# THE ROLE OF LIPOPROTEIN RECEPTORS IN THE UPTAKE OF MATERNALLY CIRCULATING BETA-CAROTENE BY THE DEVELOPING TISSUES

by

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ABSTRACT OF THE DISSERTATION

The role of lipoprotein receptors in the uptake of maternally circulating beta-carotene by the developing tissues

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In human diet, beta-carotene is the principal source of vitamin A, which is essential for normal embryonic development. Beta-carotene supplementation in pregnant women has proven to be effective to counteract the detrimental effects of vitamin A deficiency. Our lab has unequivocally shown that, in mice, intact beta-carotene supplemented during pregnancy can cross the placenta and can be efficiently metabolized by the embryo to generate retinoids. However, the mechanisms by which beta-carotene is acquired by the developing tissues from the maternal blood and key regulators involved in such uptake are unknown. Furthermore, whether maternal dietary vitamin A intake and status influence the uptake of this carotenoid by the developing tissues has never been investigated.

Our study aimed at addressing these issues by investigating whether and how maternal vitamin A status and dietary intake affect uptake of beta-carotene by the developing tissues. Furthermore, since LDL-receptor internalizes majority of beta-carotene carrying lipoproteins, we investigated whether this receptor mediates beta-carotene uptake by maternal as well as the developing tissues.

We showed that marginal vitamin A deficient status of mothers, in the case of mice lacking the retinol esterification enzyme (LRAT) and retinol-binding protein (RBP), led to an increased beta-carotene uptake in the placenta. It also led to an altered metabolism of beta-carotene in the developing tissues. Furthermore, we demonstrated that upon beta-carotene supplementation of wild-type dams, placental transcription of chylomicron-remnant receptor LRP1 and VLDL-receptor were donwregulated. We also showed that maternal excessive dietary vitamin A intake resulted in negligible accumulation of beta-carotene in embryos. Our data indicate that the regulation of placental lipoprotein assembly and secretion could control embryonic acquisition of beta-carotene.

We demonstrated that LDL-receptor (LDLr) may partially mediate the beta-carotene uptake in maternal liver. Under the conditions of higher serum lipoprotein accumulation, in the case of mice lacking LDLr, it may facilitate placental beta-carotene uptake. Our preliminary data suggested that regulation of placental lipoprotein assembly and secretion may control the transfer of beta-carotene to the embryo in absence of both maternal and placental LDLr. Overall,

these findings expanded our knowledge of mechanisms and key regulators of beta-carotene uptake in the developing tissues.

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## List of Abbreviations

| | |
|---|---|
| ADH | alcohol dehydrogenase |
| ApoB | apolipoprotein B |
| Apobec-1 | apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1 |
| ARAT | acyl-coA:retinol acyltransferase |
| CD36 | cluster determinants 36 |
| CMOI | beta-carotene-15,15'-oxygenase (also known as BCMOI, BCO, BCMOOX, β-CD, β-diox) |
| CMOII | beta-carotene-9',10'-oxygenase (also known as BCDO2, BCO2, β-diox-II) |
| DGAT1 | diacylglycerol acyltransferase 1 |
| dpc | days *post coitum* |
| HDL | high-density lipoprotein |
| ISX | intestine-specific-homeobox |
| LDL | low-density lipoprotein |
| LPL | lipoprotein lipase |
| LRAT | lecithin:retinol acyltransferase |
| MTTP | microsomal triglyceride transfer protein |
| RA | retinoic acid |
| RAE | retinol activity equivalent |
| RALDH | retinal dehydrogenase |

| | |
|---|---|
| RAR | retinoic acid receptor |
| RARE | retinoic acid response element |
| RBP | retinol-binding protein (also known as RBP4) |
| RDH | retinol dehydrogenase |
| RE | retinyl ester |
| REH | retinyl ester hydrolase |
| ROH | retinol |
| RXR | retinoid X receptor |
| SR-BI | scavenger receptor class B Type I |
| STRA6 | stimulated by retinoic acid 6 |
| TC | total cholesterol |
| TG | triglycerides |
| TTR | transthyretin |
| VAD | vitamin A deficiency |
| VLDL | very low-density lipoprotein |

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### Chapter 1



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**Chapter 3**

# Chapter 1: Introduction and literature review

### *1.1. General introduction*

Vitamin A is an essential nutrient that maintains human health by controlling vision, immune system, reproduction, development, and cell growth (Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011) mammals, vitamin A cannot be synthesized *de novo* and, therefore, it must be obtained from the diet either as preformed vitamin A or as pro-vitamin A carotenoids, among which beta-carotene is the most abundant and best characterized precursor of vitamin A. During embryonic development, a balanced supply of vitamin A is vital as both the deficiency and excess of this micronutrient causes embryonic defects (Abu-Abed et al., 2001; Clagett-Dame and Knutson, 2011; Lohnes et al., 1994). Beta-carotene supplementation of vitamin A deficient pregnant women has been shown to be an effective dietary intervention strategy to counteract the detrimental effects of vitamin A deficiency on the developing fetus (Christian et al., 1998; Christian et al., 2000b). In addition, our lab has unequivocally shown that beta-carotene supplied to pregnant mice can cross the placental barrier to reach the embryo where beta-carotene can be used to generate vitamin A *in situ* (Kim et al., 2011). Despite these (and other) studies that establish beta-carotene as an important source of retinoids for the developing mammalian embryo, the mechanisms by which this pro-vitamin A carotenoid is taken up by the developing tissues are not known. Nevertheless, this knowledge is crucial for developing effective beta-carotene supplementation strategies during pregnancy to maintain general health and wellbeing of the pregnant women and the developing fetus.

The present studies were performed to address this important issue and to expand our knowledge in regards to the maternal-fetal metabolism of beta-carotene.

### *1.2 Classification of beta-carotene among carotenoids*

Carotenoids are $C_{40}$ tetraterpenoid pigments that accumulate in the plastids of the plants providing the characteristic bright yellow, red and orange color of many fruits and vegetables (Norris et al., 1995). In plants, these pigments function as structural and functional accessory of the photosynthetic apparatus (Bartley and Scolnik, 1995). Specifically, being able to effectively quench free radicals, singlet oxygen, and reactive oxygen species, they serve as light-harvesting pigments and protect plants against photooxidative stress (Stahl and Sies, 2012a, b). Plant carotenoids also function as precursors of various hormones and play a role in attracting pollinators and other agents that contribute to seed dispersal (Cutler and Krochko, 1999).

Over 800 carotenoids have been recognized in nature, and based on their structures, they are divided into two major classes: carotenes and xanthophylls. Carotenes are long hydrocarbon chains, some of which are hydroxylated or are devoid of any functional group that makes them non-polar. Xanthophylls contain oxygen species and are terminally dihydroxylated, which makes them more polar than carotenes (von Lintig, 2010; Wisniewska et al., 2006). Beta-carotene, alpha-carotene and beta-cryptoxanthin are common carotenes whereas lutein,

zeaxanthin, meso-zeaxanthin, astaxanthin and canthaxanthin are characterized as xanthophylls (Bendich and Olson, 1989).

Based on their ability to form vitamin A, carotenoids are also classified as pro-vitamin A and non-provitamin A carotenoids. Pro-vitamin A carotenoids, such as alpha-carotene, beta-carotene, and beta-cryptoxanthin yield vitamin A and their metabolites upon enzymatic and non-enzymatic cleavage (Eroglu and Harrison, 2013; Handelman et al., 1991; Krinsky and Yeum, 2003; Wu et al., 1999; Zorn et al., 2003). Thus, as vitamin A precursors, they exert a number of beneficial effects in mammals, regulating several crucial biological functions including vision and embryogenesis (Bendich and Olson, 1989). Lycopene and xanthophylls are non-provitamin A carotenoids as they are not known to generate any vitamin A metabolites, yet they exhibit several beneficial functions, such as lowering the risks of chronic diseases including prostate cancer, cardiovascular diseases, and age-related macular degeneration to name a few (Agarwal and Rao, 2000; Campbell et al., 2004; Chen et al., 2001; Cho et al., 2004; Lindshield et al., 2007; Loane et al., 2008a; Loane et al., 2008b; Meyers et al., 2013; Rao and Agarwal, 2000; Snodderly et al., 2004; Story et al., 2010; Yue et al., 2012).

Beta-carotene is the most abundant plant-derived carotenoid present in the human diet and tissues (Olson, 1994). It is also the best-characterized precursor of vitamin A (Krinsky and Johnson, 2005). A molecule of beta-carotene contains 40 carbon atoms with 15 conjugated double bonds and 2 beta-ionone rings at the both ends. These structural properties make beta-carotene highly

hydrophobic and non-polar in nature. Plant sources such as spinach, carrots, and sweet potatoes are excellent sources of beta-carotene. Furthermore, tissues (especially liver) of animals that have consumed food-containing beta-carotene are also excellent sources of this pro-vitamin A carotenoid in the human diet (Krinsky and Johnson, 2005; Olson, 1994; Ross, 2005).

### *1.3 Beta-carotene as a source of retinoids*

About 30% of dietary vitamin A intake in western countries is contributed by beta-carotene, but in the developing countries it represents the most abundant, and in some instances the sole source of vitamin A (Olson, 1994; West, 2000; West et al., 2011). The most predominant form of beta-carotene found in nature is all-*trans*-beta-carotene, even though other isomers such as 9-*cis*-, 13-*cis*-and 15-*cis*-beta-carotene also exist (von Lintig and Vogt, 2004). Due to its symmetrical structure, all-*trans*-beta-carotene is the only carotenoid that can yield 2 molecules of all-*trans*-retinaldehyde upon its symmetrical cleavage by the enzyme beta, beta-carotene-15, 15'-oxygenase (CMOI or BCMO1). All-*trans*-retinaldehyde formed upon such cleavage can be oxidized by the action of enzymes of the retinaldehyde dehydrogenase family (RALDHs) to generate all-*trans*-retinoic acid (atRA), the biologically active form of vitamin A (Napoli and Race, 1988). Retinoic acid is a transcriptional regulator of over 500 genes, many of which are involved in embryogenesis (Balmer and Blomhoff, 2002; Morriss-Kay and Ward, 1999; Niederreither et al., 1999). It functions as a ligand for

specific nuclear receptors, retinoic acid receptor (RAR) or retinoid X receptor (RXR) that by forming homo- or hetero- dimers regulate the transcription of the target genes (Al Tanoury et al., 2013; Chen and Evans, 1995; Kastner et al., 1995; Kurokawa et al., 1995; Leblanc and Stunnenberg, 1995; Mangelsdorf et al., 1995). Under conditions of excessive retinoic acid generation, enzymes that belong to the family of cytochrome P450 can carry out oxidative degradation of retinoic acid to generate more polar compounds, like 4-hydroxy or 4-oxo retinoic acid, which are believed to be transcriptionally inactive (D'Ambrosio et al., 2011). A member of this family, Cyp26A1, is one of the main enzymes that catalyze this reaction and is highly expressed during embryonic development (Abu-Abed et al., 2001; White et al., 1996).

Alternatively, retinaldehyde can be reversibly reduced to retinol (Pares et al., 2008), the alcohol form of vitamin A, which is most commonly referred to as “vitamin A” (D'Ambrosio et al., 2011). Retinol dehydrogenase 10 (RDH10) is one of the enzymes that catalyzes the conversion of retinol to retinaldehyde (Pares et al., 2008). Retinol can then be esterified mainly by the action of lecithin: retinol acyltransferase (LRAT) to generate retinyl ester, which is the storage form of vitamin A in various tissues, predominantly liver, but also lung, adipose tissues, heart and kidney. LRAT constitutes about 90% of the catalytic activity utilized for retinyl ester formation, especially in the liver (Batten et al., 2004; Golczak et al., 2012; Herr and Ong, 1992; O'Byrne et al., 2005; Ruiz et al., 1999; Saari and Bredberg, 1989; Yost et al., 1988; Zolfaghari and Ross, 2000). Unlike LRAT, that

uses lecithin as a fatty acid donor, another retinyl esterase activity catalyzes the formation of retinyl esters from Acyl coA and retinol (acylcoA:retinol acyltransferase, ARAT) (Ross, 1982). Such activity has been reported in rat liver microsomes (Ross, 1982), in the rat intestine (Helgerud et al., 1982), in human intestinal Caco-2 cell line (Quick and Ong, 1990) and more recently in mouse embryo (Dixon et al., 2013). However, the molecular identity of the enzyme that catalyzes this reaction has yet to be confirmed. The enzyme acylCoA:diacylglycerol acyltransferase 1 (DGAT1) has been shown to function as an ARAT that esterifies retinol to retinyl esters in murine skin (Shih et al., 2009) and intestine (Wongsiriroj et al., 2008), under conditions in which dietary retinol exceeds the capacity of LRAT to esterify it.

Beta-carotene-9',10'-oxygenase (CMOII or BCDO2) is a second mammalian carotenoid cleavage enzyme (Amengual et al., 2011b; Kiefer et al., 2001; von Lintig, 2012). CMOII cleaves beta-carotene excentrically to generate a beta-ionone ring and apocarotenals, which can be converted to one molecule of retinaldehyde possibly by chain shortening (von Lintig et al., 2005; von Lintig and Vogt, 2004). The resulting retinaldehyde can then follow the metabolic pathways described above. Figure. 1 shows a schematic representation of the metabolic pathways through which beta-carotene generates various forms of vitamin A.

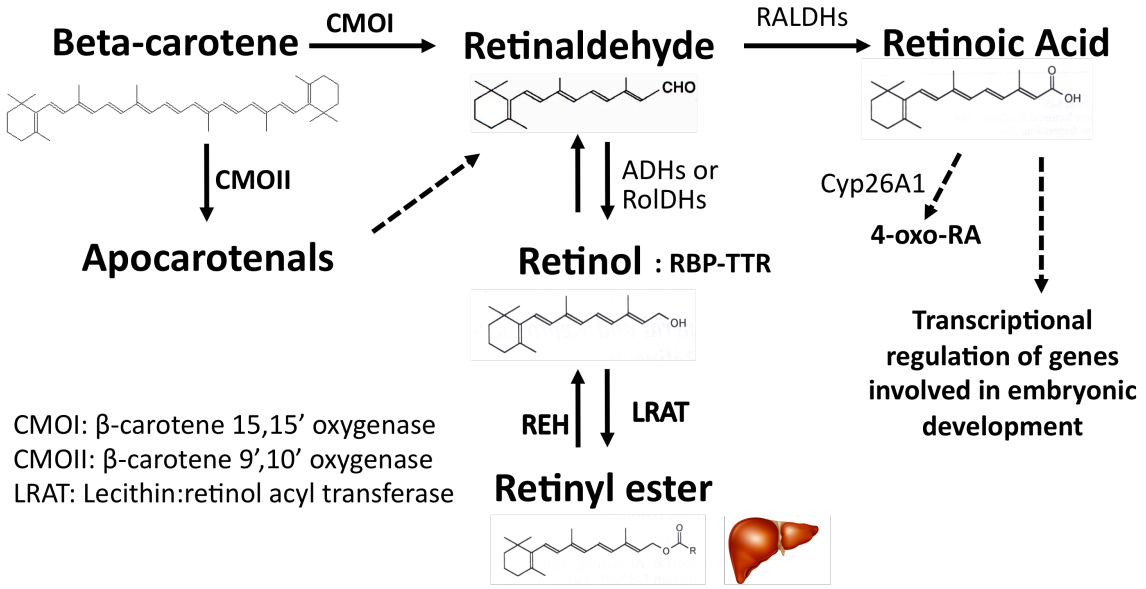



**Figure 1. Cleavage of beta-carotene to generate retinoids.** Intact beta-carotene is cleaved either symmetrically by beta, beta-carotene-15, 15'-oxygensase (CMOI) or asymmetrically by beta-carotene-9',10'-oxygenase (CMOII) to generate retinaldehyde which can be oxidized by retinaldehyde dehydrogenses (Raldhs) to form biologically active form of vitamin A i.e. retinoic acid. Retinoic acid binds to nuclear receptors RAR and RXR to control the transcription of over 500 genes, many of which are involved in embryogenesis. Excessive retinoic acid can be oxidized by the action of enzymes that belong to cytochrome p450 family. Cyp26A1 is the main enzyme that performs this catalysis. Alternatively retinaldehyde can be reduced by retinol dehydrogenase (Rdh) to retinol, which can then be esterified by the action of lecitin:retinol acyl transferase (LRAT) to retinyl esters, which contributes to majority of stores of vitamin A in tissues.

In humans and mice, both beta-carotene cleavage enzymes (CMOI and CMOII) are expressed in various adult tissues, including liver and adipose, as well as in developing tissues such as placenta, yolk sac and embryo (Kim et al., 2011; Lindqvist and Andersson, 2004; Lindqvist et al., 2005; Paik et al., 2001; Redmond et al., 2001; von Lintig et al., 2005; Wang et al., 2004)

CMOI has a cytosolic localization within the cells (Lietz et al., 2012a; von Lintig et al., 2005) and is the main enzyme that cleaves beta-carotene to generate vitamin A in the adult tissues *in vivo* (Hessel et al., 2007; Tourniaire et al., 2009). Indeed, when fed a diet rich in beta-carotene (final concentration of beta-carotene in the diet = 1 mg/g) for 16 weeks, mice lacking CMOI (CMOI-/-) were unable to cleave dietary beta-carotene and accumulated this carotenoid in large quantities in tissues, as seen by orange color of the adipose tissues (Hessel et al., 2007). In accordance with this effect, vitamin A levels fell dramatically in various tissues of the knockout mice, including lung, kidney, testis and uterus (Hessel et al., 2007).

CMOII, although able to cleave beta-carotene *in vitro*, has much broader substrate specificity. Kinetic studies on beta-carotene cleavage enzymes with other carotenoids substrates showed that CMOI has a lower Michaelis-Menten constant, *K*m value (1 to 10μM range) for beta-carotene than for other carotenoids, whereas lycopene seems to be the preferred substrate of CMOII (Ford et al., 2010; Leung et al., 2009; Lietz et al., 2010; Lindshield et al., 2008;

Paik et al., 2001; Redmond et al., 2001; von Lintig and Vogt, 2004). The contribution of asymmetric cleavage by CMOII to the generation of vitamin A *in vivo* is thought to be minor compared to that of CMOI (Amengual et al., 2011b; Lietz et al., 2012a; von Lintig, 2010). Although these enzymes are widely recognized as carotenoid cleavage enzymes, whether they also function in alternate pathways has been a subject of interest. Indeed, CMOII has been recently shown to act as a carotenoid scavenger and a gatekeeper of apoptotic pathway in mitochondria, which is the site of its subcellular localization (Amengual et al., 2011b; Lindshield et al., 2008; Lobo et al., 2012).

