcript via a splice acceptor sequence with the 5´ end of the trapped *Hsd17b*12 gene. In order to confirm the phenotype of *Hsd17b*12 mice, two different gene trap cell lines where used in this study. The major weakness of the gene trap approach is that it may not always produce a loss of function mutation because of alternative splicing, resulting in low levels of expression of the wild type transcripts and in a hypomorphic allele (Thyagarajan et al. 2003). In addition, it is possible that disruption of the intronic region, where the gene trap

vector has integrated, causes the phenotype (Gossler et al. 1989, Wurst et al. 1995, Skarnes et al. 1995, Hardouin and Nagy 2000).

6.2. HSD17Bs in mouse development

The knowledge about the processes by which a fertilized egg divides, forms a ball of cells, develops a cavity, forms the three primary germ layers of cells that will give rise to all the cell types of the body, and then generates all the specialized tissues and organs of a mature organism is far from complete (Theiler 1989). This process reflects one of the greatest mysteries of life and represents a fundamental challenge in developmental biology.

Hsd17b2 and *Hsd17b7* are expressed during mouse embryonic development, both in embryonic and in extraembryonic tissues. At E11.5 and onward, the *Hsd17b2* is expressed in several epithelial cell types in developing mouse embryo. At E11.5, the expression appears in the liver and the intestine, and is thereafter highly expressed in these tissues throughout the embryonic development (Mustonen et al. 1998a). In the present study, as in the earlier studies (Shehu et al. 2008), the expression of *Hsd17b*7 in mouse embryo was observed first in the front brain and in the heart at E9.5. Around E10.5-E11.5, the expression is found in the dorsal root ganglia, in the second and third branchial arches and in the neural tube (Marijanovic et al. 2003). In the mouse placenta from E9 onward, the *Hsd17b7* is expressed abundantly in spongiotrophoblast cells of the junctional zone (Nokelainen et al. 2000). Expression of *Hsd17b2* in the placenta appears later, around E12.5, in the labyrinth region, and towards to end of pregnancy, the expression is at strongest in this region (Mustonen et al. 1997a). In the present study, it was shown that *Hsd17b12* as well is strongly expressed during mouse development. The expression of *Hsd17b12* was observed in the embryonic ectoderm at E7.5, and especially high expression was seen at E8.5 in the neural folds at both the anterior and posterior regions of the body. At E9.5, and thereafter, the expression of *Hsd17b12* was noted along the rostrocaudal axis, with the strongest staining detected along the neural tube. In addition, *Hsd17b12* expression was also observed in the yolk sac and in the ectoplacental cone.

The expression of *Hsd17b2, 7* and *12* during the mouse development was well correlated with the embryonic phenenotypes observed in the present study. The HSD17B2KO mice exhibit fetal and perinatal mortality appearing at the age of E11.5 onwards. The embryonic lethality was associated with abnormalities in the placenta and interestingly, occurrence of the embryonic deaths in the HSD17B2KO mice well agreed with the shift in the *Hsd17b2* expression from the maternal to the fetal part of placenta (Mustonen et al. 1998b). The HSD17B2KO mice that survived through the fetal period were growth retarded and showed abnormalities in the kidney and the brain. In the end of fetal period, the HSD17B2KO mice showed signs of severe hydranencephaly, and the enlargement of ventricles progressively increased during the postnatal age. In humans, hydranencephaly is a rare condition in which the cerebral hemispheres are absent and replaced by sacs filled with cerebrospinal fluid (Tsai et al. 2008). Usually the cerebellum and brainstem are formed normally, and an infant with hydranencephaly may appear normal at birth. However, later on the symptoms of hydrocephalus will become obvious (Csabay et al. 1998, Görbe et al. 2008). In addition to hydranencephaly, the HSD17B2KO mice revealed disorganized neuronal migration. The control of the migration is highly orchestrated and

dependent on both genetic and environmental factors. In humans, the critical role of neuronal migration in brain development is evident from the variety of malformations occurring after abnormal migration (Wang et al. 2003, Görbe et al. 2008).

