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MASTERARBEIT

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„Genetic variants of the scavenger receptor class B type I and influences on human HDL metabolism“

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2 Introduction

Low plasma levels of high-density lipoprotein cholesterol (HDL-C) are related to higher risk for cardiovascular diseases. Therefore, drug maker are interested in the development of agents that elevate plasma HDL levels. It is important to understand all mechanisms and players in this disease [1].
The major HDL receptor, scavenger receptor class B type I (SR-BI) is also a potentional target for pharmalogical research and the main focal point in this study.

2.1 Lipid Metabolism

Plasma lipids are a heterogenous group of compounds including phospholipids, cholesterol, triacyglycerides and cholesteryl esters [2].
These lipids are important for steroid hormone synthesis, maintaining cell membrane structure, cell signalling and many other biological functions. Lipids are nonpolar and therefore not water-soluble. They need to be transported in water-soluble lipoproteins (Figure 1), as the bloodstream of humans and animals is aqueous. Lipoprotein-particles consist of lipids and proteins (apolipoproteins).
The inner core (nonpolar) of the particle consists mainly of cholesteryl-esters (CE) and triacylglycerides (TG), while the hydrophilic (polar) outer layer is constructed of apolipoproteins, phospholipids and unesterified cholesterol [2].

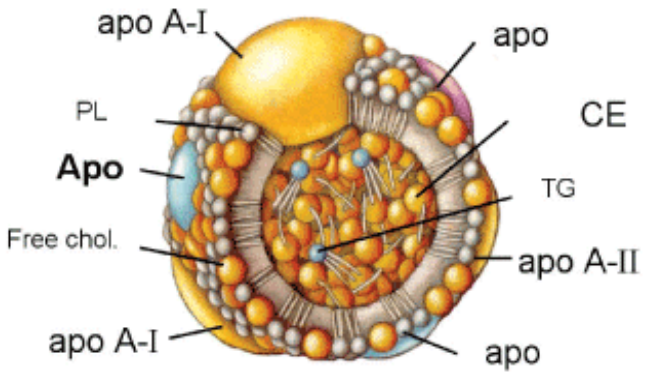


Figure 1. Composition of high-density lipoprotein (HDL). [34]

Lipoproteins are classified according to size, lipid and apolipoprotein composition and density. Each class of lipoprotein has specific functions in lipid metabolism (Table 1).

Properties	Chylomicrons	VLDL	LDL	HDL
Composition (%)				
Cholesterol	3	22	50	20
Triglycerides	90	55	5	5
Phospholipids	6	15	25	25
Proteins	1	8	20	50
Origin	Intestine	Liver, intestine	Product of VLDL metabolism	Liver, intestine
Function	Transports triglycerides from diet	Transports hepatic triglycerides	Supplies TC to cells	Performs TC reverse transport

VLDL: very low-density lipoprotein; LDL: low-density lipoprotein; HDL: high-density lipoprotein; and TC: total cholesterol.

Table 1. Functions of the principal lipoproteins. [33]

In the liver, cholesterol is converted to bile acids and released into the bile. In steroidogenic tissues, cholesterol is used as precursor for steroid hormones. Thus, it is important to provide cells with cholesterol. To supply cells with cholesterol, two ways are are distinguished:

a) The exogenous pathway: Dietary lipids are taken up by the intestine and processed for further pathways.
b) The endogenous pathway: Lipoproteins are synthesized by the liver and secreted into the blood.

Intracellularly, cholesterol is stored as cholesteryl ester in cytoplasmic lipid droplets. This process is catalyzed by the enzyme Acetyl-CoA−Acetyltransferase (ACAT). The esterification of cholesterol in HDL particles is catalyzed by the enzyme Lecithin-Cholesterin-Acyltransferase (LCAT) [3]. Two major structural apolipoproteins in HDL, apoA-I and apoA-II are responsible for the interaction with the HDL receptor.

2.2 Scavenger receptor class B type I (SR-BI)

The gene encoding SR-BI is named SCARB1 in mice and CLA-1 in humans. Scavenger receptor class B type I, an important HDL receptor, was first identified in 1994 by Krieger, Acton and colleagues in a study that used acetylated low-density lipoprotein (LDL) as a ligand [4]. SR-BI was described to be a multi-ligand receptor that binds a broad class of ligands, e.g. oxidized LDL, VLDL and HDL with high affinity [5]. Later, SR-BI was demonstrated to mediate selective uptake of HDL cholesteryl ester [6]. This indicates the important role of the receptor in lipid metabolism.

2.2.1 Structure of SR-BI

Scavenger receptor class B type I (SR-BI) is a 82 kDa glycoprotein (509 amino acids) which belongs to the CD36 family of cell surface receptors. The receptor consists of an extracellular domain and short intracellular N-terminal and C-terminal domains (Fig. 2). The extracellular domain is heavily N-glycosylated and fatty-acylated [7].

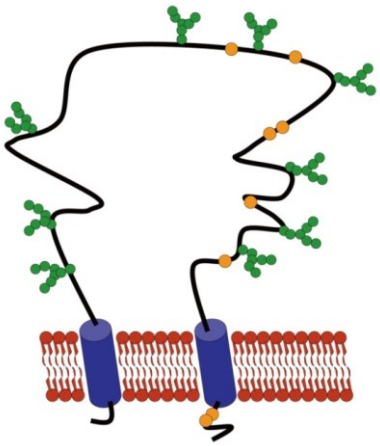


Figure 2. Model of SR-BI. [7]

The C-terminal domain of SR-BI interacts directly with the PDZK1 protein which is involved in signalling pathways. PDZK1 is discussed to stabilize SR-BI at the plasma membrane and increases HDL selective uptake and HDL association after co-expression with SR-BI [8]. SR-BI is expressed in the liver, in steroidogenic tissues, in

macrophages, in the ovaries, in astrocytes and apparently no expression was found in kidneys, brain and lungs [5].

2.2.2 Functions of SR-BI

SR-BI mediates the efflux of excess cholesterol from macrophages to high-density lipoproteins (HDL) which transports cholesterol back to the liver for catabolism. In the liver, SR-BI mediates selective uptake of cholesteryl ester (CE) from HDL (Fig. 3). This reverse cholesterol transport is an important step to prevent foam cell formation and atherosclerosis [9].

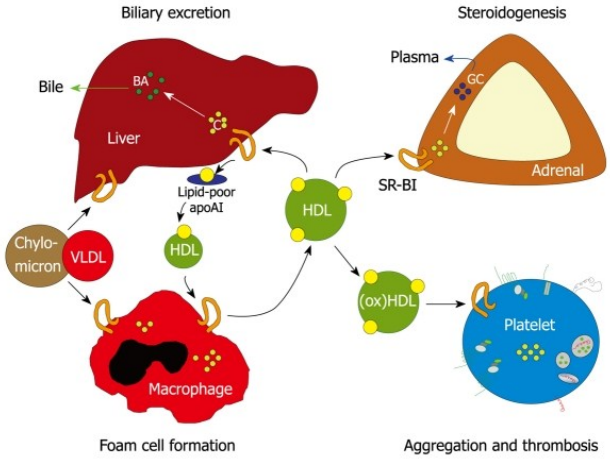


Figure 3. The role of SR-BI in lipid metabolism. [7]

Several models are discussed for the mechanism of selective uptake:
Acton et al. (1996) described this receptor-mediated uptake of cholesterol from HDL into cells [6]. The group demonstrated that SR-BI mediates selective uptake of HDL-derived cholesteryl ester without the degradation of the particle itself. Another model was mentioned by Silver and Tall (2001) who described the endocytosis of SR-BI and the HDL particle, followed by resecretion of the protein [10] [11].
SR-BI mediates both, cholesterol efflux and cholesterol influx from HDL and is therefore responsible for a bidirectional flux of cholesterol [12].

Selective cholesteryl ester uptake is an important mechanism in cholesterol metabolism. Impaired receptor activity is associated with elevated plasma cholesterol levels which underlines the fact that SR-BI is focussed as a therapeutic target.
Another important plasma protein is involved in reverse cholesterol transport, the cholesteryl ester transfer protein (CETP). It is mainly secreted by the liver and mediates the exchange of CE for triacylglycerides between lipoproteins. Cholesteryl ester is transported from HDL to LDL via CETP and taken up by the LDL-receptor in the liver. Therefore, CETP is an alternative route back to the liver [7].

2.2.3 SR-BI membrane localization

SR-BI was reported to be localized in caveolae, which are special membrane domains. These caveolae are small invaginations in the plasma membrane of cells and represent a specialized reversible plasma membrane pool of CE [5].
The main protein that is expressed in caveolae, is caveolin-1 (21 kDa). In many tissues and cells, e.g. adrenocortical cells, co-localization of SR-BI and caveolin-1 was observed. Despite, SR-BI was also found to be expressed in caveolae poor regions and caveolin-1 expression is not required for SR-BI activity [5].
SR-BI expression might be linked to changes in lipid organization of the plasma membrane [13].

2.3 Atherosclerosis

Plasma concentrations of HDL cholesterol are inversely associated with atherosclerotic vascular diseases. HDL is included in reverse cholesterol transport (RCT) as it removes excess cholesterol from peripheral cells including those in the arterial wall and transports it back to the liver [2].
This prevents foam cell formation and atherosclerosis. Foam cells are macrophages which are laden with lipids because of their unregulated lipid uptake from modified low-density lipoproteins (LDLs). Fatty streaks are formed which are considered to be the initial step leading to atherosclerotic lesions [14].
SR-BI is involved in reverse cholesterol transport and can therefore influence atherosclerosis and coronary artery disease.
In SR-BI knockout mice, increased plasma HDL cholesterol levels and larger HDL particles were observed. These mice also developed atherosclerotic lesions which underlines the anti-atherosclerotic function of SR-BI [15].

2.4 Status of Research

Mice lacking SR-BI and apoE (SR-BI/apoE double knockout [dKO] mice) develop spontaneous atherosclerotic lesions resulting in myocardial infarctions. They exhibit functional defects similar to those seen in human coronary heart disease (CHD) and are therefore an accepted model to study CHD and atherosclerosis [16].

Normally, mice are not prone to atherosclerosis as they carry most of their cholesterol in HDL particles which is due to the lack of CETP expression. In contrast, humans carry most of their cholesterol in low-density lipoproteins and display CETP expression.

Therefore, a study was published that used rabbits as a model system because of their human-like lipoprotein profile and CETP expression. Hypercholesterolemic rabbits displayed no increase in HDL cholesterol levels after SR-BI inhibition in the liver (using siRNA against SR-BI) but atherosclerosis was found to be reduced [17]. This leads to the suggestion that the alternative route back to the liver for cholesterol via CETP could be more effective than the classival route via SR-BI.

Another study focused on SR-BI knockout/CETP transgenic mice - „humanized mice“ - to gain insights into the importance of SR-BI and CETP in humans. The humanized mice displayed lower plasma total cholesterol levels compared to SR-BI knockout mice [18] [19] [20]. Furthermore, HDL particle size was slightly decreased by CETP expression [19] and adrenal HDL-cholesteryl ester uptake in SR-BI knockout mice was increased but not normalized by CETP in SR-BI knockout mice. Thus, the effect of SR-BI knockout is less severe in CETP transgenic mice [18].

Despite these studies in humanized mice, the role of SR-BI in humans is unclear. Recently, a new missense mutation of SR-BI (P297S) was found by analyzing patients with elevated levels of HDL cholesterol. The mutation carriers displayed a decreased cholesterol efflux from macrophages compared to noncarriers. Platelets of carriers contained more unesterified cholesterol and showed increased adhesion to fibrinogen. Patients with the SR-BI mutation had higher HDL levels with larger HDL-particles itself. Also the level of most urinary steroid hormones was lower in patients carrying the mutation compared to noncarriers [21].
Interestingly, the group was unable to confirm an association with increased atherosclerosis. Because of the small number of carriers (n=18) and their relatively young age (mean 45 ± 20), the statistical power of the study was low.
For functional analysis of the SR-BI_P297S mutation, the group of Kuivenhoven (2001) isolated primary hepatocytes of mice transfected with an adenovirus carrying either wildtype human SR-BI or mutant human SR-BI_P297S.
Selective uptake was markedly reduced in SR-BI_P297S transfected cells while cell association was similar in both transfected cells (wildtype and mutant SR-BI) [21].
Although several other SR-BI mutations [22] [23] located at different positions in the receptor (Fig. 4) have been described recently, the role of SR-BI in human HDL metabolism remains still unclear.