### *1.4 Intestinal absorption of beta-carotene and its plasma levels*

The small intestine is responsible for absorbing dietary lipids as well as lipid-soluble vitamins, including beta-carotene, to subsequently deliver them to the peripheral tissues. Even though the human intestine abundantly expresses the main beta-carotene cleavage enzyme CMOI, complete intestinal conversion of all of the ingested beta-carotene to vitamin A practically never happens. Indeed, about 17-45% of the ingested beta-carotene is released into the circulation in its intact, uncleaved form (Hickenbottom et al., 2002; Novotny et al., 1995; von Lintig, 2010). A variable enzymatic activity of CMOI associated with various polymorphisms in the *CMOI* gene seems to be responsible for the less efficient cleavage (Ferrucci et al., 2009; Hendrickson et al., 2012; Leung et al., 2009; Lietz et al., 2012b). In contrast, mice and other rodents cleave the majority

of the ingested beta-carotene in the intestine, and only upon intake of supra-physiological quantities, this pro-vitamin-A carotenoid can be detected in their circulation (van Vliet, 1996). Other animal models such as Mongolian gerbils (Lee et al., 1998; Pollack et al., 1994), domestic ferrets (Gugger et al., 1992; Ribaya-Mercado et al., 1989; White et al., 1993) and pre-ruminant calves (Bierer et al., 1993; Chew et al., 1993; Poor et al., 1993) also absorb dietary beta-carotene in its intact form and have plasma and tissue distribution of beta-carotene similar to humans (Parker, 1989; Renzi et al., 2012; Schmitz et al., 1991; Sy et al., 2012; Yakushina and Taranova, 1995). In humans, Individual serum beta-carotene concentrations are directly correlated to its dietary consumption and are often regarded as the biomarker of individual beta-carotene status (Borel et al., 2009). In humans, beta-carotene levels in plasma (and tissues) rise after ingestion of increased amounts of this micronutrient (Bjornson et al., 1976; Chopra et al., 2000; Johnson et al., 2000; Parker, 1996). The concentration of intact beta-carotene in the plasma is a good indicator of bioavailability of ingested beta-carotene (Dimitrov et al., 1988; Hallfrisch et al., 1994), which represents the amount of the prov-vitamin A carotenoid absorbed by the intestinal epithelia that is available for the use by the body. Certain single nucleotide polymorphisms (SNPs) in the genes involved in the lipid metabolism such as apolipoproteins B (apoB), A-IV (apoA-IV), scavenger receptor B-1 (SRB-1) and lipoprotein lipase (LPL) also have shown to affect the plasma levels of beta-carotene and individual carotenoid status (Borel et al., 2007; Herbeth et al., 2007).

In addition to genetic factors influencing the efficiency of beta-carotene cleavage activity, the bioavailability of beta-carotene is affected by various factors such as nature of food matrix (Castenmiller et al., 1999), fat content of the diet, type of fat, digestibility of fat-soluble components in the diet (Roodenburg et al., 2000), bile acids, interactions with other carotenoids and individual variations due to endogenous activity of the digestive enzymes (Dimitrov et al., 1988; van het Hof et al., 1999; van Het Hof et al., 2000).

### *1.5 Transport of beta-carotene from the intestine to target tissues*

Beta-carotene is transported in the bloodstream to be acquired and stored by various tissues within the body. In mammals, liver is a major organ that accumulates large quantities of beta-carotene. Second to liver, beta-carotene also accumulates in adipose tissue, kidney, skin and lung in significant amounts (Schmitz, Poor et al. 1991, Sy, Gleize et al. 2012). Apart from these, beta-carotene is also detected in tissues such as adrenal gland, testes, mammary gland which serve as minor stores of this provitamin-A carotenoid (Yamanushi et al., 2009).

Once in the enterocytes, intact beta-carotene is packaged into lipoproteins of intestinal origin to be secreted in the circulation and then transported to the various tissues of the body (Erdman et al., 1993; Parker, 1996; Traber et al., 1994). The incorporation of beta-carotene and other non-polar lipids in the core

of lipoprotein particles is a necessary step in order to enable the transport of these highly hydrophobic molecules in the bloodstream aqueous environment.

### *Circulating lipoproteins: structure and function*

A typical lipoprotein particle consists of a lipid core that contains less polar components such as cholesteryl esters, triglycerides and other non-polar lipids such as retinyl esters and carotenoids. The lipid core is surrounded by more polar components such as unesterified cholesterol and phospholipids. Each lipoprotein is associated with specific surface apolipoproteins that act as cofactors in various reactions involved in lipid metabolism as well as facilitate their receptor mediated endocytosis in various tissues (Fredrickson et al., 1967). Based on the structure, size and components, plasma lipoproteins can be categorized into 4 major classes such as chylomicrons and their remnants, very-low density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Fredrickson and Gordon, 1958; Fredrickson et al., 1967; Olson and Vester, 1960).

Chylomicrons are generated in the intestine and their triglyceride core is hydrolyzed to release fatty acid due to catalytic activity of the enzyme lipoprotein lipase (LPL), located on the surface of endothelial cells of capillaries especially in adipose and muscle (Higgins and Fielding, 1975; Nilsson-Ehle et al., 1980). This hydrolysis reaction, that requires apolipoprotein C-II (apoC-II) as a co-factor, results in the formation of chylomicron remnants. These are smaller in size as

compared to nascent chylomicrons, and still contain triglycerides, cholesterol and lipid soluble vitamins, such as beta-carotene and vitamin A. Chylomicron remnants also contain apolipoprotein B-48 (apoB-48) and apolipoprotein E (apoE). The apoE moiety is recognized and required for their receptor-mediated endocytosis in the liver and other tissues in order to be cleared from the bloodstream (Brown and Goldstein, 1979, 1983).

Very-low density lipoproteins (VLDL) are lipoprotein particles of hepatic origin. They are associated with three apolipoproteins namely apoE, apoB-100 and apoC-I. Upon acquiring apoC-II and additional apoE in the circulation, they transform from nascent to mature VLDL (Gibbons, 1990; Shelness and Sellers, 2001). VLDL can either be taken up by the by extrahepatic tissues or can also be transformed with the aid of LPL (which hydrolyzes the TG core of VLDL) to smaller intermediate-density particles (IDL), which are associated with apoB-100 and apoE. Further hydrolysis of core triglycerides in IDL by LPL leads to formation of lipoproteins containing greater amount of cholesteryl esters than triglycerides. These are LDL particles mainly associated with apoB-100 (Brown and Goldstein, 1979, 1983).

HDL is the smallest lipoprotein particle and is the most abundant one among all the human plasma lipoproteins (Segrest, 1986). Most macrophages and fibroblasts can form HDL. Assembly of HDL requires apoA-I and apoA-II, which can be secreted from liver and intestine to a varying degree, in mammals. Intestinal apoA-I and apoA-II can be transferred to HDL during hydrolysis of

chylomicrons, hence HDL arise largely as products of chylomicron metabolism (Segrest, 1986). HDL particles are smallest and densest because of their higher protein content and lower cholesterol content as compared to other lipoproteins. They also have the highest cholesterol:triglyceride ratio (Segrest, 1986). HDL plays a key role in reverse cholesterol transport, promoting the efflux of excess cholesterol from peripheral tissues and its returns to the liver (Fielding and Fielding, 1995; Lewis and Rader, 2005).

***Tissue uptake of lipoprotein particles via lipoprotein receptors***

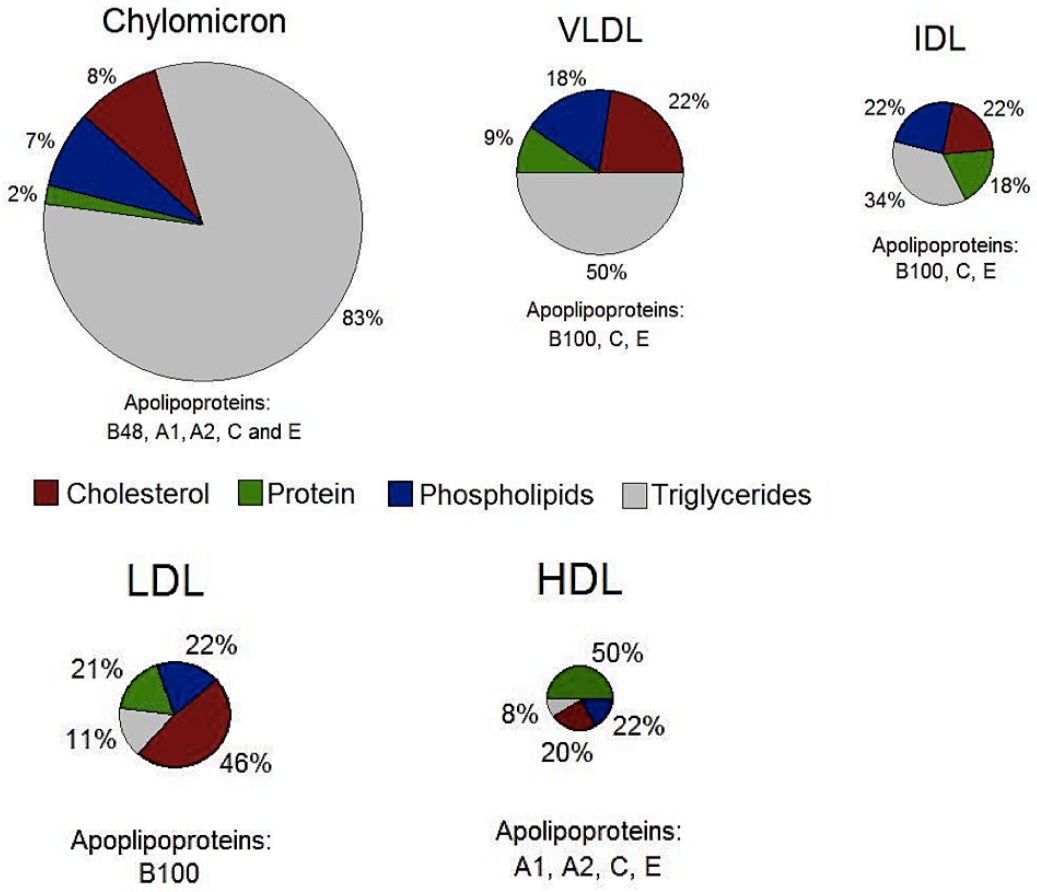
Once lipoproteins are transported in the circulation and reach the target tissues, they are taken up by specific cell surface receptors known as lipoprotein receptors. In the case of LDL, for example, cell surface receptor for LDL binds to apoB-100 and LDL is internalized via clathrin-coated pits, which fuse with early endosomes. The receptor dissociates from LDL due to changes in the pH and the LDL particles are subsequently delivered to endosomal compartments for catabolism and the receptor is recycled back to the cell surface (Goldstein et al., 1985; Mortimer et al., 1995).

The LDL-receptor family is a group of proteins that recognize apoE and apoB moieties associated with lipoproteins such as chylomicron and their remnants, VLDL, IDL and LDL, which are lipoproteins of hepatic and extra-hepatic origin (Beisiegel et al., 1989; Bjornson et al., 1976; Brown and Goldstein, 1979; Gibbons, 1990; Ishibashi et al., 1996; Lewis, 1971; Martins et al., 2000;

Parker, 1996). Circulating chylomicron remnants are mainly cleared by hepatic and extra-hepatic LDL-receptor related protein 1 (LRP1), the so-called remnant receptor (Beisiegel et al., 1989; Mahley et al., 1989; May et al., 2007). In addition, the remnants can also be taken up by LDL receptor (LDLr) *via* recognition of apoE (Martins et al., 2000). VLDL can be taken up by a cell surface receptor known as VLDL-receptor (VLDLr), which recognizes apoE. Each LDL particle contains one copy of apoB-100, which is required for clearance by LDLr.

Members of the scavenger receptor family such as cluster of differentiation 36 (CD36) and scavenger receptor type B-1 (SRB-1) are important regulators of lipid uptake across various tissues. Among these, SRB-1 is the cell surface receptor that specifically takes up HDL (Rigotti et al., 1997) and aids in reverse cholesterol transport by this lipoprotein.

The lipoprotein receptors are expressed in various tissues such as intestine, heart, skeletal muscle, adipose tissues as well as the developing tissues i.e. placenta and the embryo (Tiebel et al., 1999; Wassef and Quadro, 2011).




| **Lipoproteins** | **Receptors** |
|---|---|
| Chylomicrons and remnants (CM) | LRP1, LDLr |
| Very-low density lipoproteins (VLDL) | VLDLr, LDLr |
| Low- and intermediate-density lipoproteins (IDL, LDL) | LDLr |
| High-density lipoproteins (HDL) | SRB-1 |

**Figure. 2. Types of serum lipoproteins, their composition and the receptors mediating their tissue uptake.**

Adapted from www.robertbarrington.net and (Acton et al., 1996; Ishibashi et al., 1994; Ishibashi et al., 1996)

***Distribution of beta-carotene in circulating lipoproteins***

Owing to the non-polar nature of the molecule, beta-carotene could be mainly incorporated into the hydrophobic core of lipoproteins along with other classes of lipids such as cholesteryl esters and retinyl esters, to be transported through the lymphatic system to the systemic circulation. Distribution of beta-carotene in circulating lipoproteins in healthy human plasma has been studied, showing that beta-carotene is associated with all the classes of circulating lipoproteins to a varying degree (Bjornson et al., 1976; Ganguly et al., 1959; Johnson and Russell, 1992; Ribaya-Mercado et al., 1995; van Vliet et al., 1995). Plasma response in men upon oral beta-carotene dose was studied by Johnson and Russell in 1992. They found an early rise (3hr-6hr post consumption) in the beta-carotene concentration in the chylomicrons, these levels peaked at 6 hours and dropped afterwards due to clearance from the circulation. Beta-carotene in the VLDL fractions was elevated through day 3 post-consumption, due to hepatic resecretion of these particles. Relatively low amounts of beta-carotene were associated with IDL and the highest increase in beta-carotene concentration was observed in LDL at day 2-2.5-post consumption. HDL particles also contained beta-carotene at later timepoints. The results from this study suggested that beta-carotene can be incorporated into all the classes of lipoproteins to a varying degree and its incorporation at various time points indicates a dynamic exchange of this provitamin A carotenoid among various lipoproteins (Johnson and Russell,

1992). A similar study by Traber *et al* also showed the first appearance of the provitamin A carotenoid in the chylomicrons upon oral beta-carotene administration in 9 subjects. This was followed by a rise in beta-carotene concentrations in VLDL. Beta-carotene was detected in the HDL only during chylomicron clearance up to 11 hrs post-consumption whereas its concentrations in the LDL increased for up to 48 hrs (Traber et al., 1994). Ribaya-mercado *et al* reported that upon beta-carotene consumption, LDL fractions in the plasma of 10 women subjects showed highest rise in the beta-carotene levels followed by a rise in beta-carotene levels in HDL and VLDL fractions (Ribaya-Mercado et al., 1995). These findings were in agreement with published reports from other researchers which showed that about 60-70% of intact beta-carotene is transported in LDL in the human circulation (Bjornson et al., 1976). In the fasting circulation, beta-carotene is mainly associated with VLDL and LDL, the lipoproteins containing apoE and apoB moieties (Parker, 1996) and that in postprandial circulation beta-carotene in triglyceride-rich fraction i.e. VLDL is a marker of intestinal beta-carotene absorption (van Vliet et al., 1995).

Among other species, ruminants are considered a good model to study carotenoid transport as they are inefficient cleavers of intestinal carotenoids just like humans and have similar plasma and distribution of carotenoids. Oral administration of beta-carotene in calves followed by analysis of their plasma lipoproteins showed higher percentage of beta-carotene associated with LDL sub-fraction (Chew et al., 1993). Ashes *et al* reported HDL as the beta-carotene

carrier in the bovine circulation (Ashes et al., 1984). Overall, studies in various species in regards to beta-carotene transport emphasize that beta-carotene can be transported in association with various lipoproteins in the circulation, even though difference in the type of lipoprotein that transports the provitamin A carotenoid may exists among different species.

### *1.6 Lipoprotein receptors mediate uptake of beta-carotene by adult tissues*

*In-vivo* experimental evidence of mechanisms underlying the cellular and tissue uptake of beta-carotene are scarce, most of which pertain to only adult tissues. Among these, a study by Rigotti et al, 1997 showed that intestinal absorption of beta-carotene was regulated by scavenger receptor type B-1, the apical HDL receptor of the enterocytes (Rigotti et al., 1997). More recent studies unraveled that expression of SRB-1 is controlled by an intestinal specific homeobox (ISX), which in turn is regulated by retinoic acid (Lobo et al., 2010). In the case of excessive vitamin A intake, retinoic acid *via* RAR induces expression of ISX, which acts by attenuating expression of both SRB-1 and CMOI, thus repressing intestinal absorption and metabolism of beta-carotene (Lobo et al., 2010; Seino et al., 2008).

In addition, a few in-vitro studies have shown that carotenoid uptake could be receptor mediated in drosophila, human intestinal Caco-2 cells and human embryonic kidney (HEK) cells. Carotenoid uptake, specifically the uptake of beta-carotene, was shown to be mediated by the scavenger receptors SRB-1 and

CD36 (Borel et al., 2013; During et al., 2005; During and Harrison, 2007; Kiefer et al., 2002; Reboul and Borel, 2011; van Bennekum et al., 2005).

To date, no studies have investigated the uptake of beta-carotene by mammalian developing tissues.

### *1.7 Vitamin A and embryonic development*

In all vertebrates, including mammals, vitamin A is a critical regulator of various adult physiological processes such as vision, spermatogenesis, cell differentiation, apoptosis, and immunity, and is also an essential micronutrient for adequate growth and development (Blomhoff and Blomhoff, 2006; D'Ambrosio et al., 2011). Both, deficiency and excess of retinoids (vitamin A and its derivatives) in the developing tissues are known to cause a number of birth defects and stillbirth. Studies in rats first showed that offspring of dams bred on a vitamin A-deficient diet died before or at birth and displayed a spectrum of malformations affecting numerous organs and tissues. These malformations are collectively known as vitamin A deficiency syndrome (VAD). The features of VAD include cleft face and palate, small or absent eyes, malformation of the forelimbs and abnormalities in the urogenital system, in the heart and large vessels, as well as in the central nervous system (Dersch and Zile, 1993; Morriss-Kay and Ward, 1999; Thompson et al., 1964; Warkany and Schraffenberger, 1946; Wilson et al., 1953; Wilson and Warkany, 1948). On the other hand, teratogenic effects of excess of retinoids may also affect various developing organs, such as heart,

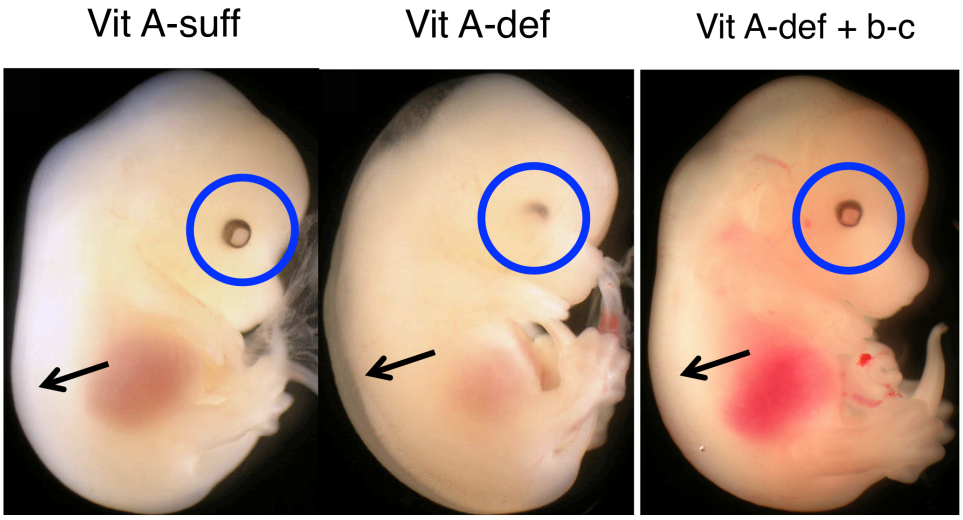
skull, skeleton, nervous system, brain, eyes and craniofacial structures (Brockes, 1989; Kochhar et al., 1993; Rosales and Kjolhede, 1993). Therefore, a balanced supply of retinoids is essential for normal embryonic development.