Both HSD17B7KO and HSD17B12KO mice showed early embryonic lethality and the morphological studies that were carried out revealed a reduced size of the HSD17BKO embryos. The embryonic lethality in the HSD17B7KO mice at E10.5 was consistent with another recent study on mice with disrupted *Hsd17b7* gene (Shehu et al. 2008). The lack of HSD17B7 activity led to a defect in the development of hemispheres of the front brain and induced apoptosis in the neuronal tissues. The lack of brain development in HSD17B7KO mice mimics the human birth defect with anencephaly and holoprosencephaly. In humans, holoprosencephaly and anencephaly are neuronal disorders that effect on normal development of the forebrain (Paidas and Cohen 1994, Wallis and Muenke 1999, Wallis and Muenke 2000, Muenke and Beachy 2000). In anencephaly, the neural tube fails to close resulting in the absence of a major portion of the brain and infants with this disorder are born without a forebrain (Csabay et al. 1998). Holoprosencephaly, on the other hand, is caused by a failure of the forebrain to divide to form bilateral cerebral hemispheres, causing defects in the development of the face and in brain structure and function (Wallis and Muenke 1999). In addition to brain malformations, the complexity of the vasculature was reduced in the *Hsd17b7* deficient embryos, particularly in the head capillary plexus and branchial arches. Furthermore, the yolk sac of the HSD17B7KO mice lacked the proper vascularization and no organized vessels were observed. At E8.5 to E9.5, the heart development and the looping of the heart appeared to be normal in the HSD17B7KO embryos. However, at E10.5, the heart development was abnormal and the HSD17B7KO embryos showed pericardial effusion, reduced number of myocardial cells, thin pericardium, and the septum of the atrium did not developed normally. At E8.5, the yolk sac vasculature fuses with the embryonic vasculature, and until the chorioallantoic placenta is established the yolk sac functions as a primitive placenta. The development of embryos during early organogenesis (E8.5 to E11.5) is critically dependent on the formation of functional yolk sac circulation and the establishment of a chorioallantoic placenta (Cross et al. 1994, Rossant and Cross 2001) and the defects in extraembryonic tissues can be the cause of embryonic lethality. However, in present study, the HSD17B7KO phenotype was not rescued by tetraploid aggregation, which indicated that the placental defect is not the reason for the observed embryonic phenotype in HSD17B7KO mice.

The HSD17B12KO mice died around E9.5 and presented severe defects in mouse embryo development. At E8.5, all the homozygous embryos were developmentally delayed, *e.g.* they had not initiated turning, and the allantois had not fused with the chorion. Homozygous HSD17B12KO embryos showed shortened anterior-posterior axis and presented with severe defects in the neuronal development. In addition, they failed to grow several mesoderm-derived structures. Analysis of the HSD17B12KO embryos revealed that they initiate gastrulation, but organogenesis was severely disrupted. In addition, the inner cell mass of KO blastocysts showed decreased proliferation capacity *in vitro*. It has been suggested that in humans the interference with gastrulation process by genetic, physical, or chemical agents can cause a range of central nervous system (CNS) and facial abnormalities (Webster et al. 1985, Webster et al. 1988).

6.3. Multifunctional role of HSD17B enzymes

Hydroxysteroid (17beta) dehydrogenases were discovered and named due to their ability to catalyze oxidation or reduction of the 17-hydroxyl or 17-keto functions of physiologically relevant sex steroids *in vitro* (Luu-The et al. 1995, Labrie et al. 1997, Peltoketo et al. 1999, Luu-The 2001, Adamski and Jakob 2001). All HSD17Bs are shearing a high structural similarity that suggests a very strict specificity of the enzyme family. However, further characterization of some family members has pointed out that the substrate specificity of HSD17B enzymes is greatly broader *in vivo* than what has been expected (Baes et al. 2000, He et al. 2002, Zhongyi et al. 2007). Although the expression of reductive (*Hsd17b2*) and oxidative (*Hsd17b7* and *Hsd17b12*) *Hsd17b* genes are present during the mouse embryonic development, the embryonic lethal phenotypes observed in the HSD17BKO2, 7 and 12 mice created in the present study did not mimic the phenotypes of mice deficient in *Esr1* (estrogen receptor-α) (Lubahn et al. 1993), *Esr2* (estrogen receptor-β) (Ke et al. 2002) or androgen receptor (AR) (Yeh et al. 2002, Matsumoto et al. 2003, Kerkhofs et al. 2009).