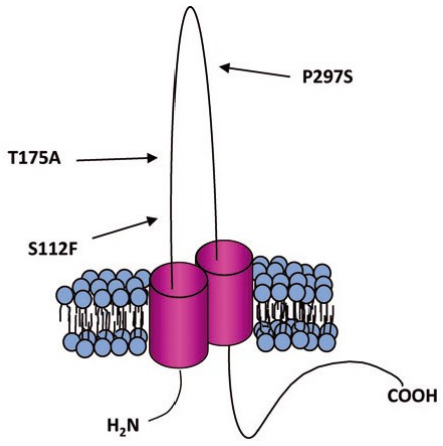


Figure 4. Mutation sites of SR-BI. All these possible mutations were found in patients and are located at the extracellular domain of the receptor. [30]

3 Aim of the study

The main focus of this study was the investigation of SR-BI in human HDL metabolism. The idea based on the paper of Kuivenhoven [21] describing a new missense mutation of SR-BI (P297S) in patients with elevated HDL cholesterol levels. We obtained wildtype SR-BI and mutant SR-BI (P297S) as a kind gift of Dr. Kuivenhoven to study the function of this mutation in human cell models. As yet this mutation was only transfected in murine primary hepatocytes. Because of the different lipoprotein profile and different CETP expression in mice and humans (see introduction) species dependent differences might be seen in human cells compared to murine hepatocytes. Therefore, two different human cell lines were used for analysis: First, HuH7 cells were chosen which are a good cell model mimicking the situation in the liver as the central organ in HDL metabolism. Second, HEK293/Flp-In cells (kidney cells) were used because of their low endogenous SR-BI expression, expecting a better effect of SR-BI overexpression in this cell line. Both cell types, HuH7 cells and HEK293/Flp-In cells were stably transfected with either wildtype human SR-BI or mutant human SR-BI (P297S). Possible differences in selective uptake, cellular association, free cholesterol distribution, lipid uptake and holo-HDL particle uptake were investigated.

4 Material and Methods

4.1 Tissue Culture

4.1.1 Media

DMEM: Dulbecco's Modified Eagle Medium, 4.5 g/L glucose (PAA, Pasching, Austria)

MEM: Minimal essential medium (PAA)

4.1.2 Cell lines

HuH7:

HuH7 cells originate from a human hepatocyte derived carcinoma cell line and were kept in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1 mM L-glutamine.

HuH7 – wt h(human)SR-BI:

HuH7 cells were stably transfected with wild-type SR-BI (plasmid: pcDNA3/hSR-BI_wt). Cells were grown in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1 mM L-glutamine and 1 mg/ml G418 (Neomycin; PAA). The generation of transfected cell lines is described below.

HuH7 – mutant hSR-BI (P297S):

HuH7 cells were stably transfected with mutated SR-BI (plasmid: pcDNA3/hSR-BI_P297S). Cells were grown in the same medium as wild-type SR-BI transfected HuH7 cells.

HEK293/Flp-In:

HEK293/Flp-In cells (Invitrogen) are stably expressing the *lacZ*-Zeocin fusion gene. Cells were grown in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1 mM L-

glutamine and 100 µg/ml Zeocin (Invitrogen). These cells are designed for use with the Flp-In System. HEK293/Flp-In cells contain a single integrated Flp Recombination Target (FRT) site.

HEK293/Flp-In – wt SR-BI:

HEK293/Flp-In cells were stably transfected with wild-type SR-BI (plasmid: pcDNA5/FRT-hSR-BI_wt). Cells were grown in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM L-glutamine and 50 µg/ml Hygromycin B (PAA).

HEK293/Flp-In – mutant SR-BI (P297S):

HEK293/Flp-In cells were stably transfected with mutated SR-BI (plasmid: pcDNA5/FRT-hSR-BI_P297S). Cells were grown in the same medium as wild-type SR-BI transfected HEK293/Flp-In cells.

4.2 Buffer

10x TBS (Tris buffered saline):

24.2 g Tris

84.8 g NaCl

Add up to 1000 ml dH_2O

ph= 7.4

Sample buffer:

90 µl glycerol

45% TRIS (8% SDS, ph=6.8)

5% mercaptoethanol

Bromphenol blue

TRIS-Glycin Buffer:

0.3% TRIS

1.4% glycin

1% SDS

Transfer Buffer:

TRIS-Glycin Buffer

20% methanol

Tris buffered saline Tween (TBS-T):

0.24% TRIS

0.84% NaCL

0.1% Tween*20

ph= 7.4

4.3 Coating of cell-culture plates

Gelatin:

0.5% gelatin (from bovine skin; Sigma) were dissolved in distilled water and autoclaved. Volumes used for cell-culture: 75 cm^2 flask: 5 ml; 25 cm^2 flask: 2 ml; 12-well plate: 1 ml/well; 24-well plate: 500 µl/well. The gelatin coated dishes were incubated at 37°C for 30 minutes.

Collagen I:

Dishes were incubated with Collagen I (from rat tail; GIBCO) at room temperature overnight (0.5 mg/ml dissolved in 0.02 M CH_3COOH). The coated dishes were washed with sterile distilled water several times and let dry at room temperature for one hour. Volumes used for cell-culture: 24-well plate: 50 µl/well; 12-well plate: 100 µl/well.

4.4 Cloning material

LB medium:

10 g Tryptone (Peptone)

5 g yeast extract

5 g NaCl

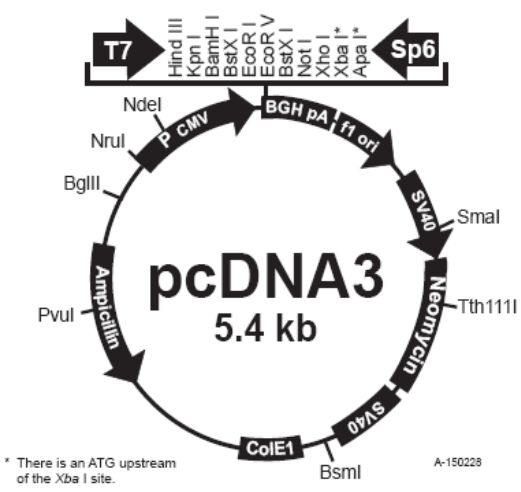
Add up to 1 L with distilled water and adjust to ph=7.5

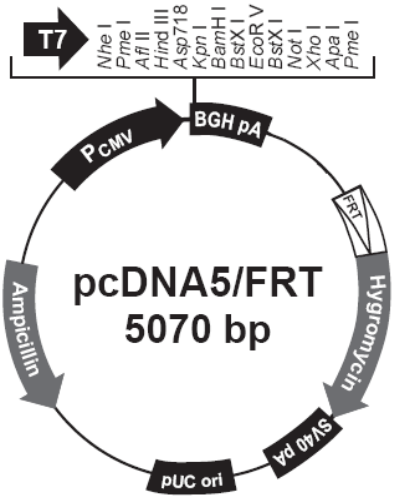
Agar-plates:

LB-medium with 1.5% agar (Sigma Aldrich)

Autoclaved and antibiotics added

4.5 Vectors used in this thesis (invitrogen)





pcDNA3 vector:

https://tools.invitrogen.com/content/sfs/vectors/pcdna3.pdf (5.11.2012)

pcDNA5/FRT vector:

http://tools.invitrogen.com/content/sfs/vectors/pcdna5frt_map.pdf (5.11.2012)

4.6 Restriction digest of DNA

For restriction digest 200 ng (analytic) to 2 µg (fragment isolation) DNA were used. The reaction mixture was prepared in a microfuge tube as follows and was incubated at 37°C for one hour.

Reagent	**µl**
Restriction enzyme	1
Restriction-Buffer (10x)	2
DNA (plasmid)	200 ng to 2 µg
distilled water	Add up to 20 µl volume

4.7 Ligation of DNA

All ligations were performed with the Fast Link ^{TM} DNA Ligation Kit (Epicentre). The ligation mixture contains all reagents listed below and was incubated at room temperature for one hour.

Vector : Insert [mol/mol]	1: 6 [100 ng vector]
10x Fast Link Ligation Buffer	2 µl
10 mM ATP	2 µl
Sterile water	Up to 20 µl
Fast-Link DNA Ligase	1.3 µl
total	**20 µl**

4.8 Bacterial Transformation

One Shot TOP10 Chemically Competent E.coli (invitrogen) were used for transformation.

Cells were defrosted on ice and incubated with 5 µl of the ligation mixture for 30 minutes. Next, a heat-shock at 42°C without shaking was performed for 30 seconds and cells were incubated directly on ice for 2 minutes afterwards.

Finally 250 µl S.O.C Medium (invitrogen) were added to the transformation mixture, which was then shaked horizontally at 37°C at 300 rpm for one hour. Each transformation (50 – 200 µl) was spread on a pre-warmed selective agar-plate and incubated at 37°C overnight.

4.9 Plasmid preparation

For small amounts of plasmid isolation the EZ-10 Spin Column Plasmid DNA Minipreps Kit (geneXpress) was used. Single-colonies were picked from selected plates and inoculated in LB-medium containing the appropriate antibiotics at 37°C overnight.

The overnight culture (1.5 ml) of E.coli in LB-medium was transferred into a new tube and centrifuged at 12000 rpm for 2 minutes. Supernatants were drained. The pellet was resuspended in 100 µl of Solution I and kept for 1 minute. Solution II (200 µl) was added to the mixture and mixed gently by inverting the tube 4-6 times. Solution III (350 µl) was added, mixed gently and incubated at room temperature for 1 minute. The mixture was centrifuged at 12000 rpm for 5 minutes. The supernatant was transferred to the EZ-10 column and centrifuged at 10000 rpm for 2 minutes. The flow-through was discarded. Wash Solution (750 µl) was added to the column and centrifuged at 10000 rpm for 2 minutes. The washing step was repeated. The tube was centrifuged for an additional minute to remove any residual Wash Solution. After centrifugation, the column was transferred to a clean 1.5 ml microfuge tube. Elution Buffer (50 µl) was added into the center of the column and let stand for 2 minutes at room temperature. The mixture was centrifuged at 10000 rpm for 2 minutes. The concentration of purified DNA was measured by Nanodrop-100 Spectrophotometer and stored at -20°C or used for further experiments.

HiSpeedMaxi Prep (Qiagen) was used for higher amounts of plasmid isolation.

4.10 Transfection

TurboFect in vitro Transfection Reagent (Fermentas) is a cationic polymer which forms compact, positively charged complexes with DNA, which is negatively charged. It protects DNA and enables gene delivery to eukaryotic cells. For stable transfections, vector-DNA was linearized by restriction digest to facilitate integration into the genome. Adherent cells were grown in a 6-well plate (HuH7: 2 x10^5 cells/well; HEK293: 4 x10^5 cells/well) in 3.6 ml growth medium. For transfection, 4 µg DNA was incubated with 6 µl TurboFect for 20 minutes at room temperature. The DNA/TurboFect mixture was added drop-wise to each well, gently rocked and incubated at 37°C in a CO_2 incubator. Transient expression was analyzed after 48 hours. For stable transfection, cells were grown in selective medium for 2 weeks.

Transfection of HuH7 cells:

HuH7 cells do not have the Flp-In system. Therefore, SR-BI was isolated from the pcDNA5/FRT-Flp-In vector using HindIII and BpiI and ligated into the multiple cloning site (MCS) of the pcDNA3 vector. HuH7 cells were then transfected with 4 µg of pcDNA3/hSR-BI wt or -P297S. The plasmid was linearized with Bgl-II before transfection. This restriction enzyme was chosen as it digests the vector outside of the SR-BI insert.