For its adequate supply of retinoids, the developing embryo relies entirely on the various retinoid forms circulating in the maternal bloodstream (Marceau et al., 2007; Spiegler et al., 2012), which need to cross the placental-fetal barrier to reach the developing tissues. The main form of vitamin A circulating in the maternal bloodstream is retinol bound to retinol-binding protein (RBP), its sole specific carrier in the circulation (Quadro et al., 2003). Retinol-RBP is secreted mainly from the liver, the principal storage site of vitamin A in the body, and serves as the main retinoid source in the fasting state (Blomhoff et al., 1990). Studies by Kawaguchi et al. (Kawaguchi, Yu et al. 2007) provided evidence that Stra6 acts as a cell surface receptor for retinol-RBP complex and mediates its intracellular flux. Even very early in development Stra6 is widely expressed in the mouse embryo, as well as in placenta (Bouillet, Sapin et al. 1997; Sapin, Bouillet et al. 2000). However, in the fed state, retinyl ester packaged in chylomicrons and their remnants represent another abundant source of vitamin A for the developing tissues (Harrison and Hussain, 2001). A recent study from our lab has shown that lipoprotein lipase expressed in the placenta acts in concert with other placental lipoprotein receptors, such as LRP1 and LDLr, to facilitate the acquisition of postprandial retinyl esters by the developing tissues (Wassef and Quadro, 2011).

Although at lower concentrations, other forms of vitamin A circulate in the bloodstream such as retinoic acid bound to albumin (W. S. Blaner, 1994), water soluble glucuronides of both retinol and retinoic acid (Barua et al., 1988) and provitamin A carotenoids in lipoproteins (Napoli and Race, 1988; Olson, 1989). These could be used as source of retinoids by the developing tissues.

### *1.8 Role of beta-carotene in mammalian embryonic development*

Recent studies from our laboratory provided a clear proof that beta-carotene circulating in the maternal bloodstream can not only cross the placental barrier to reach the embryo but can also serve as an endogenous source of retinoids essential for normal growth and development (Kim et al., 2011). By using mice lacking both RBP and CMOI (CMOI-/-RBP-/-), our laboratory demonstrated that double knockout dams maintained on a vitamin A deficient dietary regimen during pregnancy developed embryos (both CMOI-/-RBP-/- and CMOI+/-RBP-/-) with severe malformations associated with VAD syndrome. However, supplementation of beta-carotene to the double knockout dams at mid-gestation substantially reduced these malformations in embryos that expressed one copy of CMOI (CMOI+/-RBP-/- embryos) (Figure. 3). The rescue of the embryonic phenotype was also accompanied by elevated levels of retinoic acid (Figure. 3). This study confirmed that beta-carotene can be taken up by the embryos in its intact form and can be cleaved in situ by the action of embryonic CMOI to generate retinoids locally.




| C+/-R-/- | Normal | RBP-/- like | Cleft | Excen | Total E |
|---|---|---|---|---|---|
| A def | 0 % | 62 % | 36 % | 2 % | 89 |
| A def +b-c | 61 % | 32 % | 0 % | 7 % | 82 |

**Figure 3. Embryonic malformations associated with vitamin A deficient status of CMOI-/-RBP-/- (C-/-R-/-) dams is rescued upon maternal supplementation with beta-carotene.** C-/-R-/- dams were mated with RBP-/- males to conceive embryos carrying one copy of CMOI (C+/-R-/-). When the dams were fed a vitamin A deficient diet (< 0.02 IU of vitamin A/g of diet), the embryos showed malformed eye and peripheral edema, as indicated by the circle and arrow, respectively, and also displayed other signs of vitamin A deficiency, such as cleft face and palate (36%). Embryos from the dams fed a vitamin A sufficient diet (29 IU of vitamin A/g of diet) were phenotypically normal. Upon beta-carotene supplementation [a single dose (250 µl) of 20-50 µg of beta-carotene/g of body weight], malformations in the embryos of the dams fed a vitamin A deficient diet were rescued resulting in 61% normal embryos, and none

of the embryos showing cleft. The phenotype of the rescued embryos was accompanied by elevated levels of retinoic acid, measured by LC/MS.

### *1.9 Public health significance and gaps in knowledge*

Demonstrating at the molecular level that beta-carotene can serve as a local source of vitamin A for the developing tissues was an important step stone also from a public health standpoint. Vitamin A deficiency, for instance, is a global epidemic in the developing countries affecting hundreds of millions of individuals worldwide (West, 2002; West et al., 2011). Interestingly, more recently, it has also been recognized that even in the developed countries certain populations are at a greater risk of vitamin A deficiency (Grune et al., 2010; Strobel et al., 2007; Weber and Grune, 2012). More importantly, beta-carotene supplementation of pregnant women as well as of infants and children has been shown to improve neonatal development and growth and to attenuate the symptoms associated with vitamin A-deficiency (Christian et al., 1998; Christian et al., 2000a; Christian et al., 2000b; West et al., 2011). On the other hand, consumption of large doses of dietary supplements and vitamins, including beta-carotene, has become very common recently (Mulholland and Benford, 2007) generating the need to investigate the effects of high-dose carotenoid intake at different stages of the lifecycle, including pregnancy and development. Notably, 3% of all children born in the United States have a major malformation at birth, and 70% of these are of unknown etiology (Zile, 2004). Thus, it is of obvious importance to understand what are the mechanisms and factor that can influence beta-carotene availability and utilization (i.e. beta-carotene cleavage activity) in the mammalian developing tissues, i.e. its placental uptake, transfer to and

consequent uptake by the embryo. This is a crucial issue that needs to be elucidated to develop appropriate beta-carotene supplementation strategies in various populations and, more generally, to provide new insights into appropriate dietary practices during pregnancy. Surprisingly, this knowledge is currently lacking.

In adult tissues, where elevated expression levels and enzymatic activity of CMOI have been demonstrated not only in intestine, but also in other tissues, such as liver and kidney (Lindqvist and Andersson, 2002, 2004; Paik et al., 2001), it has been shown that nutrients and nutritional status, especially in regards to vitamin A, influence availability and metabolism of the provitamin A carotenoid in a tissue specific manner (During et al., 1998; Yeum and Russell, 2002). Specifically, the activity of intestinal CMOI in rats decreased with increasing vitamin A content (van Vliet et al., 1996). CMOI activity in the rat livers also increased by 70% upon beta-carotene supplementation (van Vliet et al., 1996). On the other hand, another study in rats showed that intestinal CMOI activity was 20-30% higher in the case of severe vitamin A deficiency (Villard and Bates, 1986). Furthermore, studies in the chick have shown a consistent decrease in carotenoid content in the serum, liver and skin with increased vitamin A intake through the diet (Dua et al., 1966). In support of these studies, a dose dependent decrease in intestinal CMOI activity upon oral-supplementation of beta-carotene (up to 79%), as well as retinoids such as retinyl acetate (up to 79%), apo-8'-carotenal (up to 56%) and all trans-retinoic acid (up to 79%) and 9-

cis-retinoic acid (up to 67%) was shown in rats. This study also showed that retinoic acid treatment in chickens decreased intestinal CMOI expression, thus confirming that beta-carotene and retinoids regulate intestinal CMOI expression by a transcriptional feedback mechanism *via* an interaction with retinoic acid receptors (Bachmann et al., 2002). In addition, given that this provitamin A is carried in the maternal bloodstream by lipoprotein particles, it is reasonable to postulate that its placental uptake could be mediated by the key players that regulate lipid uptake from lipoproteins in adult and developing tissues (During and Harrison, 2007; Ganguly et al., 1959; Johnson and Russell, 1992; Ribaya-Mercado et al., 1995; Voolstra et al., 2006). Indeed, maternal circulating lipids could be taken up by the placenta through receptor-mediated endocytosis of lipoprotein particles and subsequently transferred to the embryos (Duttaroy, 2009; Woollett, 2005, 2011). In humans as well as in mice, the syncytiotrophoblast layer of the placenta, which is of embryonic origin and forms the barrier between maternal circulation and the embryo (Watson and Cross, 2005) expresses lipoprotein receptors including LRP1, LDLr, SRB-1, VLDLr as well as the enzyme LPL as shown in Figure 4 (Desoye et al., 2011) Furthermore, a few studies have shown that human placenta can secret apoB-100 containing lipoproteins (LDL) and can synthesize as well as secret apolipoproteins E into the fetal circulation (Madsen et al., 2004; Richardson et al., 1996; Rindler et al., 1991).

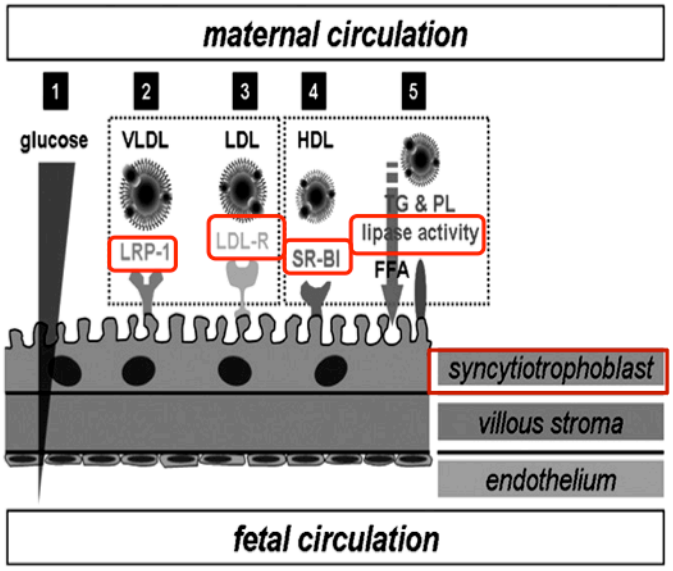



**Figure 4. Schematic representation of placental syncytiotrophoblast layer expressing various lipoprotein receptors.** Receptor for chylomicron remnants, LRP1; low-density lipoprotein receptor, LDLr; scavenger receptor type B1, SRB-1; and lipoprotein lipase, LPL.

Adapted from (Desoye et al., 2011)

### *1.10 Specific aims*

To further our understanding of maternal-fetal metabolism of beta-carotene, we set out to investigate *in vivo* the uptake of beta-carotene by the developing tissues under various maternal vitamin A intake, and to identify the key players involved in this process. Specifically, we aimed at elucidating how maternal dietary vitamin A intake/status influences beta-carotene uptake in the developing unit and the role of the lipoprotein receptors in regulating such uptake.

Based on the current literature and previous data from our laboratory, we proposed the following aims to address these questions:

1) Aim 1: To investigate the influence of maternal dietary vitamin A status and intake on the uptake of beta-carotene by the placenta and the embryo.
2) Aim 2: To investigate the role of low-density lipoprotein receptor (LDLr) in mediating the uptake of beta-carotene in maternal and the developing tissues.

# Chapter 2: The influence of maternal vitamin A status and dietary intake during pregnancy on the uptake of beta-carotene by the developing tissues i.e. the placenta and the embryo

The data shown in this chapter are the results of a collaborative project with other members of the laboratory. Some of the results included in this chapter (part A) were published as below.

Lesley Wassef, **Varsha Shete**, Alice Hong, Elizabeth Spiegler, Loredana Quadro. 2012. Beta-carotene supplementation decreases placental transcription of LDL receptor-related protein 1 in wild-type mice and stimulates placental β-carotene uptake in a mouse model of marginal vitamin A deficiency. *J. Nutr.* 142(8): 1456-62.

Maternal nutrition during gestation has a profound effect on embryonic development as the developing embryo relies entirely upon maternal supply of various macro- and micronutrients. Specifically in the case of vitamin A, a compromised maternal vitamin A status as well as dietary variations in the vitamin A content during pregnancy have shown to result in several developmental abnormalities in the embryo (Clagett-Dame and DeLuca, 2002; Clagett-Dame and Knutson, 2011; Dua et al., 1966). To counteract the detrimental effects of vitamin A deficiency on the embryonic development, beta-carotene supplementation of the mothers has been proven as an effective strategy (West et al., 2011; West et al., 1999). Furthermore, our lab has demonstrated unequivocally that beta-carotene could act as local source of retinoids in the mouse embryos (Kim et al., 2011). The mechanisms underlying the uptake of this pro-vitamin A carotenoid as well as the factors influencing this process still need to be investigated and yet they are crucial to ultimately formulate appropriate nutrition guidelines during pregnancy for various populations of pregnant women worldwide.

In this study, we aimed at gaining insights into the effects of alterations of the maternal vitamin A status and dietary intake of vitamin A on the uptake of beta-carotene by the developing tissues i.e. placenta and the embryo.

### *2-1. Introduction*

Various forms of vitamin A acquired through maternal circulation are required for the normal growth and development of the embryo (Clagett-Dame and Knutson, 2011; Spiegler et al., 2012). The main form of vitamin A circulating in the maternal bloodstream is retinol bound to retinol-binding protein (RBP), its sole specific carrier in the circulation (Quadro et al., 2003). Retinol-RBP secreted mainly from the liver serves as the main retinoid source for the embryo in the fasting state (Blomhoff et al., 1990). In the fed state, retinyl ester packaged in chylomicrons and their remnants represent another source of vitamin A for the developing tissues (Harrison and Hussain, 2001), which can be acquired by the developing tissues by a mechanism facilitated by lipoprotein lipase (LPL) in the placenta in concert with other placental lipoprotein receptors, such as LRP1 and LDLr (Wassef and Quadro, 2011).

Although at lower concentrations, other forms of vitamin A circulate in the bloodstream such as retinoic acid bound to albumin (W. S. Blaner, 1994), water soluble glucuronides of both retinol and retinoic acid (Barua et al., 1988) and provitamin A carotenoids in lipoproteins (Napoli and Race, 1988; Olson, 1989). These latter could represent an important source of retinoids to the developing tissues, given that in a large part of the world beta-carotene from food of plant origin represent the only source of dietary vitamin A (Olson, 1994; Weber and Grune, 2012).

When the supplies of retinoids (vitamin A and its derivative) are

inadequate, proper embryonic growth and development cannot be achieved. A large number of studies over the past several decades have demonstrated that maternal vitamin A deficiency during gestation generates a wide and characteristic range of embryonic malformations, collectively known as vitamin A deficiency syndrome (VAD), in animal models such as rat (Thompson et al., 1964; Warkany and Schraffenberger, 1946; Wilson et al., 1953; Wilson and Warkany, 1948), quail (Dersch and Zile, 1993) and mouse (Morriss-Kay and Ward, 1999). The features of the VAD syndrome include: cleft face, palate and lip; small or absent eye; abnormality of the urogenital system; abnormality of the heart; and malformation of the forelimb. On the other hand, excessive maternal intake of vitamin A can lead to retinoid-toxicity in the embryo causing various congenital malformations often overlapping with those described in VAD (Kochhar et al., 1993).

Beta-carotene is the principal source of vitamin A in the human diet (Olson, 1994). It is transported in the circulation in association with various lipoprotein particles (Bjornson et al., 1976; Parker, 1996). It is taken up by various tissues, including the liver and the adipose, and is cleaved predominantly by the enzyme beta-carotene-15,15'-oxygenase (CMOI) to generate retinoids *in situ* (von Lintig et al., 2005; von Lintig and Vogt, 2004). Beta-carotene can cross the placenta and reach the embryo to serve as a local source of retinoids (Kim et al., 2011).

Studies in regards to the factors regulating beta-carotene tissue uptake and metabolism are limited to the adult tissues. Bioconversion of beta-carotene to generate vitamin A by the action of the cleavage enzyme CMOI has been shown to be regulated by vitamin A status of the animal and vitamin A content of the diet, in a tissue-specific manner (During et al., 1998; Yeum and Russell, 2002). Specifically, the activity of intestinal CMOI was shown to be inversely correlated with vitamin A content of the diet in the absence of any beta-carotene supplementation in rats (van Vliet et al., 1996). Whereas, when the rats were supplemented with a diet rich in beta-carotene for 6 weeks, the intestinal CMOI activity fell significantly. This study also showed that CMOI activity in the rat livers was not affected by vitamin A content of the diet but increased by 70% upon beta-carotene supplementation (van Vliet et al., 1996). Another study in the pregnant and virgin rats showed that intestinal CMOI activity was 20-30% higher in the case of severe vitamin A deficiency (Villard and Bates, 1986). Furthermore, studies in the chick have shown a consistent decrease in carotenoid content in the serum, liver and skin with increased vitamin A intake through the diet (Dua et al., 1966). In support of these studies, a dose dependent decrease in intestinal CMOI activity upon oral-supplementation of beta-carotene (up to 79%), as well as retinoids such as retinyl acetate (up to 79%), apo-8'-carotenal (up to 56%) and all trans-retinoic acid (up to 79%) and 9-cis-retinoic acid (up to 67%) was shown in rats. This study also demonstrated that retinoic acid treatment in chickens decreased intestinal CMOI expression, thus confirming that beta-carotene and

retinoids regulate intestinal CMOI expression by a transcriptional feedback mechanism *via* an interaction with retinoic acid receptors (Bachmann et al., 2002).

The intestinal absorption of beta-carotene has been shown to be controlled by a diet-responsive regulatory network coordinated by the intestinal homeobox gene ISX. This retinoic acid-induced transcription factor represses both the uptake and metabolism of beta-carotene under the conditions of excessive retinoic acid availability (Lobo et al., 2013; Lobo et al., 2010; Seino et al., 2008).

Data in regards to whether vitamin A status of the mothers affects beta-carotene uptake in the developing unit is extremely limited. Dimenstein and colleagues suggested that, in humans, conversion of beta-carotene to retinol in the placenta is controlled by the nutritional status of the mother, being particularly effective in vitamin A depleted state when maternal serum retinol is lower than 15 µg/dL (Dimenstein et al., 1996).

To gain a better understanding of the maternal factors that may influence uptake and metabolism by the developing tissues, we set out to investigate the effects of both, a marginal deficient vitamin A status of the dams and variations in the vitamin A content of their diet on the above-mentioned processes. Based on the current literature and previous studies from our laboratory, *we hypothesized that uptake and metabolism of maternally circulating beta-carotene by the developing tissues is influenced by maternal dietary vitamin A intake and status*.

Two sets of experiments were performed:

**Part A** of the study was designed to investigate the effect of beta-carotene supplementation on the uptake of this pro-vitamin A carotenoid by the developing tissues in a mouse model of marginal vitamin A deficiency.

**Part B** of the study was designed to investigate whether variations in the maternal dietary vitamin A content during gestation influence uptake of supplemented beta-carotene by the developing tissues and the key regulators involved in such mechanism.

### *2-2. Materials and Methods*

#### *2-2.1 Animals and diet*

**Part A:** In order to investigate the effect of beta-carotene supplementation on the uptake of this provitamin A by the developing tissues depending upon the maternal vitamin A status, wild-type and LRAT-/-RBP-/- (Kim et al., 2008b; Liu and Gudas, 2005) mice were maintained on a non-purified chow diet containing 29IU/g diet of vitamin A throughout life. Other laboratories use a similar concentration of vitamin A in the mouse diet and define this diet as “copious in vitamin A” (Kane et al., 2011). Following are the specifications of the chow diet (Diet: Prolab Isopro RMH3000 5p75; Composition: energy from protein, fat, and carbohydrates: 26, 14, and 60%, respectively; vitamin A: 29IU vitamin A/g of diet; beta-carotene: trace amounts) manufactured by LabDiet (W.F. Fisher and Son). Approximately 3 months old female mice were mated with the males of the same genotype. Upon mating, a vaginal plug was detected and it was designated as 0.5 *days post-coitum* (dpc). The females were separated and were fed regular chow diet throughout life and gestation.