Expression of *Hsd17b2* in the placenta and embryo, together with the oxidative HSD17B activity associated with the enzyme, has suggested a role for the enzyme in reducing the sex steroid exposure of the fetus (Tong et al. 2005). Estrogen excess has been shown to cause placental thrombosis and spontaneous fetal loss in estrogen sulfotransferase, *Sult1e1*, KO mice (Mahendroo et al. 1997), and fetal death in mice lacking *Srd5a1*, the gene that encodes steroid 5α-reductase type 1 (Lydon et al. 1995). In the current study, it was shown that placentas of HSD17B2KO mice displayed histological malformations, but no signs of thrombosis. Mice deficient in the progesterone receptor gene exhibit several reproductive abnormalities, but homozygous embryos developed normally without defects in the placental function (Gleeson 2001, Guerrini and Carrozzo 2001). Furthermore, the treatment of pregnant female mice with antiestrogen or with progesterone did not prevent the fetal loss of the HSD17B2KO mice. Thus, the cause of HSD17B2KO embryonic deaths did not appear to be due to the lack of progesterone or increased action of estradiol.

Phylogenic analysis have indicated that HSD17B2 is a close homolog of retinoid converting enzymes in *C. elegans*, displaying a high sequence identity to retinol dehydrogenase type 1 (Chai et al. 1995a, Chai et al. 1995b, Simon et al. 1995). Furthermore, similarly to HSD17B2, several retinoic acid metabolizing enzymes belong to the family of aldo-keto reductases, both in rodents and humans (Niederreither et al. 1999a). The phenotypes identified in the transgenic mice expressing human HSD17B2 (Zhongyi et al. 2007) and in the HSD17B2KO mice are closely similar to some of the phenotypes obtained in mice with altered retinoic acid metabolism (Gaemers et al. 1998a, Gaemers et al. 1998b, Lohnes et al. 1994, Cohlan 1953). *In vitro* studies have shown that retinoids significantly induce *Hsd17b2* expression and enzyme activity in a dose- and time-dependent manner (Ito et al. 2001). It is, thus, possible that some of the phenotypes observed in HSD17B2KO mice are due to a misbalanced retinoid signaling mediated via HSD17B2.

In addition to steroids, the recombinant human and mouse HSD17B7s convert zymosterone to zymosterol *in vitro*, an essential reaction in cholesterol biosynthesis that involves a 3-ketosteroid reductase activity (Marijanovic et al. 2003). The present study showed the *in vivo* role of HSD17B7 in cholesterol biosynthesis in mice. The concentrations of the cholesterol biosynthesis intermediates measured in HSD17BKO embryos were altered in the manner