Transfection of HEK293 cells:

HEK293/Flp-In cells were co-transfected with 4 µg of pcDNA5/FRT-hSR-BI_wt or _P297S and 4 µg of pOG44 (Flp recombinase expression vector). The pcDNA5/FRT vector was linearized with Bgl-II before transfection. HEK293/Flp-In cells contain a single integrated Flp Recombination Target (FRT) site which is recognized by the Flp Recombinase. This process ensures integration of target DNA into the genome.

4.11 Agarose-Gelelectrophoresis

0.8% agarose gel (Gibco BRL, Life Technologies) containing 0.5 µg/ml ethidiumbromide was used for separating and analyzing DNA. The electrophoresis chamber was filled with 1xTAE Buffer and run with constant voltage of 80 V for 30 minutes.

4.12 Western Blot Analysis

4.12.1 Preparation of cell lysates

Cells were grown in 6-well plates (HuH7: 5 x10^5 cells/well; HEK293: 6 x10^5 cells/well) and washed twice with cold PBS on ice. Lysis Buffer (200 µl/well - 62.5 mM Tris, 20 mM EGTA, 8 M Urea, 20 mM EDTA, 1% SDS, 10% Glycerol, ph = 6.8) was added, containing 1% protease inhibitor (Sigma). Cell lysates were incubated on ice for 10 minutes, scraped off and collected in a microcentrifuge tube. The samples were then homogenized by pressing through a small syringe, treated with ultrasonic for 3 seconds followed by 1 minute shock freezing in liquid nitrogen. After thawing the cell lysates on ice, they were centrifuged at 12000 rpm at 4°C for 10 minutes. The supernatants were collected, the protein content was quantified photometrically at 280 nm and samples were stored at -20°C.

For western blot analysis the protein concentrations (30-50 µg/lane) were adjusted with PBS to a volume of 30 µl. The samples were mixed with sample buffer in a ratio of 1:4 and heated for 30 minutes at 40°C or 95°C for 5 minutes.

4.12.2 Preparing the gel

For SDS-PAGE glass plates were clamped to prepare a gel of 0.75 mm thickness. The gels were prepared according to the table below.

	Separating Gel (8%)	Stacking Gel (4%)
Distilled water	1.6 ml	1.1 ml
3 M TRIS-HCl (ph=8.45; 0,3% SDS)	1.1 ml	0.3 ml
30% acrylamid	1.3 ml	0.7 ml
10% APS	30 µl	20 µl
TEMED	3 µl	2 µl

4.12.3 Gel electrophoresis

The gels were inserted into an electrophoresis chamber which was filled with running buffer (Tris/glycin). All slots were filled with buffer to remove air bubbles and loaded with the samples (15 slots: 13 µl/lane). PageRuler Prestained Protein Ladder (Thermo Scientific) was used as a size marker (5 µl/lane).

The electrophoresis was run with constant voltage (180 V) at 4°C for 85 minutes.

4.12.4 Membrane transfer

Filter, nitrocellulose-membrane (0.45 µm; Biorad) and sponge were soaked with transfer buffer and assembled as follows: sponge / filter / nitrocellulose / gel / filter / sponge. The sandwich was inserted in the electrophoresis chamber and filled with transfer buffer. Membrane transfer was run with constant current of 400 mA at 4°C for one hour.

The membrane was stained with Ponceau Red (0.1 g Ponceau S were dissolved in 5 ml acetic acid and filled up with distilled water to a volume of 100 ml) at room temperature for 5 minutes. Ponceau red, a protein binding dye, was used to visualize the protein bands. The membrane was washed three times with washing buffer (TBS-T). SR-BI primary antibody (NB400-104, Novus Biologicals; polyclonal rabbit) was diluted 1:3000 or 1:5000 in TBS - 5% milk powder.

Beta-Actin (Sigma) was used as loading control. The membran was incubated with the primary antibodies at 4°C overnight.

The next day the membran was washed again with washing buffer and the secondary antibody (goat anti-rabbit, Pierce; goat anti-mouse, Sigma) was added at a dilution of 1:5000 (goat anti-rabbit; SR-BI) and 1:10000 (goat anti-mouse; Beta-Actin) in TBS – 5% milk powder and incubated at room temperature for one hour.

For the detection of the bands the blot was incubated with the Super Signal West Dura Substrate (Thermo Scientific) for 3 minutes and analyzed by the ChemiImager 4400. TotalLab TL120 program was used for blot analysis afterwards.

4.13 RNA isolation

Cells were grown in 6-wells (HuH7: $5x10^5$ cells/well; HEK293: $6x10^5$ cells/well) and washed twice with PBS. For RNA isolation the RNAeasy Plus Micro Kit (Quiagen) was used. Cells were lyzed with 350 µl/well Buffer RLT Plus containing 1% mercaptoethanol. The lysates were transferred into falcon tubes and centrifuged. Small needles were used for homogenization. The cells were transferred onto the gDNA Eliminator Spin Columns and centrifuged at >10.000 rpm for 30 seconds (the eluate was discarded). Ehanol (70%; 350 µl/sample) was added and mixed. The mixture was transferred onto the RNeasy MinElute Spin Column and centrifuged at >10.000 rpm for 15 seconds (the eluate was discarded). Buffer RW1 (700 µl) was added to the column and centrifuged at >10.000 rpm for 15 seconds (the eluate was discarded). Then, 500 µl of Buffer RPE was loaded onto the column and centrifuged at >10.000 rpm for 15 seconds (the eluate was discarded). The column was washed with 500 µl ethanol (80%) and centrifuged at >10.000 rpm for 2 minutes (the eluate was discarded; column was transferred into a new micorzentrifuge tube). For elution, 15 µl RNase free water was added to the center of the column and centrifuged at full speed for one minute. The microcentrifuge tube was kept on ice and RNA concentration was measured by Nanodrop-100 Spectrophotometer. RNA was stored at -80°C.

4.14 Reverse Transcription

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription of mRNA into cDNA. For the RT reaction, a master solution was mixed as listed below:

Reagent	***1x reaction***
Nuclease free water	3.2 µl
10x RT Buffer	2 µl
100 mM dNTP Mix	0.8 µl
10x RT Random Primers	2 µl
Multi Scribe Reverse Transcriptase	1 µl
RNase Inhibitor	1 µl
total	*10 µl*

RNA (1000 ng) was adjusted with RNase free water to a volume of 10 µl and mixed with 10 µl master solution. The samples were put into a Thermocycler (peQlab) and run for 2.5 hours (25°C for 10 minutes – 37°C for 2 hours – 85°C for 5 minutes).

The cDNA (1000 ng /20 µl) was adjusted with RNase free water to a total volume of 50 µl and stored at -20°C.

4.15 Quantitative Real-Time PCR

StepOne Software 2.1 was used to define the plate-layout for the PCR. Primer and sample names were assigned and the reaction volume per well was set to 15 µl. For PCR, a master solution was mixed as listed below:

Reagents	***Volume/well***
Master Mix (Applied Biosystems)	7,5 µl
Primer (SR-BI and 18s)	0,76 µl
Nuclease free water	4,75 µl
total/well	*13 µl*

Primer	***Assay ID***
18s	Hs99999901 -s1
SR-BI	Hs00969821-m1

The master solution (13 µl/well) and 2 µl/well of cDNA were mixed in a 48 well-plate, sealed with a transmissible film and centrifuged for 1 minute. For real-time PCR, StepOne Real Time PCR System (Applied Biosystems, Australia) was used. The measured Ct-values (cycle treshold) of SR-BI and 18s rRNA (endogenous control) of each sample were used for relative quantitation (RQ) calculation referring to the control sample of each PCR.

4.16 Bradford Assay

The Bradford Assay was used to determine protein concentration of cell lysates as well as lipoproteins. Upon protein binding, the absorption spectrum of Coomassie brillant blue is shifted and can be detected at 595 nm. For every assay a standard curve with different concentrations of BSA standard protein (c= 1 mg/ml) was prepared and measured simultanously with the samples. Microtiter plates were used for measurement and samples were diluted as follows:

Standard:	#1	#2	#3	#4	**Samples:**		
BSA (1 mg/ml)	0 µl	1 µl	2 µl	5 µl		Sample:	5 µl
Bradford Reagent	20 µl	20 µl	20 µl	20 µl		Bradford Reagent	20 µl
Distilled water	80 µl	79 µl	78 µl	75 µl		Distilled water	75 µl

4.17 Isolation of Lipoproteins

Human blood was collected from male and female healthy volunteers after an overnight fasting period and plasma was obtained by centrifugation (620 x g for 10 min, at 18 °C, brake 0). After centrifugation, the upper phase containing plasma was used for lipoprotein isolation. The lipoproteins were isolated by a stepwise ultrazentrifugation. First, the density of VLDL (d=1.019) was adjusted with potassium bromide (KBr).

For calculation the following formula was used:

$$g\ KBr = \frac{V(plasma) * (final\ density - initial\ density)}{1-(0{,}312 * final\ density)}$$

Tubes containing plasma were centrifuged at 50,000 rpm at 4 °C for 21 hours using an ultracentrifuge (Beckmann ultracentrifuge, rotor=55.2 Ti).
VLDL was isolated first as it represents the fraction with the lowest density. This process was repeated once to purify LDL (d=1.07) and twice for the HDL (d=1.22)

fraction. All lipoproteins were dialysed (dialysis buffer: 0.9% NaCl, 1% EDTA, ph= 7.4) for 24 hours to remove KBr. Protein content was determined by Bradford protein assay.

4.18 Iodination of human HDL

Lipoproteins were labeled with sodium [^{125}] iodide (Hartmann Analytics), which labels the apolipoproteins of HDL. For labeling procedure, IODO-BEADS iodination reagent kit (Thermo Scientific) was used. The lipoprotein solution (250 µg HDL in 250 µl PBS) was incubated with 0,5 mCi sodium [^{125}] iodide and a iodo bead at room temperature for 20 minutes. Meanwhile the storage solution of the gel chromatography column (Pierce D-Salt Dextran Desalting Column) was dripped off and equilibrated with 5 column volumes PBS (25 ml). Gel chromatography was used to separate labeled lipoproteins from free sodium [^{125}] iodide. After 20 minutes incubation time, the lipoprotein solution was applied on the gel chromatography and eluted with PBS by 5 steps: 1x 750 µl and 4x 500 µl PBS. The fractions were collected in minicentrifuge tubes. For radioactivity measurement (1 µl per fraction) the gamma-counter (COBRAII-Auto gamma; Packard Canberra Company, Austria) was used. The protein content of each fraction was quantified by the Bradford protein assay. For experiments a specific activity of about 300 cpm/ng of labeled HDL was used.

4.19 Double labeling of human HDL for selective uptake analysis

Lipoproteins were double labeled with ^{3}H-cholesteryl-oleate (Perkin Elmer; Massachusetts, USA) and sodium [^{125}] iodide (Hartmann Analytics). ^{3}H-cholesteryl-oleate (100 µl) was transferred into a clean glass tube and evaporated during rotation under nitrogen. The lipid film was dissolved in 50 µl DMSO and the diluted HDL (1 mg /450 µl PBS) was added. The glass tube was sealed with parafilm and incubated in a shaking water-bath at 40°C for 2 hours. Sodium [^{125}] iodide [1 mCi] and two iodo beads were added to the solution at room temperature for 20 minutes. Gel chromatography was used to separate labeled lipoproteins from free ^{3}H-cholesteryl-oleate and Sodium [^{125}] iodide. After 20 minutes incubation time, the iodo beads were removed and the mixture was centrifuged at 12.000 rpm for 5 minutes at room temperature. The

supernatant was applied to the column (which was equilibrated with PBS before) and eluted with 500 µl 1x PBS by 5 steps.
Each fraction (1 µl) was counted with 1 ml distilled water and 15 ml Ready-Safe (Beckman Coulter; USA) by the Beta-counter (Tri-Carb 2800TR; Perkin Elmer)
The protein content of each fraction was quantified by the Bradford protein Assay.