**Part B*:*** In order to investigate the effects of variations in the maternal vitamin A dietary regimen on the beta-carotene uptake by the developing tissues, approximately 3 months old wild-type females maintained on a regular chow diet throughout life, were mated with wild-type males. At 0.5 dpc, the female were randomly assigned to three different dietary groups: vitamin A-sufficient diet (14

IU/g diet); high vitamin A-content diet (110IU/g diet) and vitamin A-excess diet (220 U/g diet). All of the diets provided during gestation were purified diets and were purchased from Research Diet (NJ, USA). The females were fed one of the above-mentioned diets throughout pregnancy.

All mice employed in this study were from a mixed genetic background (C57/BL6 × 129sv). Both diet and water were available to the animals on an *ad libitum* basis. Mice were maintained on a 12:12 light/dark cycle with the period of darkness between 7 PM and 7 AM. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 1996) and were approved by the Rutgers University Institutional Committee on Animal Care.

### *2-2.2 Beta-carotene supplementation of the dams*

Pregnant dams under all the experimental conditions described in parts A, and B of the study were supplemented with a single dose of beta-carotene at 13.5 dpc. Unlike humans, mice are efficient cleavers of beta-carotene due to high activity of CMOI in the intestine (Lee et al., 1999). Almost all of ingested beta-carotene is cleaved in the mouse intestine to generate retinoids resulting in negligible amounts (if any) of intact beta-carotene released into the circulation. The experiments described here require significant (well measurable) amount of intact beta-carotene to be present in the maternal circulation, to mimic the human condition where intact beta-carotene is found in the bloodstream. To meet the

latter goal, we supplemented mice by means of an intraperitoneal injection of beta-carotene. Intraperitoneal injection directly delivers intact beta-carotene into the peritonea of the mouse, and from here into the circulation, thus circumventing the intestinal cleavage. Following the study performed by Glise et al (Glise, 1998), we added 50 mg of beta-carotene in a mixture of ethanol, Cremophor (Sigma, St Louis, MO) and PBS (1:11:18 ratio), under yellow light. The dams were injected with a dose of 20-50 µg of beta-carotene/g body weight (~250 µl of the emulsion). This dose gives rise to about 40-100µg of Retinol Activity Equivalent (RAE) [1µg of RAE= 12µg of beta-carotene from foods. This unit takes into account the poor intestinal absorption of carotenoids and the fact that the bioactivity of beta-carotene in foods is less than had previously thought (Ross, 2005)]. This dose, without being toxic (indeed, embryonic malformations are never detected under this regimen of supplementation in wild-type mice), allows the delivery of very well detectable levels in the maternal circulation, ranging from (200-1200 µg/dl). These circulating levels of beta-carotene also result in very well detectable levels of beta-carotene in developing tissues (Wassef et al., 2012).

In each group, dams injected with the same volume of vehicle (250 ul of the ethanol, Cremophor and PBS mixture) served as controls. The administration of beta-carotene was performed at 13.5 dpc and all animals were euthanized at 14.5 dpc by $CO_2$ inhalation. Maternal serum and liver, as well as placenta, yolk sac and embryos were collected, frozen and stored at -80°C until further

analyses.

### *2-2.3 Beta-carotene and retinoid measurements by HPLC analysis*

Reverse-phase HPLC analysis was performed to measure serum and tissue retinoid levels (Kim and Quadro, 2010) and beta-carotene levels (Kim et al., 2011). Tissues (100-200 mg) were homogenized in PBS using a PRO200 homogenizer (Oxford, CT). Half of the homogenate was used to extract retinoids (Kim and Quadro, 2010). The other half was used to extract beta-carotene by adding 0.5 ml of methanol and 1 ml of acetone, and then performing the extraction with 1 ml of petroleum ether. This latter extraction was repeated twice and the supernatant was treated as for retinoid analysis. 100 µl of serum was used for both retinoid and beta-carotene extraction. Retinoids and beta-carotene were separated on a 4.6 × 250 mm Hichrom Ultrasphere ODS analytical column (Part no. 235329, Hichrom Limited, UK) using acetonitrile, methanol, and methylene chloride (70:15:15, v/v) as the mobile phase flowing at 1.8 ml/min. A Dionex Ultimate 3000 HPLC system and a computerized data analysis workstation with Chromeleon software were used. Retinol, retinyl esters and beta-carotene were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. Retinyl acetate (Sigma, St Louis, MO; for retinoids) and echinenone (CaroteNature, Switzerland; for beta-carotene) were added as internal standards. Detection limits are as

follows: for retinoids - serum <0.1 ng/dl and tissues <1 ng/g; for beta-carotene – serum <1 ng/dl and tissues 10 ng/g).

### *2-2.4 mRNA analysis by qPCR*

Total RNA was extracted from a sample of liver and individual placentas and embryos using the RNA Bee kit (Tel-test Inc, TX) according to the manufacture's instructions. RNA concentrations were measured by using the Nanodrop 1000 and quality was ascertained by 260/280 ratio and by a formaldehyde gel. This step was followed by DNase I treatment (Roche Diagnostics, IN). One microgram of the DNase Treated RNA was reverse transcribed to cDNA using the instructions and reagents (random hexamer primers were used) from Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, IN) in a final volume of 20 µl. A no-reverse transcriptase control (NRTC) was included. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, IN) was used for QPCR. A validation test (calibration curve) was carried out for each primer pair using a pool of control samples to determine PCR efficiency and reactions were performed according to the protocol of the manufacturer with 500 nM of each primer (final concentration), cDNA and the SYBR Green I. All the analyzed amplicons of interested genes encompass at least one intron. For the QPCR experiments, 300 nM of each specific primer (final concentration), together with an amount of cDNA for each sample equivalent to 10-20 ng of the total RNA input in a final volume of 15 µl was used. Clear plates were used for the reaction in the Lightcycler 480 machine (Roche

Diagnostics, IN). All samples were run in duplicates or triplicates. Included within each experiment were the NRTC and the non-template control. After enzyme activation (10 min, 95°C), 35–40 PCR amplification cycles were performed: 10 s at 95°C, 20 s at 58°C, and 30 s at 72°C. At the end of each run, samples were heated to 95°C with a temperature transition rate of 0.11°C/s to construct dissociation curves.

From the instrument, quantification cycles (Cq) were obtained for each sample for each gene of interest. To determine changes in gene expression, the DDCT method was used. Briefly, DCT values were obtained for each sample by subtracting the Cq of the gene of interest from that of the housekeeping gene. The DDCT values were obtained from subtracting DCT for each sample from the average DCT for the calibrator group. The expression of each gene relative to the calibrator was calculated using $2^{(-DDCT)}$. All groups were expressed as fold change from the control group.

### *2-2.5 Isolation of serum lipoproteins*

Serum of the dams maintained on the diets containing various amounts of vitamin A (from Part B) was subjected to classic method of density gradient ultracentrifugation to isolate serum lipoproteins. To accomplish this, blood was collected from the dams and it was centrifuged at 13,000 rpm for 20 minutes to collect the serum. The sera were subjected to density based ultracentrifugation to separate VLDL + chylomicron fraction ($d < 1.006$ g/ml), LDL + IDL fraction ($d =$

1.023-1.06 g/ml) and HDL fraction (d = 1.063-1.21 g/ml). Briefly, this protocol was modified from the original protocol used by Goldberg *et al* (Goldberg et al., 2012). 100 µl of the serum was added to the bottom of airfuge tubes (Beckman Coulter Inc, CA) 100 µl of 0.9% saline solution (d = 1.004 g/ml) was layered over the serum. The tubes were accurately weight balanced and centrifuged at a speed of 70,000 g in Beckman TLA 120.1 rotor (Beckman Coulter Inc, CA) for 4 hours. The tubes were carefully removed and exactly 100 µl from the top layer containing VLDL + chylomicrons was removed in a clean tube using a 100 µl Hamilton glass syringe (Hamilton Company, NV). The syringe was extensively washed with 0.9% saline between the samples. The bottom 100 µl were mixed with 100 µl of KBr solution (d = 1.12 g/ml, prepared by dissolving 17.22 g of KBr in 100 ml Milli-Q water), the samples were weight balanced and centrifuged at the same speed for 18 hours. Top 100 µl of LDL + IDL fraction and bottom 100 µl of HDL fraction were transferred to separate clean tubes using Hamilton glass syringe. All the serum fractions as well as the total sera were used to determine total cholesterol (Cholestrol E kit, Wako dignostics), and triglycerides (Infinity triglyceride kit, Themo scientific) concentrations. Beta-carotene content of all the fractions was determined by HPLC analysis as described.

### *2-2.6. Statistical Analyses*

Normality of the data was established by using the Shapiro-Wilk test. Normally distributed data were analyzed by 2-way ANOVA followed by a *t*-test or

one-way ANOVA. Data with a non-normal distribution were analyzed by Kruskal Wallis test, followed by a Mann-Whitney test. Analyses were performed with SPSS statistical software (IBM SPSS Statistics, Version 19). A p-value $<0.05$ was used to establish statistical significance.

### *2-3. Results Part A*

**Part A** of this study was designed to investigate the effects of a single dose of beta-carotene, administered to the dams at mid-gestation, on beta-carotene uptake and metabolism in maternal and developing tissues of mice with a normal vitamin A status (wild-type dams) and of mice on a status of marginal vitamin A deficiency. Mice lacking both lecithin:retino alcyltransferase (LRAT) and retinol-binding protein (RBP) served as the mouse models of marginal vitamin A deficiency. Indeed, in the absence of LRAT, the major enzyme that esterifies retinol to retinyl esters (Kim et al., 2008b) and RBP, the sole specific carrier of retinol in the circulation (Quadro et al., 2003), the LRAT-/-RBP-/- mice are unable to store and mobilize vitamin A and must acquire retinoids through the diet to support vitamin A-dependent functions, including embryonic development (Kim et al., 2008b). These mice are on a status of marginal vitamin A deficiency when fed a diet containing sufficient amounts of vitamin A (liver stores are essentially depleted and serum retinol levels are extremely low) and are highly susceptible to develop a severe state of vitamin A deficiency when fed a vitamin A-deprived diet (Kim et al., 2008b). Wild-type and LRAT-/-RBP-/- mice were fed a regimen of adequate vitamin A (nonpurified chow diet containing 29 IU vitamin A/g diet) throughout life and gestation. The dams were supplemented with beta-carotene at mid-gestation (13.5 dpc), sacrificed 24 hours later and maternal and developing tissues were analyzed by HPLC and qPCR as described above.

### *2-3.1. Evaluation of dose and delivery method of beta-carotene supplementation of the dams*

In order to confirm that our method and dosage of beta-carotene supplementation was able to efficiently deliver beta-carotene into the serum and tissues of the dams, we first analyzed by HPLC maternal and developing tissues from wild-type dams supplemented with 20-50 μg/dL of beta-carotene at 13.5 dpc and sacrificed 24 hours following the supplementation. Beta-carotene was detected in its intact form at 14.5 dpc in maternal serum, liver, placenta, yolk sac, and embryos (Table 1). Maternal serum beta-carotene levels in the range of 230-660 μg/dL were achieved by employing this dosage and delivery method of beta-carotene. A gradient of beta-carotene concentration was observed across the tissues with maternal liver showing the highest and embryos showing the lowest beta-carotene concentration. Beta-carotene was undetectable in serum and liver of the dams injected with vehicle (data not shown) as expected (Kim et al., 2011). These data confirmed that our dose and delivery method of beta-carotene resulted in well-detectable levels of this provitamin A carotenoid in serum and tissues.

**Table 1. Beta-carotene (bc) levels in maternal liver and the developing tissues of WT dams fed chow-diet and supplemented with beta-carotene at 13.5 dpc**

| Maternal genotype/ treatment | Liver µg/g | Placenta µg/g | Yolk Sac µg/g | Embryo µg/g |
|---|---|---|---|---|
| WT + bc | 93 ± 42 | 0.85 ± 0.23 | 0.25 ± 0.13 | 0.018 ± 0.007 |

n = 4-8 dams; 2-3 placenta, yolk sac and embryos per dam; Values expressed as mean ± SD

### *2-3.2. Effect of beta-carotene supplementation on tissue beta-carotene and retinoid levels*

We next proceeded to compare the effect of the beta-carotene supplementation on the retinoid and beta-carotene levels in maternal and developing tissues in wild-type and LRAT-/-RBP-/- (L-/-R-/-) dams. Firstly, a single administration of beta-carotene showed no effect on maternal serum and hepatic retinoid (retinol and retinyl esters) concentrations when compared to vehicle treated dams, regardless of the genotype (Table 3). Also, serum and hepatic retinol and retinyl ester concentrations of L-/-R-/- mice were as previously reported (Kim et al., 2008a). Maternal beta-carotene supplementation in WT mice did not alter retinol or retinyl ester concentrations in embryonic or in extra-embryonic tissues compared to the vehicle treated group (Figure 5). Interestingly, while embryonic retinol concentration was similar between the two genotypes (Figure 5), yolk sac retinol concentration was significantly higher in L-/-R-/- mice compared to WT, regardless of the maternal treatment (Figure 5). In the case of placenta, a statistically significant difference in beta-carotene levels was observed in L-/-R-/- dams compared to WT dams (Table 2). As expected, due to the lack of LRAT, retinyl esters were not detectable in developing tissues of the double knockout strain, irrespective of treatment (Figure 5).

Taken together the HPLC data indicated that under a normal maternal vitamin A status, a single maternal administration of beta-carotene does not perturb retinoid concentrations in maternal and developing tissues. However, a

marginally impaired maternal vitamin A status may affect placental and yolk sac uptake of beta-carotene.

**Table 2. Beta-carotene levels in the maternal liver and developing tissues in wild-type (WT) and LRAT-/-RBP-/- (L-/-R-/-) dams fed a nonpurified diet containing sufficient amounts of vitamin A and supplemented with beta-carotene (bc) at 13.5 dpc**

| Maternal genotype/ treatment | Liver µg/g | Placenta ng/g | Yolk sac ng/g | Embryo ng/g |
|---|---|---|---|---|
| WT + bc | 93.1 ± 41.9 | 854.8 ± 236.6 | 247.3 ± 129.0 | 18.3 ± 7.5 |
| L-/-R-/- + bc | 71.0 ± 22.8 | 1650.5 ± 328.0* | 145.2 ± 37.6 | 19.9 ± 7.5 |

n = 4-8 dams; n = 6-21 placenta and embryos analyzed; Values expressed as mean ± SD; *, $p<0.05$ vs WT + bc; Student's T-test, SPSS Inc.

**Table 3. Retinol (ROH) and retinyl ester (RE) levels in the maternal serum and liver in wild-type (WT) and LRAT-/-RBP-/- (L-/-R-/-) dams fed a nonpurified diet containing sufficient amounts of vitamin A and supplemented with either the vehicle or beta-carotene (veh or bc respectively) at 13.5 dpc**

| Maternal genotype/treatment | Serum | | Liver | |
|---|---|---|---|---|
| | ROH µg/dL | RE µg/dL | ROH µg/g | RE µg/g |
| WT + veh | 5.2 ± 0.9 | n.d. - 3.7 | 1.7 ± 0.6 | 300.9 ± 57.3 |
| WT + bc | 6.9 ± 2.01 | n.d. – 2.0 | 2.9 ± 1.4 | 326.6 ± 86.0 |
| L-/-R-/- + veh | 8.6 ± 2.9 | n.d. | 0.6 ± 0.3 | n.d. |
| L-/-R-/- + bc | 5.7 ± 2.9 | n.d. | 0.6 ± 0.3 | n.d. |

n = 4-8 dams per group; Values expressed as mean ± SD; n.d. = not detected; ANOVA, SPSS Inc.

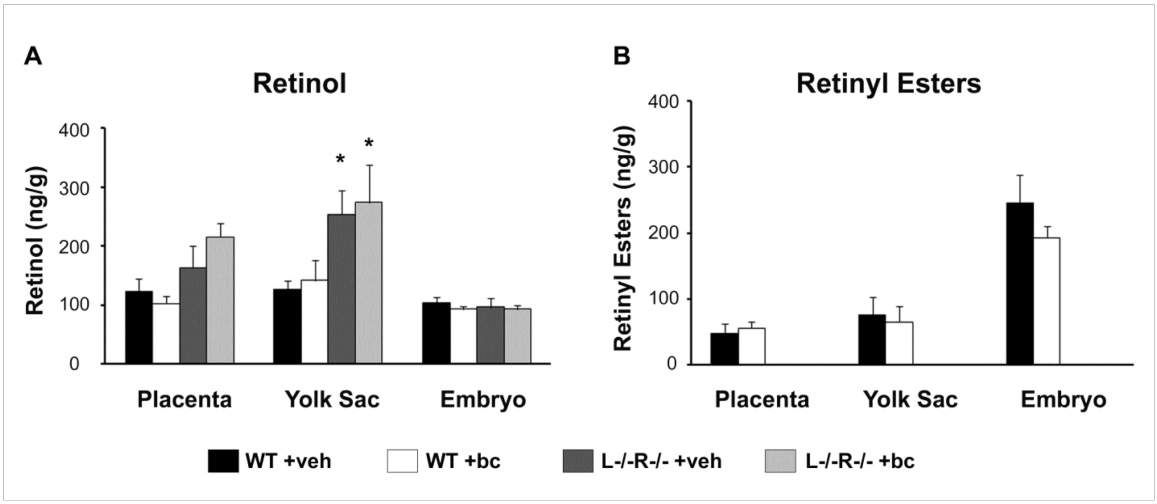



**Figure 5. Retinol (Panel A) and Retinyl Ester (Panel B) levels in the developing tissues i.e. placenta, yolk sac and the embryos in wild-type and L-/-R-/- dams supplemented with either vehicle or beta-carotene (veh or bc respectively) at 13.5 dpc.** Analysis performed by qPCR. Black bars, WT + vehicle; White bars, WT + bc; Dark Grey bars, L-/-R-/- + veh; Light grey bars, L-/-R-/- + bc. n= 6-21 placenta, yolk sac and embryos; *, $p<0.05$ vs. WT + veh; ANOVA, SPSS Inc.

### *2-3.3. Effect of maternal beta-carotene supplementation on the transcription of CMOI in maternal or developing tissues*

Since some earlier mentioned studies have shown that dietary vitamin affects activity of CMOI in the adult tissues (Dua et al., 1966; van Vliet et al., 1996; Villard and Bates, 1986; Wyss et al., 2001), we asked whether an altered vitamin A status of the dams as in the case of L-/-R-/- dams had any effect on the transcription of CMOI in maternal and developing tissues. To this end, we performed qPCR analysis. Upon a single administration of beta-carotene, maternal hepatic mRNA expression of *CMOI* was not significantly different between the experimental groups (Figure 6A). Placental (Figure 6B) and embryonic (Figure 6C) expression of *CMOI* mRNA did not show any significant difference among the WT groups. Interestingly, placental *CMOI* expression in the L-/-R-/- strain was significantly elevated compared to placenta WT, even though no further upregulation was observed upon maternal beta-carotene treatment (Figure 6B). In contrast to placenta, *CMOI* mRNA expression in embryos from L-/-R-/- + veh dams was significantly reduced compared to embryos from the WT dams injected with vehicle (Figure 6C). However, in the double knockout strain, maternal beta-carotene treatment increased embryonic mRNA expression of *CMOI* (Figure 6C).