expected by the activity for HSD17B7 assumed from *in vitro* data. The amounts of the pre-defect intermediates were accumulated and those of the post defect ones were reduced in HSD17B7KO mice. Expression level of *Hsd17b7* in heterozygous mice was diminished while the expression of the other cholesterol biosynthetic enzymes measured was unaltered in the HSD17B7KO embryos. The lack of HSD17B7 enzyme was not efficiently compensated by other enzyme activities, and *Hsd17b7* deficiency resulted in a blockage in cholesterol synthesis. Interestingly, in the E10.5 embryos, the amount of total cholesterol was normal in HSD17B7KO embryos. Results of the present study, together with other studies, suggest that pregnant female supplies most of the cholesterol present in the embryo for the first 10-12 days of pregnancy (Tint et al. 2006). However, the present data shows that the normal maternal cholesterol supply is not able to rescue the neuroectodermal survival and cardiovascular differentiation in the absence of HSD17B7 activity in the embryo. *Dhcr7* is important in the final step of cholesterol biosynthesis. Interestingly, the *Dhcr7* deficient mice survive until birth (Fitzky et al. 2001), while HSD17B7KO mice are showing embryonic lethality (Shehu et al. 2008), suggesting that, compared with 7-dehydrocholesterol, zymosterone is not able to compensate for the lack of cholesterol, or that zymosterol has other functions in addition of being a cholesterol biosynthesis intermediate. It has been recently shown that zymosterol is one of the liver X receptor (LXR) ligands (Yang et al. 2006). The LXR knockout mice, however, do not present defects in embryonic development (Alberti et al. 2001). In addition, it has to be considered that the accumulation of cholesterol precursors may possess toxic effects on the embryos as postulated, for example, for Smith-Lemli-Opitz (SLO) syndrome (Gaoua et al. 1999, Gaoua et al. 2000). However, the mechanisms of such toxic effects have not been presented and the evidence for toxicity *in vivo* is lacking.

The common phenotypic feature in several mutant mice with a defect in cholesterol biosynthesis is malformation of the nervous system (Liu et al. 1999, Tozawa et al. 1999, Fitzky et al. 2001), which was observed also in the HSD17B7KO embryos. The need of *de novo* cholesterol synthesis in the brain is well supported by the data indicating that the blood-brain barrier in mice forms at around E10 (Tint et al. 2006). The mutant mice with a defect in *Ebp, Sc5d, Dhcr24* and *Dhcr7* genes survive until birth (Derry et al. 1999, Fitzky et al. 2001, Krakowiak et al. 2003, Wechsler et al. 2003, Mirza et al. 2006), while mice deficient in *Hsd17b7* and genes involved in the previous steps (Liu et al. 1999, Tozawa et al. 1999, Caldas et al. 2005) present with embryonic lethal phenotypes.

It has been shown that cholesterol is the lipophilic moiety covalently attaching to the amino terminal signaling domain of SHH during its autoprocessing, and accordingly it has been suggested that the disrupted SHH modification might account for the defects observed in mouse models with disrupted cholesterol biosynthesis (Porter et al. 1996, Beachy et al. 1997). However, the expression pattern and levels of *Shh* and two of its target genes, *Ptch1* and *Smo*, were not altered in the HSD17B7KO embryos.

In addition to its expected role in steroid metabolism, HSD17B12 has been suggested to have an important function in lipid metabolism. Originally, the enzyme was identified as a human homolog for yeast 3-ketoacyl-CoA reductase participating in fatty acid elongation. Accordingly, studies *in vitro* have indicated that the enzyme participates in the fatty acyl-CoA elongation (Moon and Horton 2003) by catalyzing the elongation of very long chain fatty acids, particularly in the elongation of essential fatty acids (Moon and Horton 2003, Nagasaki et al.

2009b). The studies with LET-767, the *C.elegans* ortholog of HSD17B12, have shown that the enzyme is also required for production of branched-chain and long-chain fatty acids *in vivo* (Entchev et al. 2008). The expression pattern of *Hsd17b12* in early mouse embryo, shown in this study, further supports its role in lipid metabolism. Both in the mouse and human, the *Hsd17b12* is expressed universally (Blanchard and Luu-The 2007, Sakurai et al. 2006), but the highest levels have been detected in the tissues related to lipid metabolism, especially in the liver, kidney, heart and skeletal muscle (Sakurai et al. 2006).