4.20 Cellular Association Experiments

Cells were grown in 12-well plates (HuH7: 1,5 x10^5 cells/well – gelatin coated; HEK293: 2,5 x10^5 cells/well –collagen I coated) for two days. Then they were washed with PBS and incubated with DMEM (+ 1 mM L-Gluthamin, +1% Antibiotics, without FBS; +10% lipoprotein deficient serum) in the incubator (37°C) overnight. On day 3, the cells were washed twice with PBS (37°C) and 500 µl/well medium (MEM + 2 mg/ml faf BSA (fatty acid free bovine serum albumin)) was added. To calculate unspecific binding, a 40-fold excess (200 µg) of unlabeled HDL was added to every fourth well. Sodium [^{125}] iodide labeled HDL was added to each well with a concentration of 10 µg/ml. To determine the specific activity, five aliquots (10 µl/well) from the medium were measured. After one hour incubation time at 37°C, the media was aspirated and cells were washed twice with cold PBS +BSA (2 mg/ml) and twice with cold PBS only. The cells were then lysed with 1 ml NaOH [0.1 M] at room temperature for one hour. The radioactivity of all samples (800 µl/well) was counted by the gamma-counter (COBRAII Auto-gamma; Packard Canberra Company) and the Bradford protein assay was again used to determine the protein amount of each well.
The blank was subtracted from the respective data points referring to total protein concentrations. The values were expressed in ng lipoprotein/mg cell protein.

4.21 Selective Uptake – Experiments

Cells were grown in 12-well plates (HuH7: 1,5 x10^5 cells/well – gelantin coated; HEK293: 2,5 x10^5 cells/well – collagen I coated) for two days. The experimental set-up was similar to the cellular association experiment, except for the counting procedure (Beta-counter). For determining specific activity five aliquots (10 µl/well) from the medium were measured with 1 ml distilled water and 15ml Ready-Safe. After NaOH [0.1 M] incubation, the radioactivity of the cell-lysates (800 µl) was measured with 15ml Ready-Safe. The Bradford protein assay was again used to determine protein amount of each well. Specific binding [ng lipoprotein/mg cell protein] was analyzed similar to cellular association experiments and selective uptake was calculated:

^{3}H-cholesteryl ester (specific association) - [^{125}] iodide specific association

4.22 Alexa568 labeling of human HDL

HDL was dissolved in PBS (1 mg /440µl) and mixed with 50 µl 1M $NaHCO_3$. Alexa568 dye (Invitrogen) was added (100 µg) and incubated at room temperature for one hour on the belly dancer. Afterwards, a Sephadex column (Sephadex G-25; 0.7 g in 7 ml PBS) was prepared and used to separate labeled HDL from free labeling dye. Labeled HDL was stored at 4°C. This labeling procedure was the same for Alexa488 labeling of human HDL.

4.23 Bodipy-cholesterol labeling of human HDL

Bodipy-cholesterol (a kind gift of Robert Bittman, Queens College of the City University of New York) was diluted to a final concentration of 1 mg/ml in Chlorophorm/Methanol (2:1). The diluted cholesterol (25 µl) was transferred into a clean glass tube and evaporated under nitrogen while rotating the tube. The lipid film was then dissolved in 50 µl DMSO. Diluted HDL (1 mg /450 µl) was added and incubated at 37°C in a rocking water-bath for two hours. To purify labeled HDL, a Sephadex column (Sephadex G-25; 0.7 g in 7 ml PBS) was used to separate labeled HDL from free labeling dye. The solution was stored at 4°C.

4.24 Holo-HDL particle uptake

Cells were grown in 24-well plates on glass cover slips (HuH7: $5x10^4$ cells/well – gelatin coated; HEK293: $5x10^4$ cells/well – collagen I coated) for two days. On day 3 the medium was aspirated and cells were washed twice with PBS. The cells were incubated with DMEM (+ 1mM L-gluthamin, +1% Antibiotics, without FBS; +10% lipoprotein deficient serum) in the incubator (37°C) overnight. On day 4 the cells were washed twice with PBS and incubated with 300 µl/well new medium (MEM + 2 mg/ml faf BSA. Alexa568 labeled HDL (50 µg/ml) was added to each well and incubated at 37°C for one hour. After incubation-time, all wells were washed twice with PBS and fixed with 4% formaldehyde (400µl/well) at 4°C for 30 minutes. The cells were washed again twice with PBS and incubated with 500 µl/well DAPI dye [(4',6-diamidino-2-phenylindole); diluted in PBS (1:100); light-excluded] which binds to dsDNA of cells and is used to stain the nucleus. The glass cover slips were washed twice with PBS. One drop of FluoPrep (bioMerieux sa, France) was placed on a microscope slide (Thermo Scientific) and covered with the cover slip carrying the cells. The slides were dried at room temperature for 30 minutes and stored at 4°C overnight. The next day, holo-HDL particle uptake was analyzed by fluorescence microscopy. The peak emission wavelength of Alexa568 is detected at 580 nm.

4.25 Lipid uptake analysis

The experimental set-up was similar to the holo-HDL particle uptake experiment, except 50 µg/ml Bodipy labeled HDL were used for cell treatment. The slides were again analyzed by fluorescence microscopy. The peak emission wavelength of Bodipy labeld HDL is detected at 520 nm. The excitation wavelength is 480 nm.

4.26 Distribution of free cholesterol in cells (Filipin III staining)

Filipin III (Sigma Aldrich) was used to stain free cholesterol in cells to analyze its distribution. Cells were grown in 24-well plates on glass cover slips (HuH7: $5x10^4$ cells/well – gelatin coated; HEK293: $5x10^4$ cells/well – collagen I coated) for two days. Cells were fixed at 4°C for 30 minutes using 4% formaldehyde. The Filipin staining solution was prepared: 10% lipoprotein deficient serum, 5% Filipin diluted in PBS. All wells were washed twice with PBS and incubated with the staining solution (400

µl/well) at room temperature for 30 minutes (protected from light). After incubation the cells were washed twice with PBS and placed on the microscope slide (with one drop of FluoPrep). The slides were dried at room temperature for 30 minutes and stored at 4°C overnight. The next day, free cholesterol distribution was analyzed by fluorescence microscopy. The peak emission wavelength of Filipin is detected at 400 nm and absorption at 350 nm.

5 Results

SR-BI plays an important role in HDL cholesterol metabolism and it is discussed to have anti-atherogenic activity in vivo. The group of Kuivenhoven (2011) analyzed patients with elevated levels of HDL cholesterol and found a new mutation in SR-BI (P297S) [21].

Despite of this, the role of SR-BI in humans is not fully understood. We aimed to investigate the role of SR-BI in human HDL metabolism using two different human cell types for generating stable cell lines overexpressing either wildtype or mutant SR-BI (P297S). HuH7 cells were chosen as a model for liver cells while HEK293 cells were chosen because of their low level of endogenous SR-BI expression.

5.1 Results in subcloned HuH7 cells

We obtained SR-BI constructs from Kuivenhoven in the pcDNA5/FRT vector. For stably transfection into HuH7 cells, SR-BI was digested from this vector and cloned into pcDNA3.

5.1.1 pcDNA3-hSR-BI_P297S/ and –hSR-BI_wt Ligation

The ligated vector DNA (pcDNA3-hSR-BI_P297S/ and –hSR-BI_wt) of the bacterial colonies was obtained by mini plasmid preparation and analyzed by agarose gel electrophoresis. The following enzymes were used for restriction digest:

Vector	**Enzyme**	**Restriction sites**
pcDNA3-hSR-BI_P297S	ApaI	2 sites
	XhoI	1 site
	BpiI	1 site
	HindIII	1 site
pcDNA3-hSR-BI_wt	ApaI	3 sites
	XhoI	2 sites
	BpiI	1 site
	HindIII	1 site

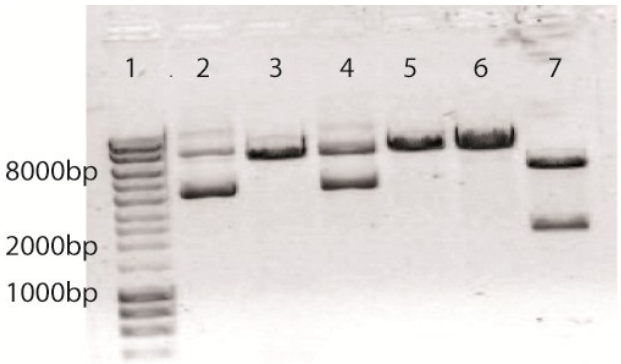


Figure 5. Restriction digest of pcDNA3-hSR-BI_p297S ligation samples. Lane 1: Hyperladder (Bioline), lane 2: uncut ligation sample (control), lane 3: ApaI, lane 4: XhoI, lane 5: HindIII, lane 6: BpiI and lane 7: double digest with HindIII and BpiI.

All enzymes digested at the right positions and gave correct results except XhoI and ApaI (Fig. 5). The bands of lane 4 (XhoI) and lane 2 (uncut vector) are similar which implies that XhoI did not work. ApaI digestion (lane 3) does not display a second fragment as expected. Importantly, HindIII and BpiI successfully digested the vector resulting in fragments of about 6 kbp (vector) and about 1.7 kbp (SR-BI). The correct configuration was confirmed by sequencing (VbC Biotech).

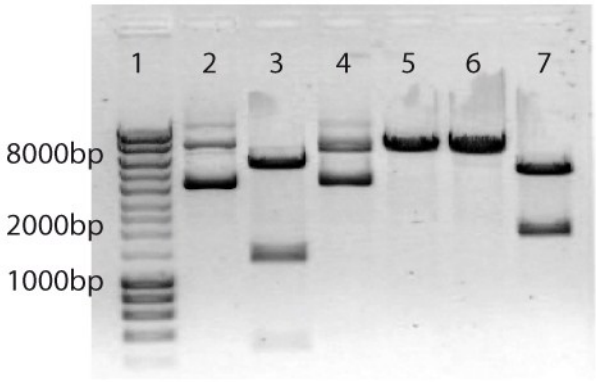


Figure 6. Restriction digest of pcDNA3-hSR-BI_wt ligation samples. Lane 1: Hyperladder (Bioline), lane 2: uncut ligation sample (control), lane 3: ApaI, lane 4: XhoI, lane 5: HindIII, lane 6: BpiI and lane 7: double digest with HindIII and BpiI.

The result of the pcDNA3-hSR-BI_wt digest (Fig. 6) was similar to the pcDNA3-hSR-BI_P297S ligation sample. XhoI (lane 4) did not work as it was similar to the uncut vector control (lane 2). ApaI digest worked in this vector. Double degestion with HindIII and BpiI was successfull in this vector (1.5 kb SR-BI). The correct configuration was also confirmed by sequencing (VbC Biotech).

5.1.2 SR-BI mRNA expression in subcloned HuH7 cells

HuH7 cells were stably transfected with the pcDNA3-hSR-BI_P297S/ and –hSR-BI_wt vector (see transfection). RNA of each cell line was isolated, transcribed into cDNA and expression levels of SR-BI and 18s were analyzed by qRT-PCR.

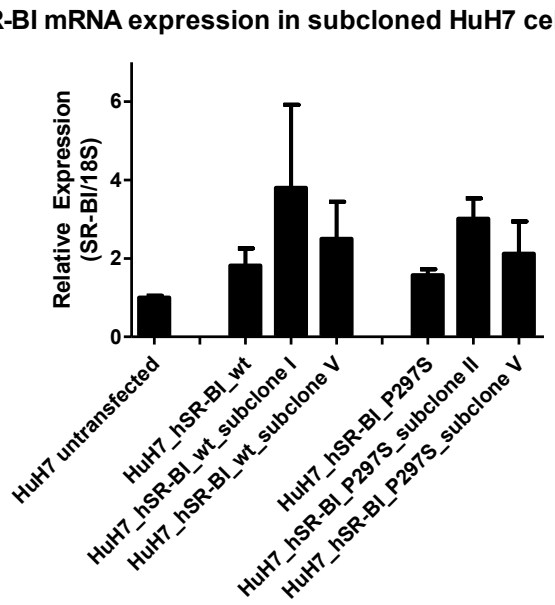


Figure 7. SR-BI mRNA expression levels in subcloned HuH7 cells. Untransfected cells were used as control. Stably transfected HuH7 cell lines are shown left beside the resprective subclones.