Taken together, these results suggest that an acute administration of beta-carotene does not perturb the pro-vitamin A cleavage in the placenta and embryo from dams under a normal vitamin A status.

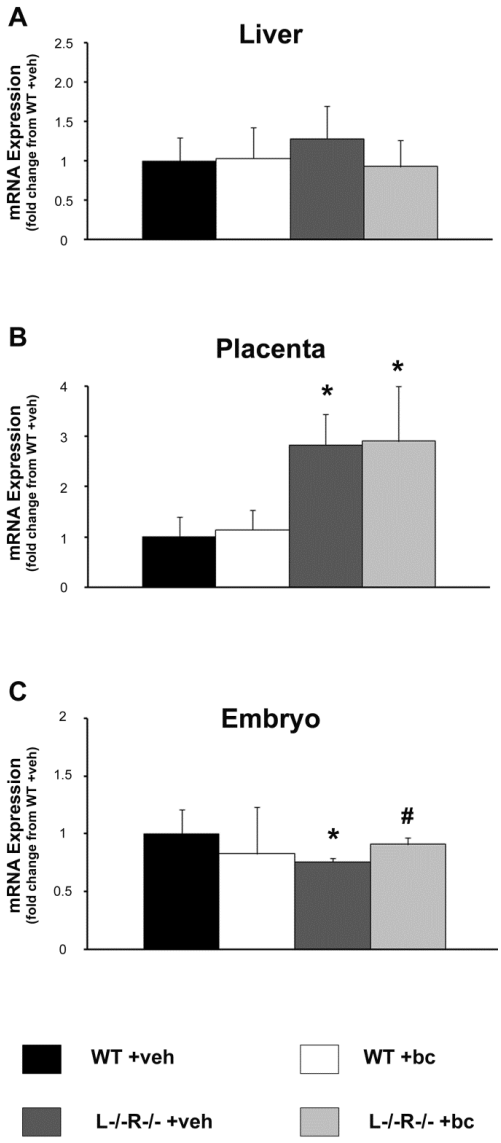



**Figure 6. mRNA expression levels of the cleavage enzyme CMOI in the maternal liver and the developing tissues i.e. placenta and the embryos in wild-type and L-/-R-/- dams supplemented with either vehicle or beta-carotene at 13.5 dpc.** Analysis by qPCR. Black bars, WT + veh; White bars, WT + bc; Dark Grey bars, L-/-R-/- + veh; Light grey bars, L-/-R-/- + bc; n = 3-5 per group; *, $p<0.05$ vs. WT + veh; #, $p<0.05$ vs. L-/-R-/- + veh; ANOVA, SPSS Inc.

### *2-3.4. Effect of beta-carotene treatment on placental transcription of lipoprotein receptors*

HPLC analysis and qPCR analysis of the placenta showed that this tissue has a capacity to accumulate high levels of beta-carotene as compared to other developing tissues and may regulate the amount of this provitamin A that is transported to the embryo. Since maternal circulating beta-carotene is present in association with various lipoproteins such as chylomicron remnants, VLDL, LDL and HDL (Bjornson et al., 1976; Erdman et al., 1993; Parker, 1996), we asked whether the receptors responsible for uptake of these lipoproteins regulate the amount of beta-carotene taken up by the placenta. To answer this question, we conducted qPCR analysis of the placental lipoprotein receptors such as scavenger receptor type B-1 (SRB-1, the HDL receptor)(Acton et al., 1996), LDL-receptor (LDLr)(Brown and Goldstein, 1979; Goldstein et al., 1985), LRP1 (the receptor for chylomicron remnants)(Ishibashi et al., 1994; Mahley et al., 1989), and lipoprotein lipase (LPL, the enzyme that hydrolyzes triglyceride core of the lipoproteins)(Wassef and Quadro, 2011). mRNA expression of *LRP1* was significantly reduced in the placenta of WT dam supplemented with beta-carotene as compared to the control group (Figure 7). This down-regulation did not take place in placenta from L-/-R-/- dams (Figure 7). However, baseline (vehicle group) placental expression of *LRP1* was lower in L-/-R-/- dams compared to that in WT (Figure 7). Interestingly, *LPL* mRNA expression was upregulated in the placenta from L-/-R-/- dams upon beta-carotene

supplementation and was also significantly higher than that of placenta from WT dams treatment with this provitamin A (Figure 7). Expression of *LDL receptor Ldlr and Srb1* mRNA remained steady, regardless of genotype and treatment (Figure 7).

Overall these data suggest a potential role of LRP1 and LPL in mediating the uptake of maternal circulating beta-carotene by the placenta.

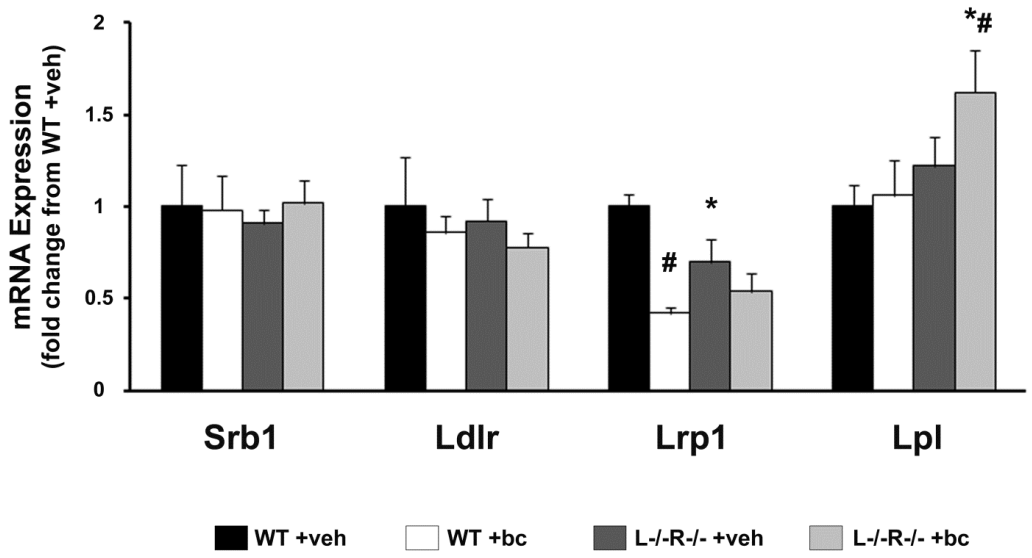



**Figure 7. mRNA expression levels of the lipoprotein receptors in the placenta of wild-type and L-/-R-/- dams supplemented with either vehicle or beta-carotene (veh or bc respectively) at 13.5 dpc.** Analysis by qPCR. Black bars, WT + veh; White bars, WT + bc; Dark Grey bars, L-/-R-/- + veh; Light grey bars, L-/-R-/- + bc; n = 3-5 placenta per group; #, $p<0.05$ vs. WT + veh; *, $p<0.05$ vs. WT + veh; ANOVA, SPSS Inc.

### *2-4. Discussion Part A*

The Part A of this study was designed to specifically evaluate in *vivo* the effects of a single acute supplementation of beta-carotene at mid-gestation on the uptake and metabolism of this vitamin A precursor in maternal and developing tissues under a normal vitamin A status and in a condition of marginal vitamin A deficiency. Specifically, we employed WT mice as well as LRAT-/-RBP-/- mice as a model of marginal vitamin A deficiency (Kim et al., 2008a). To study the beta-carotene uptake in the developing mouse embryos, the mid-gestation stage of the dams (i.e. 14.5 dpc) was chosen. In the embryo at mid-gestation the majority of the organogenesis is completed and the embryo is capable of regulating its own retinoid metabolism (Kim et al., 2008b; Spiegler et al., 2012; Wassef and Quadro, 2011). In addition, the beta-carotene cleavage enzymes CMOI and CMOII (Kim et al., 2011) as well as the genes involved in tissue lipoprotein uptake (Wassef and Quadro, 2011) are expressed at this stage of development in both embryonic and extra-embryonic tissues.

We confirmed an efficient beta-carotene supplementation of the dams with our dosage and delivery method since well detectable levels of intact beta-carotene were observed in maternal serum, liver as well as the developing tissues upon HPLC analysis. A gradient of tissue beta-carotene concentration was observed from maternal liver to embryo, with maternal liver being the one with the highest concentration of the provitamin A carotenoid. These results support the notion that the liver is the main site of beta-carotene uptake (Gugger

et al., 1992; Parker, 1996; Shmarakov et al., 2010), and are in agreement with literature data suggesting that placenta and yolk sac act as a barrier to regulate the uptake and transfer of nutrients to the fetus, including retinoids and its carotenoid precursor (Marceau et al., 2007). In contrast to previous reports on adult tissues of mice chronically fed with beta-carotene (Amengual et al., 2011a; Fierce et al., 2008), our data showed that a single dose of beta-carotene administered to the dams did not affect retinoid homeostasis either in maternal or in developing tissues of the WT mice. We therefore asked whether this was the result of a regulatory mechanism to suppress beta-carotene conversion into retinoid *via* CMOI action, under a normal vitamin A status. Data from the literature indicate that beta-carotene supplementation regulates intestinal *CMOI* expression by a transcriptional feedback mechanism mediated by retinoic acid (Lobo et al., 2010). In our study, we demonstrate that beta-carotene supplementation does not perturb the transcription of *CMOI* either in maternal (liver) or in developing (placenta or embryo) tissues of WT mice. We hypothesize that this discrepancy may be due to the length of our supplementation protocol compared to previously published reports (i.e., acute *vs.* chronic). Nevertheless, our data do not completely rule out an effect of beta-carotene on CMOI enzymatic activity (van Vliet et al., 1996).

Current knowledge regarding the tissue uptake of beta-carotene from the circulation pertains to adult tissues (During et al., 2008; Harrison, 2012; van Bennekum et al., 2005) and the molecular mechanisms that regulate the uptake

of beta-carotene at the placental barrier have not been investigated to date. Being a lipophilic molecule, beta-carotene circulates in the bloodstream in association with lipoprotein particles like chylomicrons and their remnants, VLDL, LDL and HDL (Harrison, 2012; Johnson and Russell, 1992; Ribaya-Mercado et al., 1993) to a varying degree. Thus, cell surface receptors that mediate the uptake of these lipoproteins could also be the regulators of beta-carotene uptake in tissues (Harrison, 2012; Johnson and Russell, 1992; Ribaya-Mercado et al., 1993). The transcriptional downregulation of *LRP1*, in the placenta of beta-carotene supplemented WT dams, strongly suggest the involvement of this receptor in placental beta-carotene uptake. Indeed, LRP1, the so-called receptor for chylomicron remnants has a high affinity for lipoproteins that contain apolipoprotein E (Beisiegel et al., 1989; Lillis et al., 2008). A similar effect on *LRP1* transcription was not observed on maternal liver (data not shown), suggesting a tissue-specific response. We interpret this down-regulation as a regulatory mechanism that may control placental uptake of this provitamin A carotenoid, under the conditions of normal vitamin A status of the dams, when no additional precursor of vitamin A (such as beta-carotene) may need to be taken up or metabolized by the placenta.

Once acquired by the placenta, beta-carotene can be also transferred in its intact form to the embryo to be used as a “local” source of retinoic acid (Kim et al., 2011). By qPCR analysis, we examined the mRNA levels of lipoprotein receptors in WT embryos to determine if they would respond to maternal beta-

carotene supplementation and thus show an involvement in the uptake of beta-carotene by the embryo. Our analysis did not reveal any statistically significant difference in the embryonic mRNA expression of *LPL, SRB-1, LRP1* and *LDLr* between our experimental groups (data not shown), suggesting that the regulation of beta-carotene uptake may not be taking place at the embryonic level. It remains to be established whether the transfer of beta-carotene from placenta to embryo through the secretion of placental lipoproteins into the fetal circulation (Madsen et al., 2004) could also be affected by beta-carotene availability.

Due to their extremely low concentration of hepatic retinol stores, the lack of serum RBP and thus their increased susceptibility to develop signs of vitamin A deficiency, the L-/-R-/- mice can be considered a model of marginal vitamin A deficiency when maintained on a dietary regimen of sufficient vitamin A intake (Kim et al., 2008a; Liu and Gudas, 2005). Only placental beta-carotene concentration was elevated in L-/-R-/- mice compared to WT, suggesting that the maternal vitamin A status may control the uptake of intact beta-carotene in the placenta. Despite more beta-carotene being taken up by the placenta of L-/-R-/- mice compared to WT mice, placental retinol concentration did not change upon supplementation in this strain. In addition, placental mRNA expression of *CMOI* was elevated in the double knockout mice, regardless of the maternal treatment. We interpret our data as the result of a compensatory mechanism that, under a marginal vitamin A deficient status in L-/-R-/- mice (Liu and Gudas, 2005; Liu et

al., 2008), would maximize the conversion of the beta-carotene into retinol *via* CMOI action, to ultimately increase the amount of retinol available for transfer to the embryo. This is in agreement with the earlier mentioned study by Dimenstein and colleagues, which suggested that beta-carotene could be a precursor of retinol in human placenta and that its conversion could be dependent on the nutritional status of the mother, being particularly efficient in a more depleted state (Dimenstein et al., 1996). Nonetheless, embryonic retinol concentrations were similar among our experimental groups, regardless of genotype or treatment. We propose two possibilities to account for the difference in extraembryonic retinol available for transfer towards the developing tissues vs. embryonic retinol concentration in the L-/-R-/- strain. Firstly, since these mice lack RBP, the transport of retinol out of the placenta trophoblast towards the embryo could be impaired (Wassef and Quadro, 2011). Secondly, we previously showed that despite their inability to store vitamin A, increased retinoic acid oxidation *via* Cyp26A1, and intracellular retinol efflux via Stra6 ensure homeostatic levels of retinoids in the developing tissues of the L-/-R-/- mice (Kim et al., 2008a). So, these highly effective homeostatic mechanisms could result in similar embryonic retinol concentrations despite different metabolic pathways of tissue retinoid utilization.

Although more beta-carotene was taken up by the placenta of the L-/-R-/- mice compared to WT, similar beta-carotene concentrations were detected in WT and double knockout embryos from supplemented mothers. Interestingly,

embryonic transcription of *CMOI* was upregulated upon maternal beta-carotene treatment in the double knockout strain, suggesting that an increased rate of utilization of beta-carotene in the embryo could account for the above-mentioned discrepancy.

Overall, Part A of this study revealed that placental beta-carotene uptake and its processing by the developing tissues are influenced by beta-carotene availability and maternal vitamin A status. It also identifies, for the first time, LRP1 as a potential molecular mediator of the pro-vitamin A uptake at the placental level.

### *2-5. Results Part B*

**Part B** of this study was designed to investigate the effects of variations in the vitamin A content of the maternal diet on the uptake of beta-carotene by the developing tissues, upon a single administration of beta-carotene to the dams. Animals and diets employed in this set of experiments, the beta-carotene supplementation strategy and analytical methods are described earlier. Briefly, from the beginning of gestation, wild-type dams were maintained on three different purified diets containing vitamin A sufficient diet (14 IU/g), a high vitamin A content diet (110 IU/g) or vitamin A excess diet (220 IU/g). The dams were supplemented with beta-carotene at 13.5 dpc, and sacrificed 24 hours later to collect maternal and developing tissues for further analyses.

#### *2-5.1. Effect of maternal dietary vitamin A content on the tissue retinoid levels*

To confirm the effect of different dietary vitamin A levels on the vitamin A status of maternal and developing tissues in wild-type mice, we first analyzed by HPLC tissue retinol and retinyl ester levels. As expected, retinoid content in all the tissues analyzed increased with greater amount of dietary vitamin A (Table 4 and 5). Under all the dietary regimens, beta-carotene supplementation did not alter retinoid levels in any of the tissues analyzed (Table 4 and 5).

**Table 4: Serum and hepatic retinol (ROH) and retinyl ester (RE) concentrations of WT pregnant mice fed diets containing varying amounts of vitamin A with (bc) or without (veh) beta-carotene supplementation**

| Maternal diet/ treatment | | Serum | | Liver | |
|---|---|---|---|---|---|
| | n | ROH (µg/dL) | RE (µg/dL) | ROH (µg/g) | RE (µg/g) |
| 14IU + veh | 9 | 7.1 ± 2.6 | 4.0 (n.d.-8.5) | 2.3 ± 0.9 | 265 ± 58 |
| 14IU + bc | 4 | 5.2 ± 0.9 | 1.1 (n.d.-2.0) | 2.5 ± 0.3 | 187 ± 12 |
| 110IU + veh | 6 | 12.8 ± 4.1* | 4.1 (3.6-78.2)* | 6.2 ± 0.8* | 610 ± 124* |
| 110IU + bc | 4 | 12.4 ± 3.3* | 3.8 (0.8-12.8)* | 4.5 ± 1.8* | 598 ± 115* |
| 220IU + veh | 5 | 13.5 ± 4.4* | 16.4 (6.2-56.7)* | 7.6 ± 2.2* | 1392 ± 332*§ |
| 220IU + bc | 3 | 16.3 ± 2.9* | 9.0 (3.8-10.2)* | 8.4 ± 2.1*§ | 1609 ± 383*§ |

n=3-4 females per group; Values expressed as mean ± SD except Serum RE; Values expressed as geometric mean (range) for Serum RE; *, $p<0.05$ vs. 14 IU group respective treatment; §, $p<0.05$ vs. 110 IU group respective treatment; ANOVA and Non-parametric tests Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc.

**Table 5: Placental and embryonic retinol (ROH) and retinyl ester (RE) concentrations from WT mice fed diets containing varying amounts of vitamin A with or without beta-carotene supplementation (veh and bc respectively)**

| Diet/treatment | | Placenta | | Embryo | |
|---|---|---|---|---|---|
| | n | ROH (ng/g) | RE (ng/g) | ROH (ng/g) | RE (ng/g) |
| 14IU + veh | 9 | 94 ± 31 | 43 ± 13 | 88 ± 18 | 178 ± 42 |
| 14IU + bc | 5 | 95 ± 23 | 33 ± 9 | 92 ± 2 | 177 ± 18 |
| 110IU + veh | 8 | 161 ± 84* | 120 ± 25* | 115 ± 24* | 384 ± 149* |
| 110IU + bc | 5 | 163 ± 59* | 162 ± 28* | 122 ± 16* | 430 ± 108* |
| 220IU + veh | 5 | 293 ± 19*§ | 240 ± 12*§ | 214 ± 53*§ | 761 ± 112*§ |
| 220IU + bc | 5 | 268 ± 51*§ | 234 ± 20*§ | 190 ± 33*§ | 716 ± 112*§ |

n=5-12 placenta/embryos per group; Values expressed as mean ± SD;
*, $p<0.05$ vs. 14 IU group respective treatment; §, $p<0.05$ vs. 110 IU diet respective treatment, ANOVA, SPSS Inc.

### *2-5.2. Effect of maternal dietary vitamin A content on the beta-carotene uptake in the maternal and developing tissues*

To investigate whether different concentration of vitamin A in the maternal diet would influence the amount of beta-carotene taken up by the maternal as well as developing tissues, we compared beta-carotene levels in tissues collected from the wild-type dams supplemented with beta-carotene as described above. Well detectable levels of beta-carotene were present in serum, liver, placenta and embryos of all the beta-carotene supplemented dams under all of the dietary regimes (Table 6). Maternal serum and liver beta-carotene levels as well as placental beta-carotene concentrations were not different under any of the dietary regimens. However, beta-carotene levels were dramatically reduced in embryos from dams fed the vitamin A excess diet compared to other dietary groups, therefore suggesting that acquisition of beta-carotene by the embryo may be regulated according to the maternal vitamin A status/intake.