Essential fatty acids (EFAs) determine the fluidity of neuronal membranes and control various physiological functions in the brain (Yehuda et al. 2005). Brain is rich in structural lipids, and AA and docosahexaenoic acid (DHA) are the predominant EFAs in the mammalian brain (Crawford et al. 1976, Crawford 1993). An equal amount of AA and DHA is essential for proper development and function of the brain. Studies in humans and experimental animals have demonstrated that the deficiency of AA leads to neurodevelopmental disorders accompanied by impaired fetal growth and behavioral retardation (Sinclair and Crawford 1973, Galli and Spagnuolo 1976, Leaf et al. 1992, Wainwright et al. 1997, Birch et al. 1998, Birch et al. 2000). AA is derived from one of the EFAs, linoleic acid, through a series of metabolic steps involving desaturation and subsequent chain elongation (Cook et al. 1991). AA, in turn, gives rise to a whole group of biologically important substances known as the eicosanoids, including prostaglandins, leukotrienes and thromboxanes. These compounds are important for the regulation of various metabolic and physiological processes (Chatzipanteli et al. 1996, Tapiero et al. 2002). Although during the present study, the homozygous ES cells were not obtained, the current study showed that AA synthesis was already reduced in HSD17B12HE ES cells suggesting that HSD17B12 is involved in AA synthesis either directly or indirectly, and consequently essential for the neuronal development in mice. Other recent studies have also indicated a role for HSD17B12 in arachidonic acid synthesis, as knocking down HSD17B12 via siRNA in the breast cancer cells resulted in growth inhibition that was completely recovered by supplementation of AA (Nagasaki et al. 2009b).

6.4. Future prospects

Results of this study bring up new questions about the role of Hsd17b enzymes during the development. There seems to be a critical developmental window, especially for neuronal development, were the embryonic expression of *Hsd17bs*, particularly *Hsd17b7* and *Hsd17b12*, is required. Although the present study was carried out in mice, the novel findings open up the possibility that *Hsd17b2, 7* and *12* could be the missing or defective genes behind some of the neuronal disorders in humans. Future studies must be carried out to clarify the role of Hsd17b enzymes in gastrulation and in signaling evolved in neural induction as retarded gastrulation will subsequently alter the neuronal development. The role of Hsd17b enzymes in highly coordinated network of regulatory pathways that affects normal neuronal development, still remains to be determined. There are also other interesting phenotypes to be solved in more detail, like the effect of Hsd17b7 deficiency in heart. It would be especially interesting to find out, if the primary phenotype is the defected vascularization, as the heart actually derived from the developing vascular system. Mutation studies in human could provide the link between Hsd17b7 deficiency and the abnormalities that occur during early cardiac development.

The molecular mechanisms behind the phenotypes observed in this study still need to be evaluated in more detail. Many of the Hsd17b enzymes, among those studied in this research seem to have a considerably wider role in biological signalling than has been anticipated based on the *in vitro* studies only. In fact, an extensive effort should be made to challenge and evaluate all the *in vitro* data of Hsd17b enzymes using *in vivo* models to clarify the actual primary metabolic pathways that Hsd17b enzymes are physiologically involved in.

7 CONCLUSIONS

The local activation and inactivation of nuclear receptor ligands is anticipated to have a great impact in determining the level of receptor activation. Hydroxysteroid (17-beta) dehydrogenases (HSD17Bs) are among the enzymes activating or inactivating the signaling through the nuclear receptors. However, the physiologically relevant substrates of most of the HSD17Bs *in vivo* are only partly known. The main conclusions of the present study are the following:

1. Our data and the literature showed that *Hsd17b2, 7* and *12* are all expressed during mouse embryonic development in various embryonic and extraembryonic tissues, with some spatial and temporal differences.

2. There is a critical, developmental window, especially for the neuronal development, where the embryonic expression of *Hsd17b2, 7* and *12*, is required. Disruption of *Hsd17b2, 7* and *12* results in severe malformations of the developing central nervous and cardiovascular systems, and ultimately leads to embryonic death.

3. The main functions of HSD17B2, 7 and 12 during mouse embryonic development are not in the steroid metabolism but in other basic metabolic pathways like cholesterol and fatty acid.

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In Turku

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