Despite the big standard deviation of the HuH7_pcDNA3-hSR-BI_wt subclone I it is shown that SR-BI levels are more than twofold higher in subcloned cells compared to HuH7 untransfected cells (Fig. 7). In contrast, subcloning was less efficient in SR-BI_P297S transfected cells. HuH7-pcDNA3-hSR-BI_wt_subclone I and HuH7-pcDNA3-hSR-BI_P297S_subclone II were used for further experiments.

5.1.3 SR-BI protein levels in subcloned HuH7 cells

Proteins of stably transfected HuH7 cells were isolated and analyzed by western blotting. Each cell line was measured in duplicates.

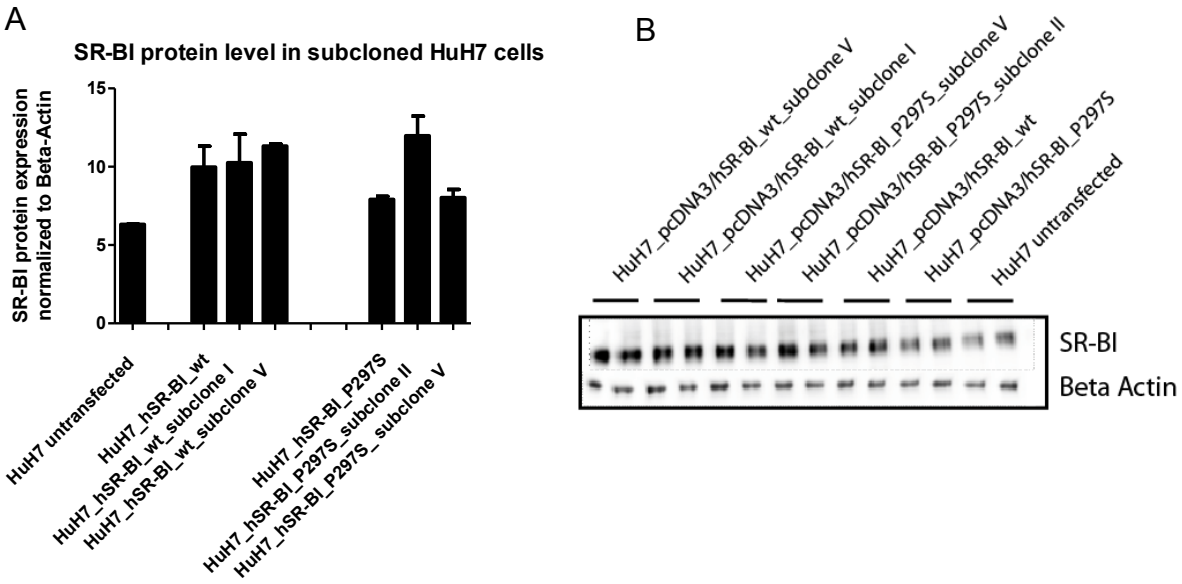


Figure 8. SR-BI protein level in subcloned HuH7 cells. Untransfected cells were used as control. Stably transfected cell lines are shown left beside the respective subclones (A). For western Blot analysis, TotalLab TL120 program was used to measure the band volume of each sample (B). SR-BI (82kDa) values were normalized to Beta-Actin (42kDa; endogenous control)

The protein level of SR-BI is twofold higher in HuH7-pcDNA3-hSR-BI_wt_subclone I and HuH7-pcDNA3-hSR-BI_P297S_subclone II compared to untransfected cells (Figure 8). Subcloning was only effective in SR-BI_P297S transfected HuH7 cells.

5.1.4 Cellular Association Experiments in subcloned HuH7 cells

In this study, subcloned HuH7 cells (human cell line) were used to investigate cellular association of human HDL. Subcloned HuH7 cells were seeded in 12-well plates and incubated with [^{125}] iodide labeled HDL (10 µg/ml) at 37°C for one hour.

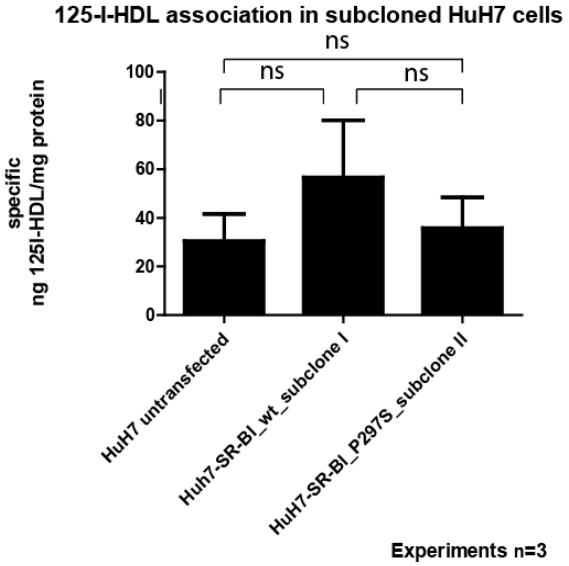


Figure 9. Cellular association of [^{125}] iodide labeled HDL in subcloned HuH7 cells. Untransfected cells were used as control. The data represent three independent experiments using the same conditions. Analysis of variance (ANOVA) was used for statistical analysis.

Specific HDL association was not significantly elevated upon SR-BI overexpression (Fig. 9). Furthermore, cell association did not differ significantly between wildtype and mutant SR-BI transfected cells. However, HDL association tended to be higher in SR-BI_wt overexpressing cells.

5.1.5 Holo-HDL particle uptake in subcloned HuH7 cells

To observe differences in HDL endocytosis, subcloned HuH7 cells stably expressing either wildtype or mutant SR-BI (P297S) were seeded in 24-well plates on glass cover slips and incubated with Alexa568 labeled HDL (50 µg/ml) at 37°C for one hour. After cell fixation, holo-HDL particle uptake was analyzed by fluorescence microscopy.

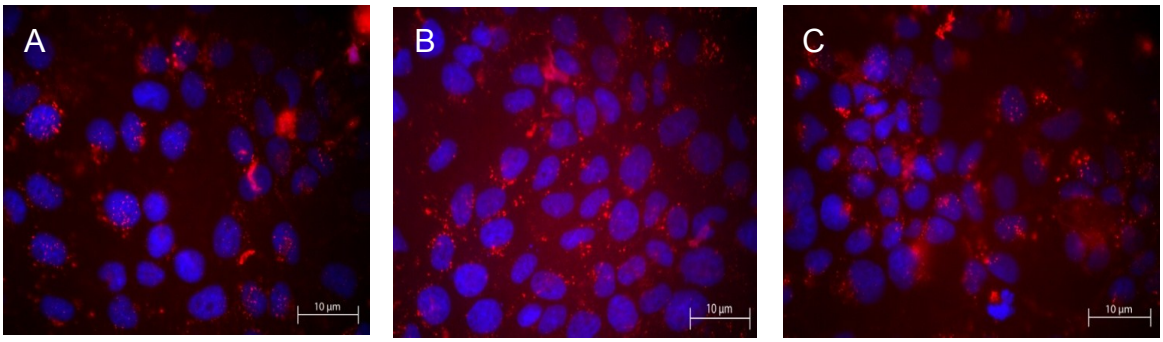


Figure 10. Holo-HDL particle uptake in subcloned HuH7 cells. Untransfected HuH7 cells (A), SR-BI wildtype transfected HuH7 cells (B) and mutant SR-BI (P297S) transfected HuH7 cells (C) were used for analysis. DAPI (blue) stains the nucleus, Alexa568 labeled HDL is shown in red.

SR-BI expression of both constructs resulted in increased HDL endocytosis (Fig. 10) but there is no difference between the SR-BI transfected cells (wildtype SR-BI and mutant SR-BI (P297S)).

5.1.6 Selective uptake in subcloned HuH7 cells

The aim of this experiment was to determine whether there is a difference in selective uptake between the wildtype and mutant form of SR-BI. HuH7 cells were cultured in 12-well plates and incubated with ^{3}H-cholesteryl ester and sodium [^{125}] iodide double labeled HDL (10 µg/ml) at 37°C for one our.

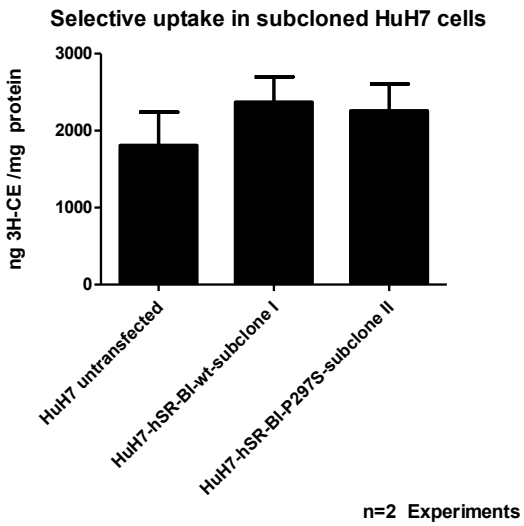


Figure 11. Selective uptake of ^{3}H-CE in subcloned HuH7 cells. Untransfected cells were used as control. The diagram represents two independent experiments using the same conditions.

As shown in figure 11, there is no significant difference between stably transfected cells. Both stably transfected cell lines (wildtype and mutant SR-BI) show a slight elevation of selective uptake.

5.1.7 Lipid uptake analysis in subcloned HuH7 cells

Subcloned HuH7 cells were grown in 24-well plates on glass cover slips and incubated with Bodipy labeled HDL (50 µg/ml) at 37°C for one hour. After cell fixation, lipid uptake was analyzed by fluorescence microscopy.

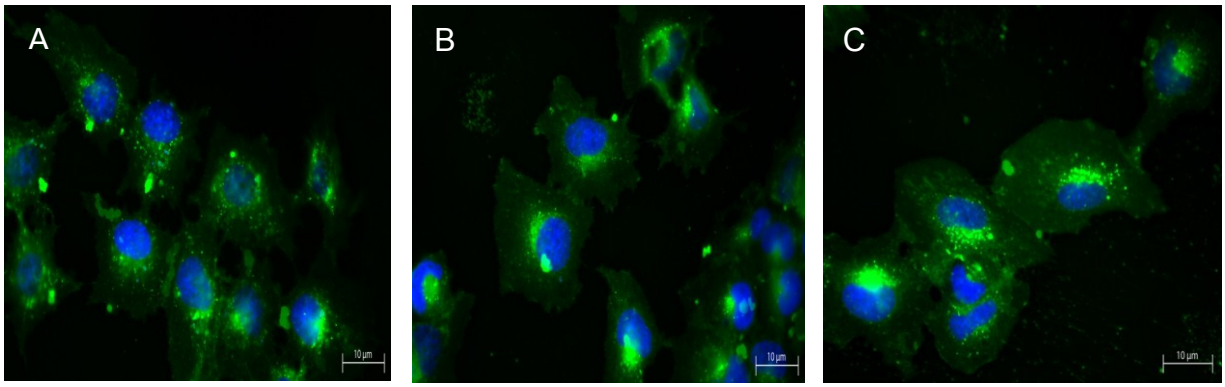


Figure 12. Bodipy cholesterol staining in subcloned HuH7 cells. Untransfected cells (A), SR-BI wildtype transfected cells (B) and mutant SR-BI (P297S9 transfected HuH7 cells (C) are depicted. DAPI (blue) was used to stain the nucleus, Bodipy cholesterol is shown in green.

There is no difference in lipid uptake comparing transfected cells (Fig. 12B/12C). In contrast to untransfected cells (Fig. 12A) it seems that in both transfected cell lines (wildtype and P297S) the amount of lipids is elevated in perinuclear compartments, probably Golgi apparatus or *transgolgi* network.

5.1.8 Distribution of free cholesterol in subcloned HuH7 cells

Next, possible differences in free cholesterol distribution comparing SR-BI wildtype transfected cells against SR-BI mutant (P297S) transfected cells were analyzed. Cells were seeded in 24-well plates on glass cover slips and incubated with Filipin III at room temperature for 30 minutes to stain free cholesterol. Fluorescence microscopy was used for analysis.