**Table 6. Beta-carotene concentrations in serum, liver, placenta and embryo from WT pregnant mice fed varying vitamin A diets and supplemented with beta-carotene.**

| Maternal diet | n | Serum (µg/dL) | Liver (µg/g) | Placenta (ng/g) | Embryo (ng/g) |
|---|---|---|---|---|---|
| Sufficient (14 IU) | 4 | 688 ± 226 | 54 ± 33 | 1427 ± 550 | 16 ± 1 |
| Excess (110 IU) | 4 | 767 ± 250 | 69 ± 13 | 1307 ± 193 | 14 ± 7 |
| Excess (220 IU) | 3 | 682 ± 288 | 60 ± 4 | 1244 ± 416 | n.d.* |

n=3-4 females per group; n=5-12 placenta/embryo per group; Values expressed as mean ± SD.; n.d, not detected; *, $p<0.05$ vs. 14 IU; ANOVA, SPSS Inc.

### *2-5.3. Beta-carotene distribution among the serum lipoproteins*

We therefore asked whether an altered transport of beta-carotene in the bloodstream of the dams fed the 220 IU vitamin A diet was responsible for the extremely low levels of beta-carotene detected in their embryos. Since it is well established that lipoprotein particles such as chylomicron remnants, VLDL, LDL and HDL are major carriers of beta-carotene in the circulation (Auletta and Gulbrandsen, 1974; Bierer et al., 1993; Erdman et al., 1993; Parker, 1996; Vogel et al., 1999), we performed serum lipoprotein isolation from the dams fed the 14 IU and the 220 IU vitamin A diet, as described in the Materials and Methods section. The lipoprotein fractions were analyzed for their cholesterol and triacylglycerol (TG) content as well as for their beta-carotene concentration. As expected, the TG:cholesterol ratios was the highest in the VLDL+chylomicron fractions and the lowest in the HDL fractions (Table 7). Beta-carotene was detected in all of the fractions with VLDL+chyolomicron containing the lowest beta-carotene levels (Table 7). Beta-carotene levels were not statistically significant different when the same lipoprotein class was compared between the two dietary groups (Table 7). Overall, maternal dietary vitamin A content did not seem to affect the distribution of beta-carotene among the various lipoproteins.

**Table 7: Maternal Serum Lipoprotein Fraction Analysis. Dams fed a vitamin A diet containing 14 IU or 220 IU and supplemented with beta-carotene. Total cholesterol (TC), triglyceride (TG) and beta-carotene (bc) levels were measured in each lipoprotein fraction.**

| Vitamin A content in the diet | Lipoprotein Fraction | TC (mg/dL) | TG (mg/dL) | bc (µg/dL) | % of total bc |
|---|---|---|---|---|---|
| 14IU | VLDL+Chylo | 19.7 ± 11.9 | 33.0 ± 15.2 | 22.2 ± 1.7 | 2.4 (2-4) |
| | LDL+IDL | 47.7 ± 15.1 | 23.0 ± 5.9 | 531.1 ± 228.7 | 61.1 (43-82) |
| | HDL | 90.8 ± 14.1 | 17.5 ± 2.6 | 271.0 ± 107.9 | 27.2 (18-34) |
| 220IU | VLDL+Chylo | 13.2 ± 6.4 | 17.5 ± 2.8 | 24.9 ± 17.7 | 2.3 (1-4) |
| | LDL+IDL | 53.2 ± 4.7 | 11.0 ± 1.4 | 418.5 ± 122.1 | 52.0 (33-80) |
| | HDL | 61.1 ± 4.6 | 9.9 ± 1.5 | 425.1 ± 417.9 | 37.9 (18-66) |

n = 3-5 dams; Values expressed as mean ± SD for cholesterol, TG and beta-carotene; % beta-carotene expressed as geometric mean (range); Total cholesterol, (TC); triglyceride, (TG) and beta-carotene (bc); Non-parametric tests, Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc.

### *2-5.4. Effect of maternal diet on embryonic transcription levels of genes involved in carotenoid and retinoid metabolism*

We next wondered whether an altered carotenoid/retinoid metabolism in the embryos from the dams fed the 220 IU vitamin A diet was responsible for their extremely low levels of beta-carotene. Since the main carotenoid cleavage enzyme (CMOI) as well as the genes that maintain embryonic retinoid homeostasis (Kim et al., 2008b) are regulated at the transcriptional level, we performed embryonic mRNA analysis by qPCR. The embryos from dams fed vitamin A sufficient diet served as the control baseline group and only embryos from dams on the 14 IU diet *versus* those from dams on the 220 IU diet supplemented with beta-carotene were compared. Transcriptional levels of the beta-carotene cleavage enzyme *CMOI* were similar among the three groups of embryos analyzed (Figure 8). mRNA levels of *LRAT* and *Raldh2,* the enzyme that catalyzes the oxidation of retinaldehyde to retinoic acid (Niederreither et al., 1997), were also unchanged between the groups. In contrast, a slight but significant reduction in mRNA levels of *RDH10*, which hydrolyzes retinol to retinaldehyde (Pares et al., 2008), was observed in embryos from dams on the 14 IU diet and supplemented with beta-carotene (Figure 8). Furthermore, mRNA levels of *RARb*, the retinoic acid receptor (Chambon, 1996), and *Cyp26A1*, the enzyme that degrades retinoic acid to biologically inactive forms (Abu-Abed et al., 2001; White et al., 1996), were both slightly elevated in the embryos from dams fed the 220 IU diet of vitamin A/g of diet and supplemented with beta-carotene (Figure 8). The transcription of embryonic

*Cyp26A1* was also slightly increased when the dams fed sufficient vitamin A diet were supplemented with beta-carotene (Figure 8).

Overall, these data suggest that the extremely low levels of beta-carotene in embryos from dams on the 220 IU diet are not likely due to increased beta-carotene metabolism within the embryo.

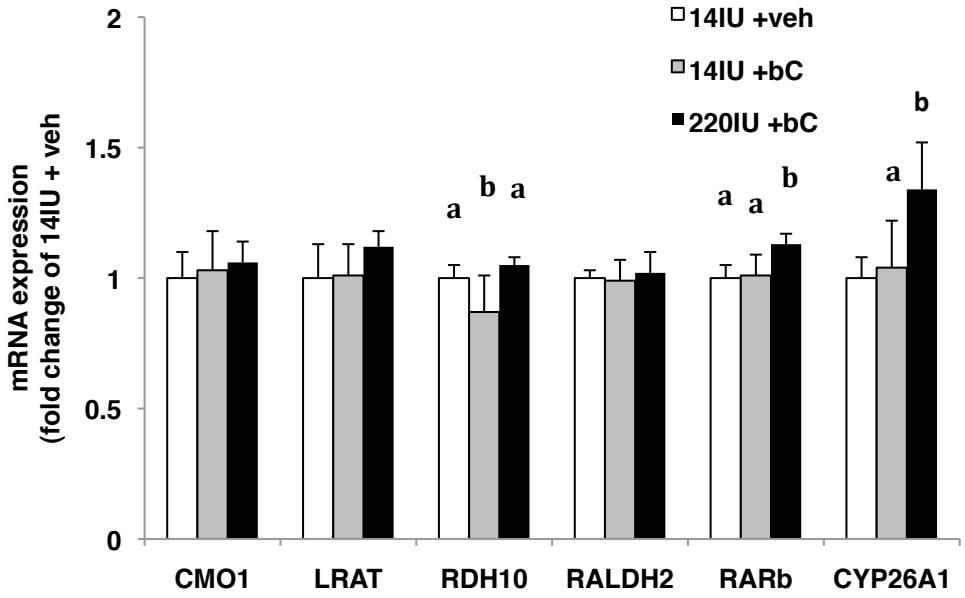



**Figure 8. mRNA expression levels of beta-carotene cleavage enzyme CMOI and key regulators of retinoid homeostasis in the embryo.** Analysis performed by qPCR. White bars, embryos of the dams fed the 14 IU vitamin A sufficient diet and injected with vehicle (control group); Grey bars, embryos of the dams fed the 14 IU vitamin A sufficient diet and supplemented with beta-carotene; Black bars, embryos of the dams fed the 220 IU vitamin A diet and supplemented with beta-carotene; n= 3-5 embryos per group. Groups with similar statistical symbols show no difference; $p<0.05$, ANOVA, SPSS Inc.

### *2-5.5. Effect of maternal diet on placental transcription of genes regulating beta-carotene uptake, metabolism and transport*

We next asked whether there the placenta could regulate the amount of beta-carotene transported to the embryo depending upon maternal dietary vitamin A and status. Beta-carotene levels were not different between placenta from dams maintained on different dietary regimens (Table 6), suggesting that the placenta acquires similar amounts of beta-carotene regardless of the maternal dietary regimen and ultimately status. However, since we could not exclude that a different rate of placental accumulation and/or secretion of beta-carotene could occur depending upon the maternal diet, we examined the expression of placental genes known to be crucial in the regulation of beta-carotene cleavage and retinoid metabolism as well as in assembly of various lipoproteins that could transport this pro-vitamin A carotenoid from the placenta to the embryo. Placenta from the same three groups of dams described above were analyzed by qPCR. Under sufficient vitamin A conditions, beta-carotene supplementation (14IU + beta-carotene) seemed to reduce mRNA levels of *CMOI*, *RARb* and *Cyp26A1* compared to the baseline group (Figure 9), suggesting a suppressed retinoic acid production by reduced cleavage of supplemented beta-carotene. In addition, mRNA levels of *CMOII* were upregulated in this same group of placenta (Figure 9), suggesting that the beta-carotene that is not cleaved to generate retinoid could be scavenged by the asymmetric cleavage enzyme instead (Lobo et al., 2012).

In placenta from dams on the 220 IU diet and supplemented with beta-carotene, mRNA levels of *RARb* and *Cyp26A1* were upregulated, (Figure 9), in agreement with the higher levels of retinoids of this tissue and the potential for increased production of retinoic acid, and similar to what was also seen in the case of the embryos. However, mRNA levels of both *CMOI* and *CMOII* were not different from the baseline in this group (Figure 9).

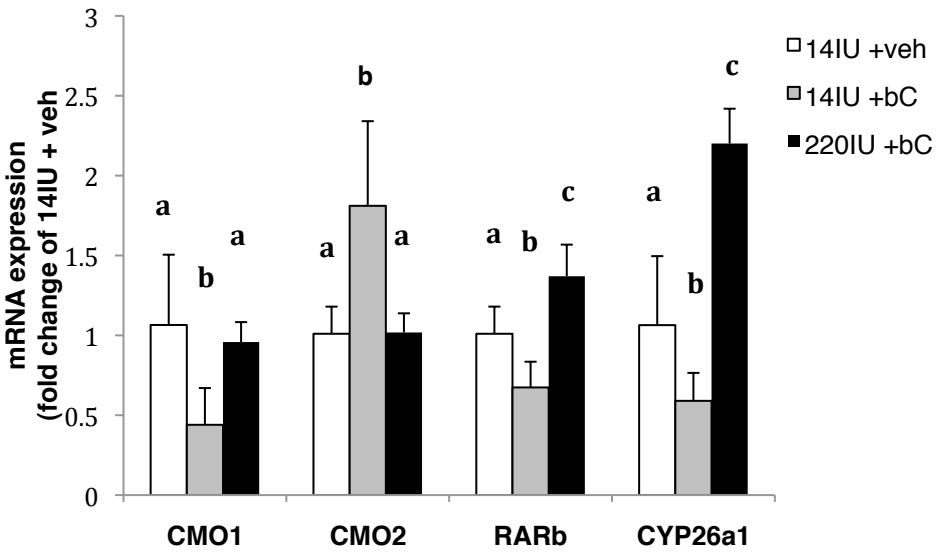



**Figure 9. mRNA expression levels of beta-carotene cleavage enzyme CMOI, CMOII and key regulators of retinoid homeostasis in the placenta.** Analysis performed by qPCR. White bars, placenta of the dams fed vitamin A sufficient diet 14 IU and injected with vehicle (control group); Grey bars, placenta of the dams fed vitamin A sufficient diet 14 IU and supplemented with beta-carotene; Black bars, placenta of the dams fed vitamin A sufficient diet 220 IU and supplemented with beta-carotene; n = 3-5 placenta per group; groups with similar statistical symbols show no difference; $p<0.05$, ANOVA, SPSS Inc.

We next conducted mRNA analysis of the lipoproteins receptors that mediate the tissue uptake of lipoproteins, and thus potentially also the uptake of the beta-carotene associated with them. Interestingly, and similar to the placenta of the dams fed regular chow diet (in Part A of this chapter), mRNA levels of placental receptor *LRP1* were significantly downregulated upon beta-carotene supplementation in placenta from dams maintained on sufficient diet (Figure 10). In addition, mRNA levels of VLDL-receptor (*VLDLr*) were significantly reduced in the placenta from the 14IU + beta-carotene group (Figure 10). However, in the placenta from dams fed the 200 IU diet and supplemented with beta-carotene, the mRNA levels of these two receptors were similar to those of the baseline group (Figure 10). mRNA levels of *LDLr, SRB-1, ApoEr* (also known as *LRP8),* and *CD36* were not altered by either beta-carotene supplementation or excess vitamin A in the diet (Figure 10).

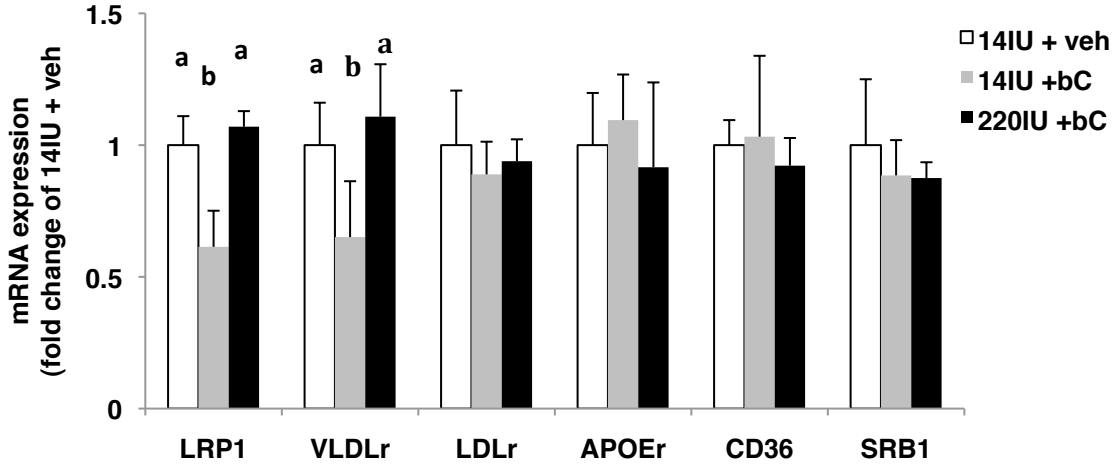



**Figure 10. mRNA expression levels of lipoprotein receptors in the placenta.** Analysis by qPCR. White bars, placenta of the dams fed vitamin A sufficient diet 14IU and injected with vehicle (control group); Grey bars, placenta of the dams fed vitamin A sufficient diet 14IU and supplemented with beta-carotene; Black bars, placenta of the dams fed vitamin A sufficient diet 220IU and supplemented with beta-carotene; n = 3-5 placenta per group; groups with similar statistical symbols show no difference; $p<0.05$, ANOVA, SPSS Inc.

Based on the data discussed so far, we wondered whether the mechanism of transfer of beta-carotene from the placenta to the embryo was also affected in the absence of maternal and placental LDLr. We therefore performed mRNA analysis of the genes involved in the assembly of the lipoproteins, which are known to be secreted from placenta in mammals (Madsen et al., 2004). Placental mRNA analysis showed an upregulation of *MTTP* in the 14IU group upon beta-carotene supplementation (Figure 11). *MTTP* encodes the protein that acts a chaperone in transport of neutral lipids to nascent lipoproteins and hence is a rate determining protein in assembly of apoB-containing lipoproteins (Hussain et al., 2012). When comparing only the supplemented groups, *MTTP* mRNA levels were reduced under vitamin A excess conditions (Figure 11). Similarly, *ApoB* that encodes apolipoprotein B-100, which is acquired by LDL during assembly and is required for its tissue uptake (Brown and Goldstein, 1979), was also upregulated upon beta-carotene supplementation at vitamin A sufficient conditions (Figure 11). When compared with the vitamin A excess group, *ApoB* mRNA levels were reduced (Figure 11). On the other hand, *ApoE*, which encodes apolipoprotein E associated with chylomicron remnants and VLDL (Gibbons, 1990; Mansbach and Siddiqi, 2010; Shelness and Sellers, 2001) did not show any transcriptional changes in any of the groups (Figure 11). mRNA levels of *Apobec-1*, which encodes the enzyme that edits apoB-100 mRNA in the small intestine resulting in the production of apoB-48 that is crucial for assembly of chylomicron remnants

(Blanc et al., 2012) were reduced upon beta-carotene supplementation and when compared with the vitamin A excess group, they were upregulated (Figure 11).

Overall, placental mRNA analysis of genes involved in the lipoprotein assembly suggested that placental assembly of apoB-containing lipoproteins may be reduced when beta-carotene is provided to dams maintained on a regimen of excess vitamin A intake.

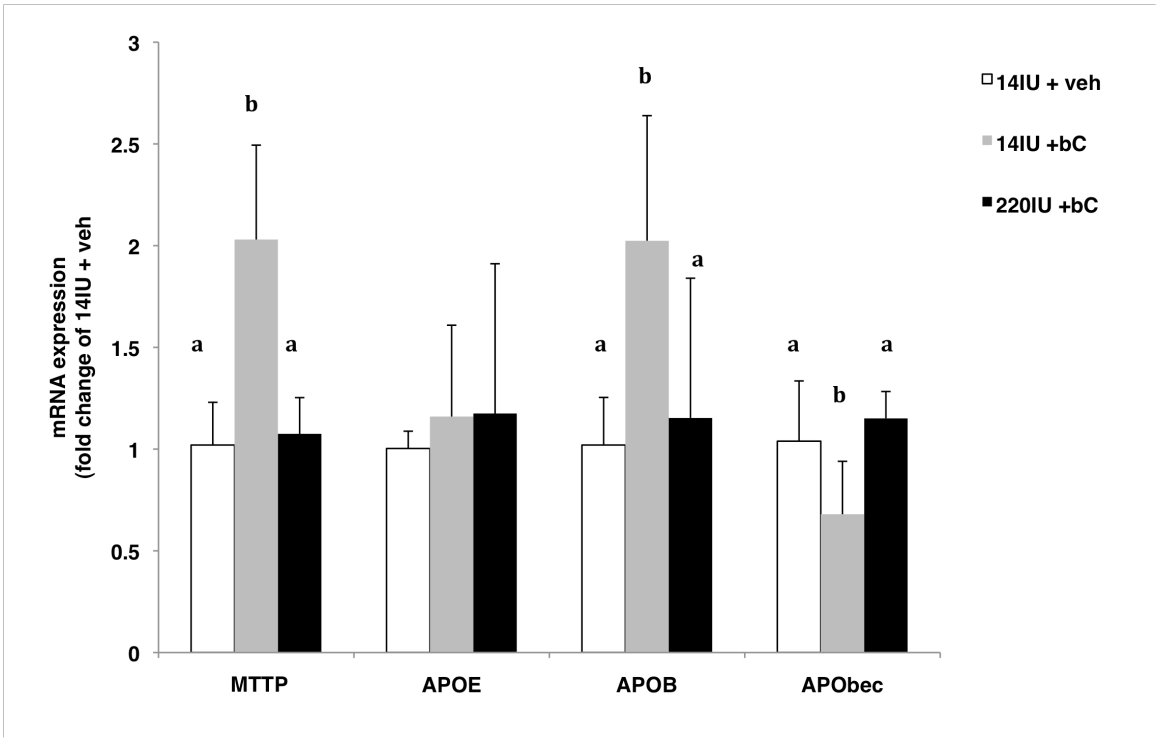



**Figure 11. mRNA expression levels of genes involved in the lipoprotein assembly in the placenta.** Analysis by qPCR. White bars, placenta of the dams fed vitamin A sufficient diet 14 IU and injected with vehicle (control group); Grey bars, placenta of the dams fed vitamin A sufficient diet 14 IU and supplemented with beta-carotene; Black bars, placenta of the dams fed vitamin A sufficient diet 220 IU and supplemented with beta-carotene; n = 3-5 placenta per group; groups with similar statistical symbols show no difference; $p<0.05$, ANOVA, SPSS Inc.