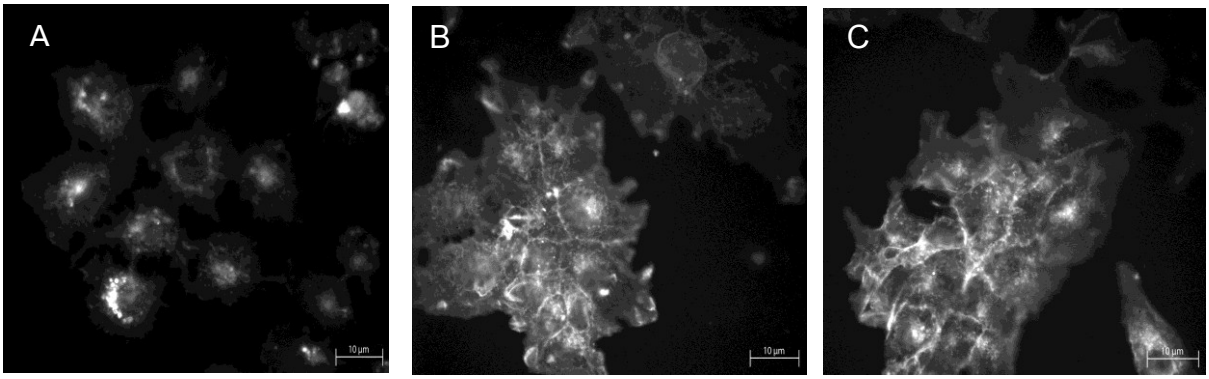


Figure 13. Free cholesterol distribution in subcloned HuH7 cells. Untransfected cells (A), SR-BI wildtype transfected cells (B) and SR-BI mutant (P297S) transfected cells (C).

Figure 13 demonstrates the differing distribution of free cholesterol in transfected cells (13B/13C) and untransfected cells (13A). Untransfected cells show a central accumulation of free cholesterol while transfected cells display a high amount of free cholesterol in the plasma membrane. Comparing SR-BI_wt and SR-BI_P297S transfected cells, no differences in free cholesterol distribution were observed.

5.2 Results in HEK2993/Flp-In cells

Experiments in HuH7 cells did not definitely allow to describe differences between SR-BI_wt and SR-BI_P297S. This might be a result of the weak SR-BI over-expression, probably because of high endogenous SR-BI or due to promotor down-regulation (see discussion). Thus, HEK293 cells were used for analysis because of their low endogenous SR-BI level. Better effects of overexpression were expected in these cells.

5.2.1 SR-BI mRNA expression in HEK293/Flp-In cells

HEK293/Flp-In cells, stably expressing pcDNA5/FRT-hSR-BI_wt or pcDNA5/FRT-hSR-BI_P297S (see transfection) were cultured in 6-well plates and used for RNA isolation. Expression levels of SR-BI and 18s rRNA were analyzed by qRT-PCR.

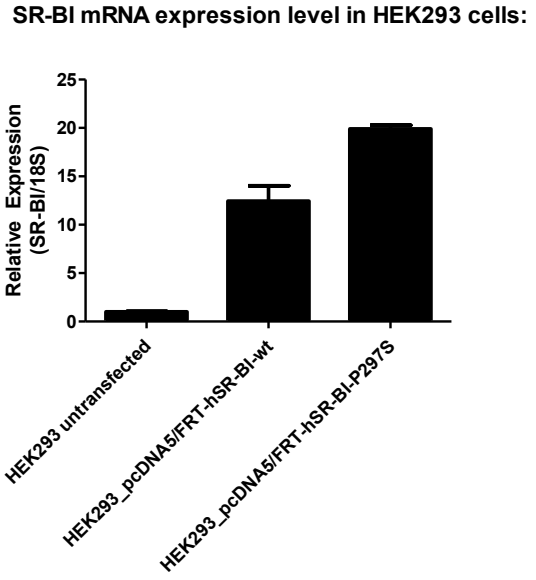


Figure 14. SR-BI mRNA expression level in HEK293/Flp-In cells. Untransfected cells were used as control. SR-BI levels were normalized to 18s expression.

Transfected cells display a 10 to 20-fold higher SR-BI expression compared to untransfected cells (Fig. 14). This experiment confirms a successful transfection with a better effect than seen in subcloned HuH7 cells.

5.2.2 SR-BI protein levels in HEK293/Flp-In cells

Next, successfull SR-BI overexpression was confirmed by western blotting.

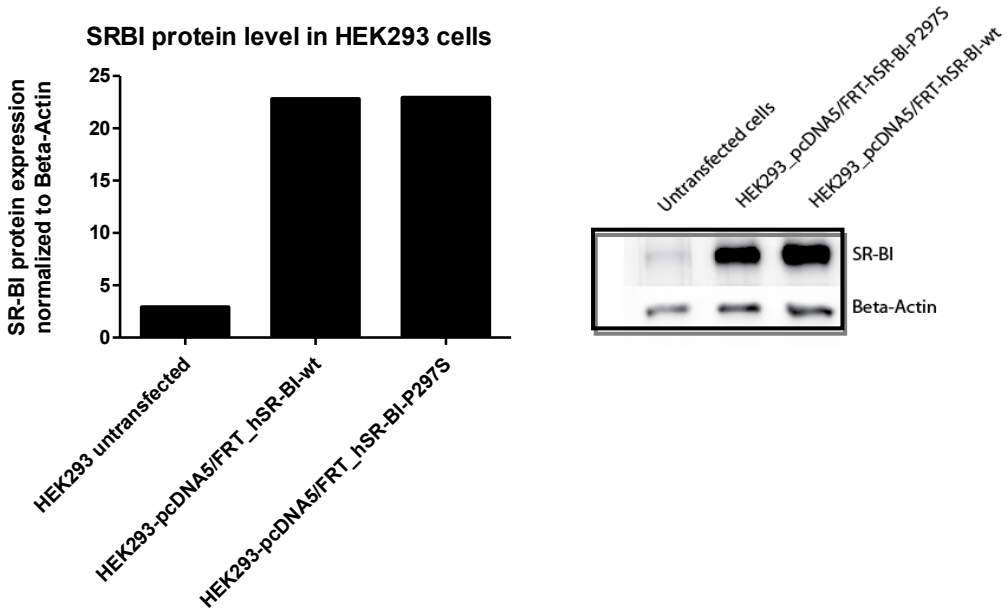


Figure 15. SR-BI protein level in HEK293/Flp-In cells. Untransfected cells were used as control. In the graph no standard deviation is shown because one sample per cell line was used for analysis. SR-BI (82kDa) values were normalized to Beta-Actin (42kDa; endogenous control).

SR-BI protein levels are 8-fold increased in transfected cell lines (Fig. 15). This effect is clearer seen in HEK293/Flp-In cells than in subcloned HuH7 cells as the protein level of untransfected HEK293 cells is much lower than in HuH7 cells. Of note, expression of both mutant and wildtype SR-BI resulted in equal expression levels. This makes both cell lines an ideal tool for studying SR-BI function.

5.2.3 Cellular Association Experiments in HEK293/Flp-In cells

HEK293/Flp-In cells were cultured in 12-well plates and incubated with Sodium [^{125}] iodide labeled HDL (10 µg/ml) for one hour at 37°C. The experimental set-up was the same as in the subcloned HuH7 cellular association experiments.

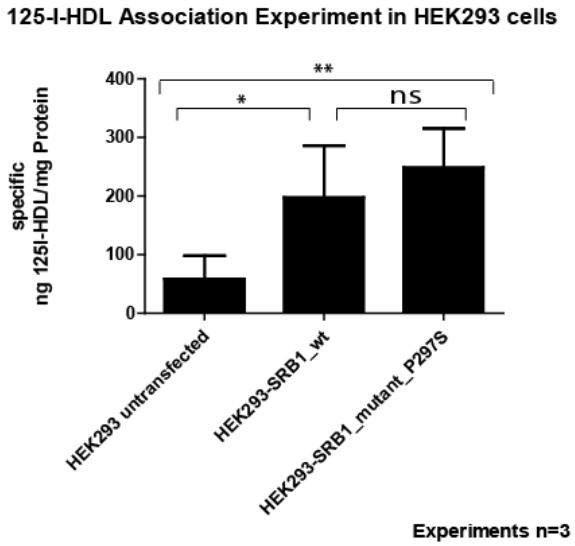


Figure 16. Cellular association of [^{125}] iodide labeled HDL in HEK293/Flp-In cells. Untransfected cells were used as control. These data represent three independent experiments with the same experimental set-up. For statistical analysis, ANOVA (analysis of variance) was used.

Transfected cells show higher cellular HDL association compared to untransfected HEK293/Flp-In cells (Fig. 16). Comparing the cells transfected with either wildtype SR-BI or mutant SR-BI (P297S) no significant differences in cellular association were found. In contrast to the results in HuH7 cells (compare Fig. 9), SR-BI overexpression significantly increased HDL association.

5.2.4 Holo-HDL particle uptake in HEK293/Flp-In cells

Stably transfected HEK293 cells (SR-BI_wt and SR-BI_P297S) were used to observe differences in HDL endocytosis. They were seeded in 24-well plates on glass cover slips and incubated with Alexa568 labeled HDL (50 µg/ml) at 37°C for one hour. Cells were fixed and holo-HDL particle uptake was analyzed by fluorescence microscopy.

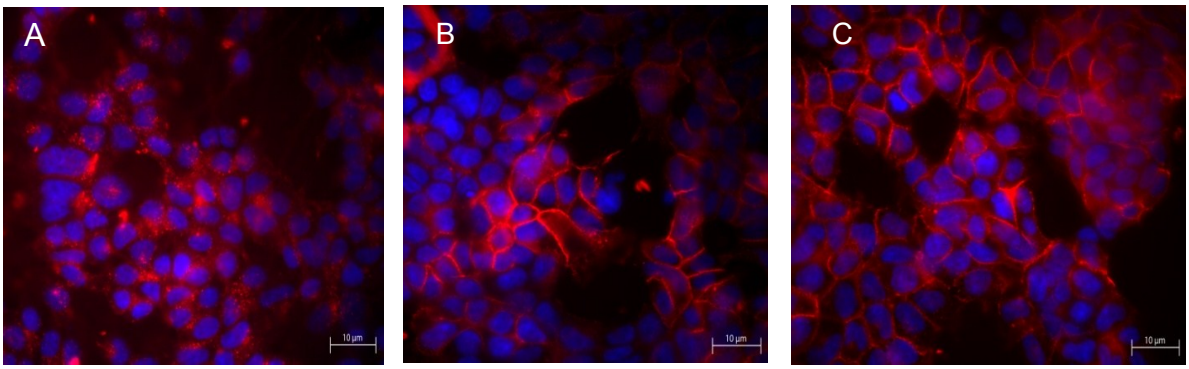


Figure 17. Holo-HDL particle uptake in HEK293/Flp-In cells. Untransfected cells (A), SR-BI wildtype transfected cells (B) and SR-BI mutant (P297S) transfected cells were used for analysis. Alexa568 labeled HDL is shown in red, nuclei are stained with DAPI (blue).

As shown in figure 17, HDL was mainly localized intracellularly in untransfected cells (Fig. 17 A). In addition, both transfected cell lines displayed intense plasma membrane staining, indicating increased HDL binding. No differences between wildtype and mutant SR-BI were observed. These data indicate, that increased HDL association upon SR-BI transfection (compare Fig. 17) is mainly due to increased HDL binding and not because of HDL uptake.

5.2.5 Selective uptake in HEK293/Fpl-In cells

Selective upake analysis was performed simultanously with HuH7 cells using the same conditions. Cells were cultured in 12-well plates and incubated with ^{3}H-cholesteryl ester and sodium [^{125}] iodide double labeled HDL (10 µg/ml) at 37°C for one hour.