### *2-6. Discussion Part B*

Recent studies from our laboratory have unequivocally shown that the developing tissues can metabolize beta-carotene *in situ* (Kim et al., 2011). However, very little is known in regards to the uptake mechanisms of this provitamin A carotenoid and to the factors regulating such uptake in the developing tissues. We aimed at expanding our current knowledge in this area by addressing two specific questions as described below.

In **Part B** of the study, we sought to investigate whether variations in maternal dietary vitamin A content during gestation influence the uptake of beta-carotene in the maternal as well as the developing tissues. In order to accomplish this, upon mating, we randomly assigned wild-type females to three different dietary groups containing variable amounts of vitamin A starting at 0.5 dpc until they were sacrificed at mid-gestation (14.5 dpc). These diets were: vitamin A-sufficient diet (14 IU/g diet); high vitamin A-content diet (110 IU/g diet) and vitamin A-excess diet (220 IU/g diet). Unlike the studies performed in Part A, all the diets employed for the studies in Part B were purified diets. The advantage of using purified diets for these experiments was that these diets did not contain any traces of carotenoids as in the case of the chow diet. The diet containing 14 IU of vitamin A/g of diet provides sufficient amounts of vitamin A (Ross, 2010a).

The dams were fed the above-mentioned diets for 2 weeks i.e. throughout pregnancy and sacrificed at mid-gestation to collect serum and the tissues. The retinoid analysis of the maternal and developing tissues confirmed that maternal

dietary vitamin A content positively correlated with the serum and tissue retinoid concentrations, with the lowest retinoid levels detected in tissues from dams under the 14 IU vitamin A regimen. This positive correlation of tissue retinoid levels with dietary vitamin A content was independent of the beta-carotene supplementation of the dams. It is of note that the highest levels of retinoids in the embryos from the vitamin A excess groups were not toxic to the embryo. Indeed, all the embryos collected in this study were phenotypically normal with no signs of retinoid toxicity (Rothman et al., 1995). It is possible that since the mice employed in this study were wild-type and expressed all the genes crucial to maintain homeostatic levels of retinoic acid (Kim et al., 2008b), efficient homeostatic mechanisms protected the embryos from potential retinoid toxicity.

We wanted to evaluate the effect of maternal dietary vitamin A on the distribution of beta-carotene in the lipoproteins, which are the major carriers of this pro-vitamin A in the circulation (Erdman et al., 1993; Johnson and Russell, 1992; Vogel et al., 1999) and alterations of which may lead to a regulated supply of this carotenoid to the developing tissues. We performed lipoprotein isolation of the serum of the dams from sufficient and excess groups that were supplemented with beta-carotene and subjected the fractions to HPLC analysis to measure their beta-carotene content. Beta-carotene was detected in all of the fractions with VLDL+chyolomicron containing the lowest beta-carotene levels. Beta-carotene levels in the LDL+IDL and HDL fractions were comparable. Thus, our study confirms that beta-carotene is associated with chylomicron remnants,

VLDL, IDL, LDL and HDL in the circulation to a varying degree (Auletta and Gulbrandsen, 1974; Erdman et al., 1993; Traber et al., 1994). In humans, beta-carotene is mainly associated with LDL (Bjornson et al., 1976; Johnson and Russell, 1992; Traber et al., 1994) Our data, however, indicated that beta-carotene distribution in LDL and HDL was similar. We interpreted that discrepancy between the human and the mouse data as the result of the fact that mice are generally recognized as HDL-animals (Clevidence and Bieri, 1993). Therefore, the supplemented beta-carotene distributes evenly in both LDL and HDL leading to similar levels. Overall, maternal dietary vitamin A content did not seem to affect the distribution of beta-carotene among the various lipoproteins.

We next investigated the effect of various maternal dietary vitamin A contents on the beta-carotene uptake by the tissues. Maternal serum, liver and the placenta, which is the barrier between maternal and embryonic circulation, did not show any difference in the beta-carotene levels irrespective of the maternal dietary vitamin A regimen. The most striking effect was seen in the embryos under vitamin A excess conditions (220 IU) where beta-carotene levels were mostly undetectable unlike in the embryos from other dietary groups. These results suggested that excessive retinoid availability in the maternal as well as the developing tissues leads to a reduced embryonic beta-carotene accumulation. Intriguingly, this effect was not seen in the embryos of the dams fed a diet with high vitamin A content (110 IU). Beta-carotene levels in maternal and developing tissues under this dietary regimen (110 IU) were similar to the

ones in the vitamin A sufficient group, despite more retinoid availability in the maternal and developing tissues as compared to the vitamin A sufficient group. We hypothesize that this dietary condition, although high in vitamin A, is still below a ”threshold” of retinoid availability in the tissues, which is required to trigger a reduction in the embryonic beta-carotene levels. We could hypothesize that under dietary vitamin A excess conditions, when the embryos already have elevated levels of retinoids, uptake of maternal circulating beta-carotene by the developing tissues could be unnecessary or even detrimental to the developing embryo. This may be the possible physiological reason for the reduced levels of beta-carotene by the embryo from dams on the 220 IU vitamin A diet.

In order to identify potential molecular mechanisms underlying the reduced accumulation of beta-carotene in embryos from dams on the 220 IU diet, we first analyzed the transcription levels of several key genes of the retinoid and carotenoid metabolism. The transcription of the beta-carotene cleavage enzyme *CMOI* was not similar in the various groups of embryos, irrespective of the maternal dietary vitamin A intake, thus excluding increased metabolism of beta-carotene as the potential cause of reduced embryonic beta-carotene in the 220 IU group. Although, we can not rule out that there could be an increased CMOI activity, this possibility is unlikely since excessive retinoid availability in the embryos would most likely lead to a suppressed activity of *CMOI* as previously shown by other studies performed in extraembryonic tissues (Dua et al., 1966; Lobo et al., 2010; Seino et al., 2008). Additionally, mRNA analysis of the key

players involved in embryonic retinoid metabolism (Kim et al., 2008b) showed that beta-carotene supplementation in the case of excessive retinoid availability (220 IU) results in upregulation of *RARb* and *Cyp26A1* compared to embryos from 14 IU + beta-carotene dams. Since we also observed an increased of the transcription of the above-mentioned genes when the dams were injected with vehicle (data not shown), we interpret these data as the consequence of elevated retinoic acid levels generated in these tissues with higher retinoid content. These changes would therefore represent an attempt to degrade excessive retinoic acid to protect the embryo from potential toxicity. At the moment, we do not understand the reasons for the small but significant drop in *RDH10* mRNA levels in embryos from 14IU + beta-carotene dams. This could likely represent the first attempt of the embryo to regulate endogenous retinoid levels in response to the beta-carotene supplementation (RDH10 would generate RAL and then RA). However, this possibility needs to be confirmed.

Overall, the embryonic mRNA analysis indicated reduced accumulation of beta-carotene in embryos from dams under the vitamin A excess regimen are not likely due to adaptation of retinoid and carotenoid metabolism taking place in the embryo itself. We also did not see any changes in the transcriptional levels of lipoprotein receptors in the embryo suggesting the uptake mechanisms of beta-carotene in this tissue are not affected by retinoid availability (data not shown).

Since placenta acts as a barrier between maternal and embryonic circulation and is responsible for the exchange of gas nutrients between the

mother and the embryo (Watson and Cross, 2005), we next asked whether placenta could be regulating the amount of beta-carotene that is ultimately transported to the embryo and could be responsible for the reduced accumulation of beta-carotene in the embryos from dams on the excess vitamin A regimen. qPCR analysis of the placenta showed that when this tissue is under a vitamin A sufficient state (14 IU), beta-carotene availability resulted in the transcriptional downregulation of the cleavage enzyme *CMOI* and of the retinoic acid responsive genes *RARb* and *Cyp26A1*, all together suggesting a suppression of retinoic acid production. However mRNA levels of *CMOII* were upregulated. CMOII has been shown to posses a carotenoid scavenging activity (Amengual et al., 2011b; Lobo et al., 2012) suggesting its transcriptional upregulation could be a response to beta-carotene supplementation under vitamin A sufficient conditions leading to its increased scavenging rather than its asymmetric cleavage. Furthermore, when beta-carotene supplemented groups were compared, under vitamin A excess conditions, placental mRNA levels of *CMOI, RARb* and *Cyp26A1* were upregulated in placenta from dams 220 IU + beta-carotene. Transcriptional changes in *RARb* and *Cyp26A1* indicated a response to increased retinoic acid availability in this tissue, similar to what was also seen in the case of the embryos. However, upregulation of mRNA levels of *CMOI* was intriguing since under the conditions of excessive retinoid availability it is hard to explain why beta-carotene cleavage enzyme would be upregulated transcriptionally. However, recent studies from our laboratory have indicated that CMOI may have

additional functions independent from its carotenoid cleavage action and may be responsible for facilitating the esterification of retinol to retinyl esters (Dixon et al., 2013). Under excessive retinol availability, it is possible that *CMOI* is upregulated to increase the formation of retinyl esters, the storage form of vitamin A. On the other hand, if this is not the case, it is also possible that the cleavage of beta-carotene is indeed increased in the placenta and resulting retinoic acid actively degraded by Cyp26A1, thus inhibiting the transport of beta-carotene or retinoids to the embryo under a vitamin A excess state. The latter possibility may also partially explain the reduced embryonic beta-carotene levels under a maternal regimen of vitamin A excessive intake.

Since lipoproteins are major carriers of beta-carotene in the circulation, we sought to investigate whether the lipoprotein receptors in the placenta were affected by the maternal dietary vitamin A content, thus potentially regulating the amount of beta-carotene taken up by the placenta and ultimately transported to the embryo. To this aim, we next conducted mRNA analysis of the lipoproteins receptors that mediate the tissue uptake of lipoproteins. Similar to what was observed in Part A of the study, placental transcription of *LRP1* was significantly downregulated upon beta-carotene supplementation in vitamin A sufficient group. In addition, mRNA levels of *VLDLr* were also reduced in this group (14IU + beta-carotene). These data confirm the receptors LRP1 (and VLDLr) as potential molecular players regulating placental uptake of beta-carotene, under the conditions of normal vitamin A status of the dams, when the embryo already

receives sufficient vitamin A from the maternal bloodstream. Interestingly, in the case of placenta from dams fed the 220 IU diet and supplemented with beta-carotene, the mRNA levels of *LRP1* and *VLDLr* were similar to those of the baseline group (14 IU without beta-carotene supplementation). These results suggest that in the case of chronic excessive retinoid availability, a single dose of beta-carotene of the dams does not cause any changes in the placental transcription of these receptors. Although the reasons underlying this data are not completely clear at the moment, it is possible that these receptors could be already regulated in the placenta upon excessive retinoid availability and that beta-carotene supplementation does not cause any further changes. Whether the transcription of *LRP1* and *VLDLr* is controlled by retinoic acid is not known and presents an interesting area of research for further studies.

Since it has been shown that the embryo acquires lipids *via* the placental secretion of lipoproteins (Woollett, 2005, 2011), we hypothesized that beta-carotene could follow a similar pathway to be transported from the placenta to the embryo. We therefore wondered whether maternal dietary vitamin A intake/status influences the placental assembly of lipoproteins. Placental mRNA analysis showed the transcriptional levels of *MTTP* were upregulated under vitamin A sufficient conditions upon beta-carotene supplementation. *MTTP* encodes for a protein that facilitates the transport of neutral lipids to nascent lipoproteins and is a rate limiting protein in the assembly of apoB-containing lipoproteins (Hussain et al., 2012). In addition, it has been reported that regulation of *MTTP* occurs at

transcriptional level, indicating that usually the changes in mRNA levels of this gene correlate well with the expression and activity of the protein that it encodes (Hussain et al., 2011). Similarly, mRNA levels of *ApoB* that encodes apolipoprotein B, which is acquired by lipoproteins during assembly and is required for their tissue uptake (Brown and Goldstein, 1979) was also upregulated upon beta-carotene supplementation at vitamin A sufficient conditions. Thus, the upregulation of *MTTP* (and *ApoB)* may suggest an increased assembly of apo-B containing lipoproteins in the placenta when dams under vitamin A sufficient conditions were supplemented with beta-carotene. Interestingly, when beta-carotene supplemented groups were compared, *MTTP* and *ApoB* mRNA levels were reduced in the placenta in the vitamin A excess group (220 IU + beta-carotene), and more importantly similar to those of the baseline group (14 IU + beta-carotene). Overall, the difference in *MTTP* levels in the placenta from the supplemented groups could result in a different rate of beta-carotene secretion from the placenta towards the fetus, specifically higher in the 14 IU + beta-carotene group. Ultimately, these different rates of secretion could result in lower levels of beta-carotene accumulated in the embryo, despite the lack of changes in placenta beta-carotene levels between the two groups. Further studies need to be performed to unequivocally prove these hypotheses.

Overall, data from Part A of the study strongly suggest that the maternal vitamin A status influences placental uptake of beta-carotene. Data from Part A

and B suggested that both *LRP1* and *VLDLr* could be potential modulators of placental beta-carotene. Results from Part B suggested that the maternal dietary vitamin A influences placental carotenoid, retinoid and lipoprotein metabolism resulting into a regulated transport of beta-carotene *via* the placenta towards the embryo.

# Chapter 3: Role of LDL-receptor in the uptake of beta-carotene by the maternal and the developing tissues i.e. the placenta and the embryo

### *3-1. Introduction*

Vitamin A is an essential nutrient required for proper embryonic development in mammals, including humans (Clagett-Dame and Knutson, 2011). The developing embryo relies on various forms of retinoids (vitamin A and its derivatives) circulating in the maternal bloodstream to satisfy its needs (Marceau et al., 2007; Wu et al., 2004). Our laboratory has recently demonstrated that, among these forms, the provitamin A precursor beta-carotene serves as a "local" source of retinoids in the developing tissues (Kim et al., 2011). Beta-carotene, which is the most abundant vitamin A precursor present in the human diet (mainly in food of plant origin) and tissues (Olson, 1994; Ross, 2005) can generate retinoids predominantly upon its symmetric enzymatic cleavage by the enzyme beta, beta-carotene 15, 15' oxygenase (CMOI), also expressed in the developing tissues (Kim et al., 2011; Paik et al., 2001; Wyss et al., 2001).

The mechanisms by which this provitamin A carotenoid is taken up by the developing tissues are yet to be elucidated. Nevertheless, this question is of utmost importance for human health. Understanding the pathways of beta-carotene uptake by the developing tissues and the key regulators involved in such uptake will help in designing more effective beta-carotene supplementation strategies during pregnancy.

It has long been established that beta-carotene, being a highly lipophilic and non-polar molecule, is transported in the circulation in association with lipoproteins, such as chylomicron remnants, VLDL, IDL and LDL (Auletta and

Gulbrandsen, 1974; Traber et al., 1994; Vogel et al., 1999). Lipoproteins are micellar particles synthesized within the body that function as the major transporters of lipids and lipid soluble nutrients of exogenous (from the diet) and endogenous sources (from tissues) in the circulation (Bragdon et al., 1956; Gibbons, 1990; Mansbach and Siddiqi, 2010). Specific apolipoproteins are acquired by the lipoproteins during their assembly and are crucial for their receptor-mediated uptake. The main apolipoprotein associated with LDL is synthesized in the liver and is known as apoB-100 (Nakajima et al., 2011), whereas VLDL is associated with apoE (Gibbons, 1990) and chylomicron remnants contain apoE and apoB-48 (Mansbach and Siddiqi, 2010). LDLr is a member of LDL-receptor family of proteins, which also includes other receptors such as VLDL-receptor (VLDLr) and isoforms of LDLr-related protein (LRPs) including LRP1, LRP2, ApoER2 (also known as LRP8), and LRP5/6 (Go and Mani, 2012; Willnow et al., 2007). The members of LDLr family share several common motifs and the types of lipoproteins that can bind to these receptors often overlap (Figure 12). In the pathways mediating cellular lipoprotein uptake, these receptors cluster into the clathrin coated pits on the cell surface and bind to the lipoproteins *via* the recognition of specific apolipoproteins. The lipoproteins are then transported to early and late endosomes and then to the lysosomes for their hydrolysis, to release the core lipids into the cytoplasm. The receptors dissociate themselves from the lipoproteins and are recycled back to the cell

surface for further binding to newly transported lipoprotein particles (Brown and Goldstein, 1979, 1983; Go and Mani, 2012).

LDL-receptor recognizes apoB-100 and mediates the endocytosis of LDL (Brown and Goldstein, 1979, 1983). Moreover, LDL-receptor also binds to apoE albeit with a greater affinity than to apoB-100 and thus it also mediates the uptake of apoE containing lipoproteins such as VLDL and chylomicron remnants. As a result of affinity to both apoB and apoE containing lipoproteins, overall, LDLr aids in endocytosis of majority of lipoproteins in the circulation such as chylomicron remnants VLDL, IDL and LDL particles (Martins et al., 2000).

Regulation of tissue uptake and metabolism of lipids circulating in lipoproteins, such as cholesterol, by various lipoprotein receptors has been extensively studied in adult as well as the developing tissues (Go and Mani, 2012; Herrera and Amusquivar, 2000; Ishibashi et al., 1996; Lewis and Rader, 2005). Specifically, mice lacking LDL-receptor (LDLr knockout) have an altered plasma lipoprotein profile due accumulation of LDL and are hypercholesterolemic (Ishibashi et al., 1993; Yokode et al., 1990). They also develop familial hyperlipidemia and an accelerated atherosclerosis. Furthermore, LDLr knockout mice are susceptible to developing various features of metabolic syndrome such as obesity, insulin resistance and systemic inflammation, the risks of which rise several-fold upon consumption of a diet high in cholesterol (Schreyer et al., 2002; Subramanian et al., 2008). When fed a Western type diet (high-cholesterol diet), the livers of these mice show increased inflammatory lesions, apoptosis and

higher oxidative stress (Subramanian et al., 2011). These and several other lines of evidence show that lack of LDL-receptor leads to acute and chronic disorders associated with disrupted lipid metabolism.