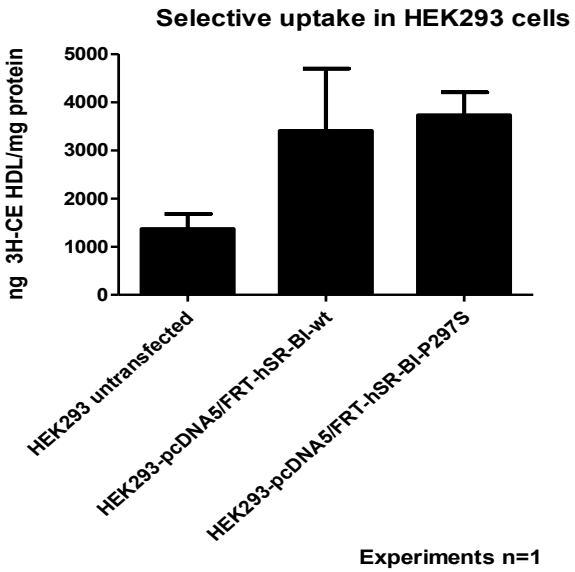


Figure 18. Selective uptake in HEK293/Flp-In cells. Untransfected cells were used as control. SR-BI wildtype and mutant (P297S) transfected cells are shown beside. The data represents one experiment.

Both transfected cell lines display a higher selective uptake compared to untransfected cells (Fig. 18). SR-BI wildtype and SR-BI mutant (P297S) transfected cells show no difference in selective uptake.

5.2.6 Lipid uptake analysis in HEK293/Fpl-In cells

HEK293 cells were grown in 24-well plates on glass cover slips and incubated with 50 µg/ml Bodipy labeled HDL at 37°C for one hour. Cells were fixed and lipid uptake was analyzed by fluorescence microscopy.

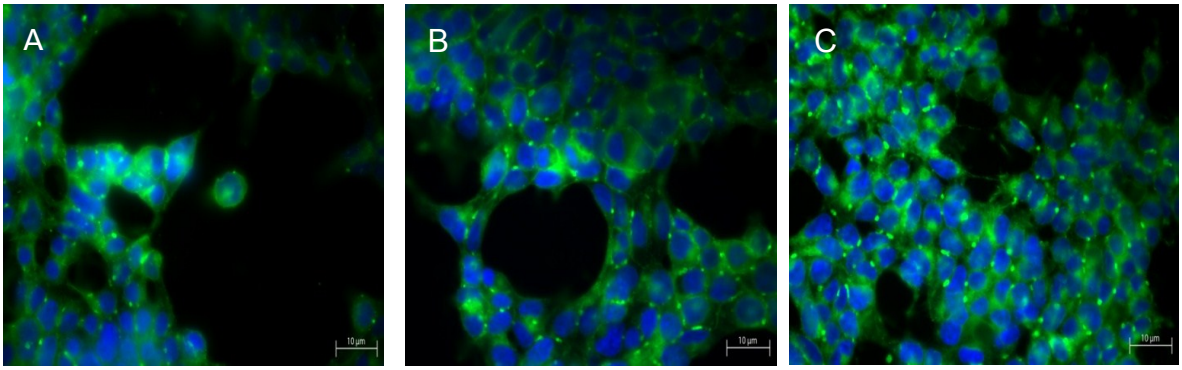


Figure 19. Bodipy cholesterol staining in HEK293/Flp-In cells. Untransfected cells (A), wildtype SR-BI transfected cells (B) and SR-BI mutant (P297S) transfected cells (C) are depicted. DAPI was used for nucleus staining (blue), Bodipy labeled HDL-cholesterol is shown in green.

There is no difference in the amount of lipid uptake among transfected cells (Fig. 19 B/C) and compared to untransfected cells (Fig. 19 A). The intracellular distribution of HDL-cholesterol also tends to be similar in all cell lines.

5.2.7 Distribution of free cholesterol in HEK293/Flp-In cells

Possible differences in free cholesterol distribution comparing SR-BI_wt and SR-BI_P297S transfected cells were investigated by Filipin III staining. Cells were grown in 24-well plates on glass cover slips and incubated with Filipin III at room temperature for 30 minutes (protected from light). Fluorescence microscopy was used for analysis.

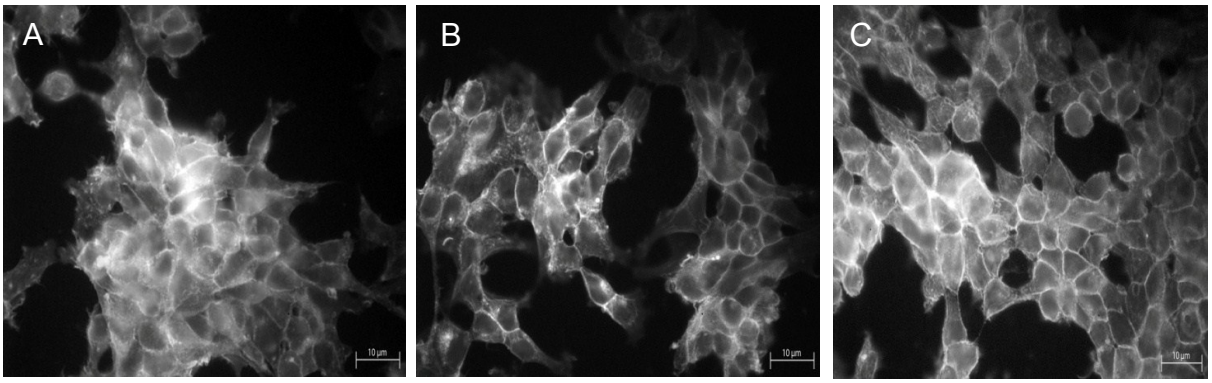


Figure 20. Free cholesterol distribution of HEK293/Flp-In cells. Untransfected cells (A), SR-BI wildtype transfected cells (B) and SR-BI mutant (P297S) transfected cells (C) wer used for analysis.

Figure 20 demonstrates elevated levels of free cholesterol levels in both transfected cell lines compared to untransfected cells.

6 Discussion

SR-BI is mainly expressed in the liver, steroidogenic organs, macrophages and ovaries [21]. In mice lacking SR-BI and apoE, myocardial infarctions were observed as a consequence of atherosclerotic lesions in their coronary ateries [16].
In SR-BI knockout mice, HDL-cholesterol levels are increased. Cholesteryl-ester-rich HDL particles were larger already on chow diet [15].
In addition, mice carrying a disrupted SR-BI promotor displayed a 53% decreased expression of the receptor in the liver. Therefore, a lower hepatic selective uptake of HDL-cholesteryl ester uptake and higher plasma HDL-cholesterol levels were observed [24].
Despite the meaningful studies in SR-BI knockout mice, the situation in humans is different. Human subjects express, in contrast to rodents, cholesteryl ester transfer protein (CETP). As mentioned in the introduction, humans carry most of their cholesterol in LDL fractions while mice transport cholesterol mainly in HDL particles. This route via CETP is an alternative for HDL-cholesteryl esters to be delivered from the blood circulation to the liver [7].
Studies using SR-BI knockout/CETP transgenic mice („humanized mice“) focused on the importance of SR-BI and CETP in humans. The obtained results did not clarify the role of SR-BI in human metabolism and need further investigation.

The newly discovered SR-BI mutation P297S found in patients with elevated levels of HDL cholesterol was the main focus in the paper of Dr. Kuivenhoven [21]. Despite the small statistical power of this study, the obtained results add to SR-BI research. Most interesting was the fact, that no association with increased atherosclerosis was found although HDL cholesterol levels were elevated in carriers. Further, reduced CE selective uptake was seen in murine primary hepatocytes transfected with SR-BI_P297S but HDL cellular association was similar in wildtype and mutant (P297S) transfected cells [21].

The aim of my study was to investigate the role of SR-BI in human HDL metabolism. Therefore, human cell lines were used for stable transfection with either wildtype human SR-BI or mutant human SR-BI_P297S. Possible differences in selective

uptake, cellular association, free cholesterol distribution, lipid uptake and holo-HDL particle uptake were investigated.

Stable HuH7 cells were generated by transfection with the pcDNA3 vector containing either wildtype or mutant (P297S) SR-BI. The SR-BI gene is expressed under the control of a CMV promotor in this vector. HuH7 cells were used for analysis as they represent hepatic cells. Prior to subcloning, the effects of transfection on SR-BI expression and holo-HDL particle uptake were insignificant (data not shown). High endogenous SR-BI expression in these hepatic cells might account for this finding. Further, these low effects could possibly be a consequence of CMV promoter down-regulation in hepatic cells [25]. To test this hypothesis, we treated transfected cells with phorbol myristate acetate (PMA), which was reported to increase CMV promoter activity [25]. Indeed, western blot analysis of treated cells showed slightly elevated SR-BI expression after PMA treatment (data not shown). However, this effect was not significant and accordingly, no alteration in holo-HDL particle uptake was observed.
To gain better effects, HuH7 cells were subcloned. SR-BI mRNA expression levels were twofold higher in SR-BI_wt_subclone I HuH7 cells. In SR-BI_P297S transfected cells, a lower effect of subcloning was seen (Fig. 7). On protein level, subcloning was only efficient in SR-BI_P297S_subclone II HuH7 cells and SR-BI_wt transfected cells dispayed nearly no difference before and after subcloning (Fig. 8).
HDL cell association was not significantly elevated in subcloned HuH7 cells after SR-BI overexpression. Interestingly, untransfected HuH7 cells and SR-BI_P297S transfected cells show a similar cellular association status (Fig. 9). This could point to the fact that the mutation (P297S) in the extra-cellular domain has a functional role in HDL-interaction.
In both transfected cell lines, holo-HDL particle uptake was higher compared to untransfected cells. Between the transfected cell lines, no significant difference was observed (Fig. 10). These results agree with the data of the cellular association experiments.
Besides SR-BI, CD36 is also an important receptor in this context as it mediates the uptake of HDL in the liver. CD36-knockout mice display increased plasma HDL cholesterol levels [26]. The presence of this receptor in HuH7 cells might be also a reason why there are no differences in transfected cells (wt SR-BI and SR-BI_P297S) looking on cellular association and holo-HDL particle uptake experiments. CD36 can

also mediate the uptake of HDL and can therefore influence cellular association or HDL-uptake experiments.

The efficiency of selective uptake was different from expectations as it was nearly the same in all HuH7 cell lines. Both transfected cell lines displayed a slightly increased selective uptake compared to untransfected cells, even in SR-BI_P297S transfected cells (Fig. 11). Due to this low effect of SR-BI overexpression between transfected HuH7 cells and untransfected cells, other mechanism for cholesteryl ester uptake are considered.

The intracellular transport of cholesteryl ester begins in caveolae, a specialized form of lipid rafts. Caveolin-1 (21 kDa) and SR-BI partially co-localize in many tissues. Caveolin reduces the concentration gradient of cholesterol between cells and the surface of lipoproteins by stabilizing the membrane cholesterol. This process may be responsible for a reduced cholesteryl ester (CE) uptake [5].

Especially in hepatocytes, the normally low levels of caveolin-1 do not influence CE uptake [11].

As in HEK293 and HuH7 cells the caveolin-1 levels are also low, caveolin is excluded from the idea being responsible for the low CE uptake in HuH7 cells.

Some other studies show that cells which do not express SR-BI do also have CE selective uptake activity. As an example, LDL-receptor-related protein (LRP) can mediate selective uptake of CE in SW872 adipocytes [27].

Both cell lines, HuH7 and HEK293 do express LRP. Maybe this protein is also involved in selective CE uptake in HuH7 cells as the CE content in untransfected cells is relatively high compared to transfected cells.

Kuivenhoven [21] described a markedly decreased [^{3}H]cholesteryl ester uptake from HDL in primary hepatocytes transfected with SR-BI_P297S compared to wildtype SR-BI.

In our study, this difference was not clearly seen. One point of criticism in Kuivenhoven's paper is the missing of untransfected cells which are not mentioned in the published data.

Gas chromatography (GC) was also used to analyze cholesteryl ester (CE) content of subcloned HuH7 cells. The data came up to our expectation as CE levels were more than two-fold increased in SR-BI_wt transfected HuH7 cells compared to untransfected cells. SR-BI_P297S transfected cells and untransfected cells nearly displayed the

same level of CE (data not shown). These results comply with the data of Kuivenhoven [21] but require repetition to evaluate their significance.
Furthermore, lipid uptake was investigated comparing all HuH7 cell lines but no differences were observed in Bodipy labeled cholesterol levels, irrespective to the P297S mutation in SR-BI (Fig. 12). Additionally, no different localization of Bodipy cholesterol was notized. HDL particles are also able to exchange cholesterol directly through the plasma membrane of cells without the help of a receptor.
Another approach in this content was the analysis of the cholesterol content via GC analysis of subcloned HuH7 cells cultured in medium containing FBS.
GC results showed a clearly increased cholesterol content in SR-BI_wt transfected cells compared to untransfected cells. SR-BI_P297S transfected cells and untransfected cells displayed nearly the same cholesterol level, indicating a functional role of P297S mutation of SR-BI (data not shown). As this experiment was done once it needs to be repeated to determine the significance of the resulting data.
Free cholesterol distribution was different in subcloned HuH7 cells (Fig. 13). In untransfected HuH7 cells, free cholesterol was located near the nucleus (central) while in both transfected cell lines most of free cholesterol was located in the plasma membrane (peripher). Between transfected cells, no differences were observed.
In another study it was obtained that SR-BI influences cellular lipid organization and deposition. SR-BI was shown to be linked to changes in plasma membrane composition [13]. This suggests cholesterol redistribution in our HuH7 cell models upon SR-BI overexpression, irrespective of the P297S mutation.
Total cholesterol levels, investigated via GC analysis, were compatible with cholesterol levels and CE levels in subcloned HuH7 cells (data not shown).
Triglyceride (TG) levels were also a focus of GC analysis and resulted in ten-fold increased TG levels in HuH7 cells transfected with SR-BI_wt compared to untransfected cells. Mutant SR-BI (P297S) transfected cells to the contrary, displayed similar TG levels as untransfected cells (data not shown). The reason for the high TG levels in HuH7 cells transfected with SR-BI_wt is unknown. One possible explanation might be the interaction of SR-BI with VLDL or LDL which was available in the serum.

To sum up, no significant differences between SR-BI_wt and SR-BI_P297S transfected cells were observed. Due to endogenous SR-BI expression or the down-regulation of the CMV promotor in hepatic cells, the effect of SR-BI overexpression was not clearly demonstrated. Other receptors, e.g. CD36 and LRP were also mentioned to affect cholesterol uptake in hepatic cells.
The normal expression, function and localization of the murine SR-BI in hepatocytes in vivo is dependend on a cytoplasmatic adaptor protein PDZK1. This protein contains four PDZ domains (PDZ1 – PDZ4) and influences expression and function of the SCARB1 [28].
In PDZK1 knock-out mice, SR-BI protein expression was suppressed in hepatocytes [29] and mice exhibited large HDL particles and elevated plasma cholesterol levels [28].
Maybe better effects would be obtained in my study after co-transfection of the PZDK1 gene into HuH7 cells, as is controls SCARB1 expression in the liver. This approach has not been investigated now.

Awaiting better effects of SR-BI overexpression, HEK293/Flp-In cells were used for further experiments. These cell line is advantageous, as they have low levels of endogenous SR-BI expression and no down-regulation of the CMV promotor is expected.
Successful overexpression was confirmed by qRT-PCR (Fig. 14) and western blotting (Fig. 15). SR-BI overexpression (wildtype and P297S) was equal in HEK293 cells.
Also HDL association was significantly increased in both transfected HEK293 cells, which comes up to our expectation (Fig. 16). Irrespective of the P297S mutation, no significant difference was observed compared to SR-BI_wt transfected cells. These results comply with the HuH7 data and lead to the suggestion that the mutation has no functional role in HDL binding.
Furthermore, an effect was seen in holo-HDL particle uptake in HEK293 cells. Untransfected cells displayed higher levels of intracellularly HDL, in both transfected cell lines intense plasma membrane staining was observed indicating HDL binding (Fig. 17). The uptake pattern was different from HuH7 cells, where most HDL was found endocytosed. The reason for the high HDL binding status is unknown. One possible explanation might be that the kidney does not metabolize as much cholesterol as the liver and cholesterol cannot be secreted by the kidney.

Therefore, cholesterol is delivered back to the HDL particle leading to a longer cellular interaction.
In HEK293 cells, no differences in selective uptake (Fig. 18), lipid uptake (Fig. 19) and free cholesterol distribution (Fig. 20) were obtained in both transfected cell lines. We were suprised to find a higher CE content via GC analysis in SR-BI_P297S transfected HEK293 cells than compared to SR-BI_wt transfected cells (data not shown). However, these data need to be repeated carefully, as this experiment was performed only once.
Free cholesterol distribution was the same in all HEK293 cell lines and only the free cholesterol levels were elevated in both transfected cell lines (Fig. 20). Why Filipin III staining is localized at the plasma membrane in untransfected cells as well as both transfected cell lines, is unknown. These data do not indicate cholesterol redistribution by SR-BI which was better seen in HuH7 cells.
GC analyis was also used to determine TG content in HEK293 cells. Surprisingly, SR-BI_P297S transfected cells displayed higher TG levels compared to SR-BI_wt transfected cells (data not shown). Total cholesterol levels, investigated via GC analysis, were compatible with cholesterol levels and CE levels in HEK293 cells (data not shown).
The reason for the higher cholesterol and the higher TG status in cells transfected with the mutation (P297S) is unknown. These data might indicate a role of the mutation in lipid efflux as well. Future studies will be required to further investigate in detail the differences of SR-BI function in liver cells and kidney cells.

The importance of SR-BI in cholesterol metabolism stimulates researchers to investigate other SR-BI mutations as well.
The large extracellular domain of SR-BI contains several cysteine residues. One mutation in the C323 residue was investigated to reduce HDL binding activity of the receptor and it also decreases CE selective uptake compared to SR-BI_wt cells [30]. Cys384 is also a mutation of a cysteine residue of SR-BI leading to reduced receptor lipid uptake activity [31].
Another mutation in the C-terminal transmembrane domain of the SR-BI receptor (Q445A) displays similar effects of HDL binding and HDL CE selective uptake compared to SR-BI_wt transfected cells [22].

Two novel mutations of SR-BI were identified: S112F and T175A. Both occur in the large extracellular loop of SR-BI and are related to high HDL- cholesterol levels [32]. Interestingly, the mother of one proband was found to carry S112F but has low HDL cholesterol levels. This leads to the suggestion, that this individual might carry an additional mutation keeping HDL cholesterol levels low [32].

In summary, no significant differences of SR-BI_wt and SR-BI_P297S in subcloned HuH7 and HEK293/Flp-In cells were observed. This leads to the suggestion, that P297S mutation is no disturbing factor in HDL cellular association and CE selective uptake of SR-BI which is in opposite to the data of Kuivenhoven [21].
My data together with the conflicting data from the literature on the effect of SR-BI mutations, argue for careful interpretation of the results in the context of the model systems used. Beside consideration of species dependent differences, the presence of other HDL receptors as well as SR-BI cofactors needs to be taken into account.

Possible future approaches to further study the effect of the SR-BI mutation P297S would include a stable co-transfection with the PDZK1 gene to stabilize the HDL receptor.
Further, the bidirectional flux of cholesterol is suggested to be higher in HEK293/Flp-In cells compared to HuH7 cells. Therefore, oleic acid could be used to suppress efflux of cholesterol as it mediates esterification of cholesterol which then remains intracellularly. This would enable a better analysis of CE selective uptake.

7 Abstract

Scavenger receptor class B type I (SR-BI) plays a central role in high-density lipoprotein (HDL) metabolism. Its major role in cholesterol uptake and reverse cholesterol transport (RCT) has been confirmed in several mouse studies. Importantly, SR-BI overexpression in mice has anti-atherogenic effects preventing cardiovascular diseases, such as coronary heart disease (CHD). However, mice insufficiently reflect human lipid metabolism. Thus, the role of SR-BI in humans is unclear.
A newly described missense mutation of SR-BI (P297S) was found in patients with elevated levels of HDL cholesterol. We aimed to investigate the influence of this mutation on the interaction between SR-BI and HDL.

I established cell lines derived from human hepatic HuH7 cells stably over-expressing wildtype and mutant SR-BI. As the effect of over-expression in Huh7 cells was modest (2-fold), stable cell lines were also established in HEK293 cells. Here, we achieved 8-fold over-expression. No difference in HDL association or selective uptake was observed in wildtype vs mutant SR-BI expressing cells using both model systems. Further, fluorescence microscopy revealed increased holo-HDL particle uptake upon expression of both wildtype and mutant SR-BI in HuH7 cells. In contrast, only HDL binding to the plasma membrane was increased in both transfected HEK293 cells irrespective of the SR-BI mutation. Finally, SR-BI over-expression induced re-distribution of free cholesterol to the plasma membrane in Huh7, but not in HEK293 cells. Again, no difference between wildtype and mutant SR-BI was observed.
Taken together, our study indicates that the P297S mutation in SR-BI does not influence HDL binding, uptake or selective uptake in stably over-expressing HuH7 and HEK293 cells. Some of the findings in this study are partially in contrast with prior published data. However, species dependent differences and the participation of other HDL receptors are important to be considered for interpretation in the respective model system.

8 Zusammenfassung

Scavenger receptor class B type I (SR-BI) spielt eine zentrale Rolle im HDL-Stoffwechsel. Seine Funktion in der zellulären Cholesterinaufnahme und im „Reversen Cholesterin Transport“ (RCT) – ein Prozess, der vor Gefäßerkrankungen schützt - wurde in Studien an Mäusen bestätigt. Mäuse spiegeln den menschlichen Fettstoffwechsel jedoch nur ungenügend wider und daher ist die Rolle von SR-BI im humanen Lipidstoffwechsel noch unklar.
Eine rezente Publikation beschreibt eine Mutation des SR-BI Rezeptors (P297S) in Patienten mit erhöhtem HDL-Cholesterin. Das Ziel unserer Studie war, den Einfluss dieser SR-BI Mutation (P297S) auf die Interaktion zwischen SR-BI und HDL zu untersuchen.
Ich habe Zelllinien aus humanen HuH7 Leberparenchymzellen etabliert, die einerseits die wildtyp und andererseits die mutierte Form von SR-BI stabil exprimieren. Da der Effekt der Überexprimierung in diesen Zellen sehr gering war (doppelte SR-BI Expression), wurden auch stabile Zelllinien aus HEK293 Zellen generiert. In diesen Zellen erreichten wir eine 8-fache Überexpression. In beiden Zellmodellen konnten wir jedoch keine Unterschiede zwischen wildtyp und mutiertem SR-BI bezüglich der Bindung von HDL an die Zellen oder der selektiven Aufnahme von Cholestertinestern messen. Mittels Fluoreszenzmikroskopie konnten wir zeigen, dass die Expression von sowohl wildtyp als auch mutiertem SR-BI die HDL-Aufnahme in HuH7 erhöht. Im Gegensatz wird in den HEK293 Zellen ein erhöhtes Binden von HDL an die Plasmamembran durch SR-BI Expression hervorgerufen, ungeachtet der SR-BI Mutation. Weiters induziert die SR-BI Überexprimierung eine Umverteilung von freiem Cholesterin in Richtung Plasmamembranin HuH7 Zellen, nicht jedoch in HEK293 Zellen. Auch hier wurde kein Unterschied zwischen Wildtyp und mutiertem SR-BI festgestellt.
Zusammenfassend zeigen meine Daten keinen Effekt der SR-BI Mutation P297S auf die selektive Cholesterinesteraufnahme, die zelluläre Bindung von HDL oder die Aufnahme von HDL in beiden Zellmodellen auf. Einige Ergebnisse dieser Studie widersprechenden bisher veröffentlichten Resultaten. Daher ist es wichtig, speziesabhängige Unterschiede und die Beteiligung anderer HDL Rezeptoren für die Interpretation im jeweiligen Zellmodell zu berücksichtigen.

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