As already stated, in the human circulation, beta-carotene can be found in association with all the types of lipoproteins. Particularly, the majority of beta-carotene in the fed state is associated with chylomicron remnants, VLDL and LDL (Bjornson et al., 1976; Ganguly et al., 1959; Johnson and Russell, 1992; Ribaya-Mercado et al., 1995), whereas in the fasting state about 60-70% of beta-carotene is present in LDL-particles (Parker, 1996). Therefore, it was postulated that lipoprotein receptors could mediate tissue uptake of beta-carotene (Borel et al., 2013; Harrison, 2012). However, studies exploring whether this is indeed the case are very few and most of them pertain only to the adult tissues (Borel et al., 2013; Moussa et al., 2011; Reboul and Borel, 2011; van Bennekum et al., 2005; Woollett, 2011).

LDL-receptor is expressed in the mouse placenta/uterus and its expression pattern drops significantly from mid to late gestation (Overbergh et al., 1995). In humans and mice, along with many other lipoprotein receptors, LDLr is expressed in the syncytiotrophoblast layer of the placenta which is of embryonic origin and forms the barrier between maternal circulation and the embryo (Desoye et al., 2011) (Figure 4 from chapter1).

Given the presence of beta-carotene in all the classes of lipoprotein particles, the fact that LDLr performs an efficient cellular internalization of these

apolipoproteins B- or E- containing lipoproteins, and the expression of LDLr in maternal and developing tissues, we sought to investigate whether this receptor controls the tissue uptake of beta-carotene during pregnancy and development.

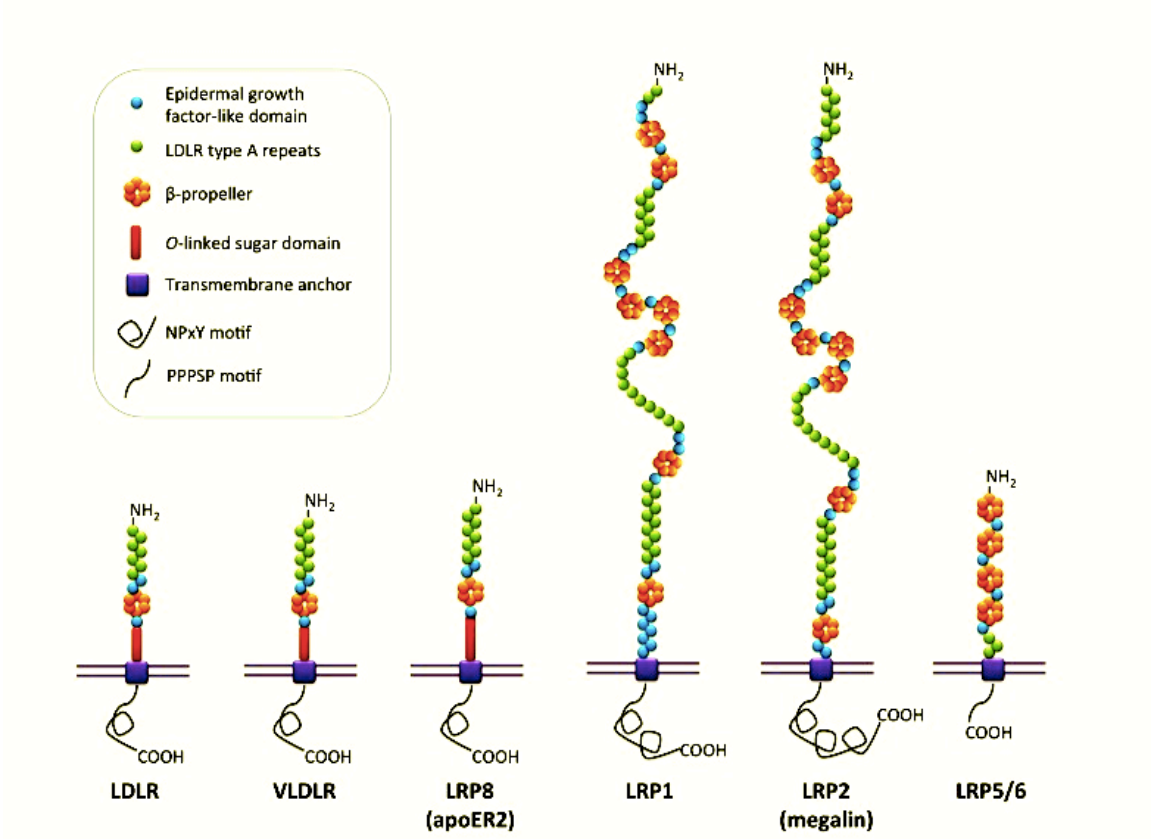



**Figure. 12. Low-density lipoprotein receptor family of proteins: Members of LDLr family share several common structural motifs listed in the inset box**

Adapted from Gwang-woong Go and Arya Mani (Go and Mani, 2012)

### *3-2. Materials and Methods*

#### *3-2.1. Animals and mating scheme:*

Mice lacking either one or both copies of LDL-receptor gene were employed in this study (LDLr+/- or LDLr-/-). Two females and one male homozygous for LDLr knockout allele (LDLr-/-) (Strain name: B6.129S7-*Ldlr*$^{tm1Her}$/J, Background: C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). After initial breeding of the homozygous pairs, second generation homozygous females were mated with wild-type males of mixed background (C57BL/6J x 129sv) and vice-versa. The colony was maintained in heterozygousity thereafter and each litter was genotyped according to published procedures (Gaw et al., 1995). To perform experiments, two different mating schemes were utilized: heterozygous (LDLr+/-) females were mated with homozygous (LDLr-/-) males; and homozygous (LDLr-/-) females were mated with heterozygous (LDLr+/-) males. This experimental design resulted in both homozygous and heterozygous embryos (LDLr+/- and LDLr-/-) from both homozygous and heterozygous dams. Approximately three-month old females were used for mating with males of similar age. Onset of pregnancy (0.5 *days post coitum*, dpc) was established by the occurrence of a vaginal plug, the morning after the mating. This 2X2 experimental design described above has two variables: maternal genotype and placental/embryonic genotype, giving rise to 4 experimental groups with homozygous and heterozygous embryos from both homozygous and heterozygous dams. This experimental design allowed us to

examine the effects of the presence or absence of LDLr on beta-carotene uptake by the placenta and embryos within the same litter. Since LDLr-/- mice have been reported to have an altered lipoprotein profile (Ishibashi et al., 1993; Yokode et al., 1990) this experimental design also allowed us to study whether maternal lack of LDLr has any effect on the placental and embryonic uptake of retinoids and beta-carotene, irrespective of the embryonic genotype.

### *3-2.2. Animal diet*

All the mice used were maintained on a regular chow diet containing 29 IU of vitamin A/g diet throughout the lifespan, including gestation. Other laboratories use a similar concentration of vitamin A in the mouse diet and define this diet as “copious in vitamin A” (Kane et al., 2011). Following are the specifications of the above-mentioned chow diet (Diet: Prolab Isopro RMH3000 5p75; Composition: energy from protein, fat, and carbohydrates: 26, 14, and 60%, respectively; vitamin A: 29 IU vitamin A/g of diet; beta-carotene: trace amounts) manufactured by LabDiet (W.F. Fisher and Son).

Both diet and water were available to the animals on an *ad libitum* basis. Mice were maintained on a 12:12 light/dark cycle with the period of darkness between 7 PM and 7 AM. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council, 1996) and were approved by the Rutgers University Institutional Committee on Animal Care.

### *3-2.3. Dosage and delivery method of beta-carotene*

As described in Chapter 2, since mice are efficient cleavers of beta-carotene, almost all of ingested beta-carotene is cleaved in the mouse intestine to generate retinoids resulting in negligible amounts (if any) of intact beta-carotene released into the circulation (Lee et al., 1999). Since our experimental scheme requires well measurable levels of beta-carotene in the mouse circulation, we supplemented mice by means of an intraperitoneal injection of beta-carotene. Intraperitoneal injection directly delivers intact beta-carotene into the peritonea of the mouse, and from here into the circulation, thus circumventing the intestinal cleavage. The beta-carotene solution was prepared as described in Chapter 2. Briefly, we added 50 mg of beta-carotene in a mixture of ethanol, Cremophor (Sigma, St Louis, MO) and PBS (1:11:18 ratio), under yellow light. The dams were injected with a dose of 20-50 μg of beta-carotene/g body weight (~250 μl of the emulsion). Dams injected with the same volume of vehicle (ethanol, Cremophor and PBS mixture) served as controls. The administration of beta-carotene was performed at mid-gestation of the dams (13.5 dpc) when the embryo is metabolically independent being able to cleave beta-carotene and maintain homeostatic retinoid levels (Kim et al., 2011; Kim et al., 2008b). All animals were euthanized at 14.5 dpc by $CO_2$ inhalation. Maternal serum and liver, as well as placenta, yolk sac and embryos were collected, frozen and stored at -80°C until further analyses (Figure 13). All the dams utilized in the study were continuously fed throughout the experiments.

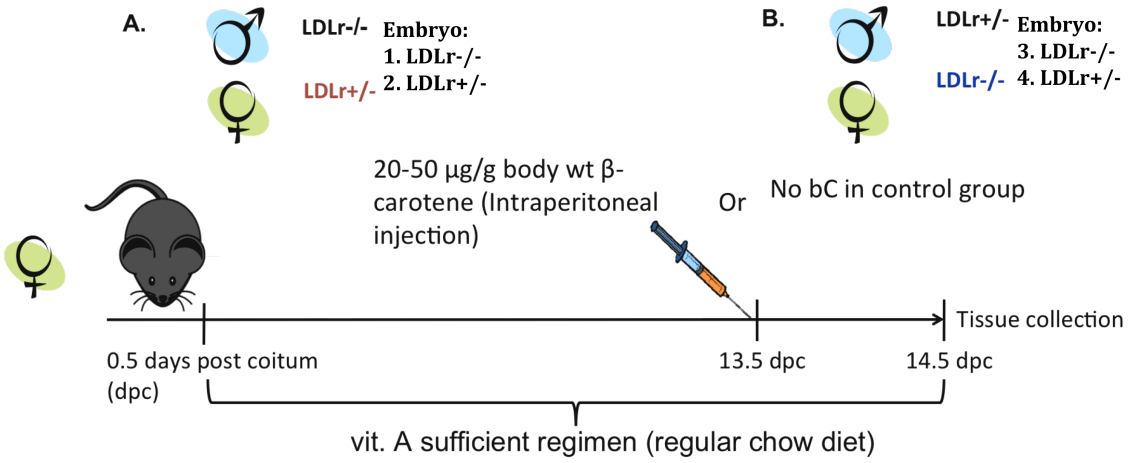



**Figure. 13. Mating scheme and treatment of the dams.** LDLr heterozygous (LDLr+/-) and knockout (LDLr-/-) dams were mated as per the scheme above, supplemented with either beta-carotene or vehicle at 13.5 dpc, and sacrificed 24 hours later. All the dams were fed chow diet providing sufficient amounts of vitamin A throughout life and gestation. Each of the mating set yielded embryos that were either heterozygous or knockout for LDLr.

### *3-2.4. Isolation of serum lipoproteins*

Serum lipoproteins of the dams were isolated using the classic method of density based ultracentrifugation as described in Chapter 2. Briefly, mouse serum was subjected to a two-step density based ultracentrifugation to separate VLDL + chylomicron fraction (d < 1.006 g/ml), LDL + IDL fraction (d = 1.023-1.06 g/ml) and HDL fraction (d = 1.063-1.21 g/ml) (Goldberg et al., 2012). To facilitate the formation of a density gradient, 0.9% saline solution (d = 1.004 g/ml) and KBr solution (d = 1.12 g/ml, prepared by dissolving 17.22 g of KBr in 100 ml Milli-Q water) were used in the first and the second step of ultracentrifuation respectively. All the serum lipoprotein fractions as well as the total sera were analyzed to determine total cholesterol (Cholestrol E kit, Wako Dignostics), and triglycerides (Infinity triglyceride kit, Thermo scientific) concentrations. Beta-carotene content of all the fractions was determined by HPLC analysis as described below.

### *3-2-5. HPLC analysis of retinol, retinyl ester and beta-carotene*

Reverse-phase HPLC analysis was performed to measure serum and tissue retinoid levels (Kim and Quadro, 2010) and beta-carotene levels (Kim et al., 2011) as described in Chapter 2. Briefly, tissues were homogenized in PBS and half of the homogenate was used to extract retinoids (Kim and Quadro, 2010) and the other half was used to extract beta-carotene. 100 µl of serum was used for both retinoid and beta-carotene extraction. 50 µl of lipoprotein fractions

were extracted using petroleum ether for beta-carotene analysis. Retinoids and beta-carotene were separated on a 4.6 × 250 mm Ultrasphere ODS analytical column (Part no. 235329, Hichrom Limited, UK) using acetonitrile, methanol, and methylene chloride (70:15:15, v/v) as the mobile phase flowing at 1.8 ml/min. Retinol, retinyl esters and beta-carotene were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. Retinyl acetate (Sigma, St Louis, MO; for retinoids) and echinenone (CaroteNature, Switzerland; for beta-carotene) were added as internal standards. Detection limits are as follows: for retinoids - serum <0.1 ng/dl and tissues <1 ng/g; for beta-carotene – serum <1 ng/dl and tissues 10 ng/g).

### *3-2.6. RNA extraction, cDNA synthesis, and quantitative real-time PCR*

qPCR analysis of the tissues was performed to determine the mRNA levels of various genes as described in Chapter 2. Briefly, total RNA was extracted from tissues and their concentrations were measured by using the Nanodrop 1000 and quality was ascertained by 260/280 ratio. The RNA were then treated DNase I (Roche Diagnostics, IN) and were then reverse transcribed to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, IN). The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, IN) was used for qPCR. 300 nM of each specific primer (final concentration), together with 10ng of cDNA were used for the RT-PCR reaction in Lightcycler 480 machine (Roche Diagnostics, IN). All samples were run in

duplicates or triplicates. After enzyme activation (10 min, 95°C), 35–40 PCR amplification cycles were performed: 10 s at 95°C, 20 s at 58°C, and 30 s at 72°C. At the end of each run, samples were heated to 95°C with a temperature transition rate of 0.11°C/s to construct dissociation curves.

To determine changes in gene expression, the DDCT method was used by comparing Cq of the gene of interest from that of the housekeeping gene (beta-actin). All groups were expressed as fold change from the control group.

### *3-2.7. Placenta organ culture*

Heterozygous and knockout (LDLr+/- and LDLr-/-) dams were mated and supplemented with beta-carotene as described above. The dams were sacrificed 4 hours following beta-carotene supplementation and fresh placentae were collected under sterile conditions. The placentae were rinsed once with sterile 1X PBS (phosphate buffer saline) and once with Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) maintained at 37°C. Subsequently, individual placenta were placed in individual wells of a 24-well sterile cell-culture plate (BD Biosciences, CA, USA), filled with with 600 µl of DMEM containing antibiotics 0.5% penicillin and streptomycin. The placenta with the medium were then incubated at 37°C. The media from each placenta were collected at 3hr, 6hr, 18hr, 24hr and 48hr from the plating. At each time point (except the 48hr time point), fresh media was added and the placenta were incubated at 37°C until the next time point. The collected media were extracted

using petroleum ether and the extracts were analyzed by HPLC for their beta-carotene content as described above. A small piece from the respective embryos was genotyped to determine the placental LDLr genotype.

### *3-2.8. Statistical Analyses*

Normality of the data was established by using the Shapiro-Wilk test. Normally distributed data were analyzed by 2-way ANOVA followed by a *t*-test or one-way ANOVA. Data with a non-normal distribution were analyzed by Kruskal Wallis test, followed by a Mann-Whitney test. Analyses were performed with SPSS statistical software (IBM SPSS Statistics, Version 19). A p-value $<0.05$ was used to establish statistical significance.

### *3-3. Results*

#### *3-3.1. Effects of the lack of LDLr on maternal serum and hepatic beta-carotene content*

As presented and discussed in Chapter 2, upon beta-carotene supplementation by IP injection of wild-type dams, beta-carotene circulates in the maternal bloodstream in association with lipoproteins as follows: ~2% in VLDL+Chylomicron fraction, ~50-60% in LDL+IDL fraction and ~30-40% in HDL fraction. Since the LDLr is responsible for the majority of the lipoprotein clearance from the circulation, mice lacking this receptor display higher accumulation of lipoproteins such as chylomicron remnants, VLDL and LDL in the serum, leading to elevated cholesterol levels and overall an altered serum lipid profile as compared to the wild-type mice (Brown and Goldstein, 1979, 1983; Go and Mani, 2012; Goldberg et al., 2012; Goldstein et al., 1985). To verify whether this abnormal lipoprotein profile would lead to an altered distribution of beta-carotene among the lipoproteins in LDLr-/- mice, serum lipoproteins from LDLr heterozygous and knockout mice were isolated using the classic method of density gradient ultracentrifugation, as described in the Material and Methods section. Giving that the dams used for this study were continuously fed until sacrifice, three lipoprotein fractions were obtained: VLDL + chylomicrons; LDL + IDL and HDL fractions.

All of the lipoprotein fractions were analyzed for their total cholesterol (TC) and triglycerides (TG) content, as well as for their beta-carotene concentrations,

as described in the Material and Methods section. As shown in Table 8, and as expected based on literature data (Go and Mani, 2012; Goldstein et al., 1985; Ishibashi et al., 1993; Ishibashi et al., 1996; Martins et al., 2000; Yokode et al., 1990), total cholesterol levels in VLDL+chylomicron (VLDL+Chyl) fraction and LDL+IDL fraction of LDLr-/- dams were higher than those in the respective lipoprotein fractions of LDLr+/- dams, with no significant differences in TG content. Furthermore, in agreement with the literature (Brown and Goldstein, 1983; Go and Mani, 2012; Ishibashi et al., 1996; Martins et al., 2000) whole serum total cholesterol was elevated in knockout dams compared to LDLr+/- females, while whole serum triglyceride levels were not different between the two groups of dams (Table 8).

HPLC analysis of beta-carotene levels in lipoprotein fractions firstly confirmed that, as for the wild-type dams, also in the case of the beta-carotene supplemented LDLr-/- dams, beta-carotene was incorporated in all the three different lipoprotein fractions, with the lowest concentration in VLDL+Chyl and the highest concentration in HDL (Table 8). Moreover, the beta-carotene concentration in the LDL+IDL fractions of LDLr-/- dams trended towards a higher mean value as compared to that in the LDLr+/- dams, even though this difference failed to reach statistically significance (Table 8). Interestingly, the distribution of beta-carotene seemed to be similar to that of cholesterol (Table 8).

Beta-carotene was detected in the whole serum of all the supplemented dams, as expected (Kim et al., 2011; Wassef et al., 2012). Serum beta-carotene

levels were slightly elevated in LDLr-/- dams as compared to LDLr+/- dams, however this difference did not reach statistical significance (Table 9).

We also measured by HPLC, maternal serum retinoid levels (Table 9). While serum retinol levels did not differ between dams of the two genotypes, retinyl ester concentration was significantly higher in LDLr-/- dams as compared to LDLr+/- dams, irrespective of the treatment. This difference was expected due to the reduced lipoprotein clearance of the mice lacking LDL (Ishibashi et al., 1996).

Since LDLr is abundantly expressed in the adult liver (Goldstein et al., 1985), we next investigated whether hepatic beta-carotene uptake was affected by lack of LDLr, by performing HPLC analysis of maternal livers from both genotypes. As shown in Table 10, hepatic beta-carotene levels were reduced by ~50% in the LDLr-/- dams as compared to the LDLr+/- dams, while liver retinol and retinyl esters were not different between the two genotypes.

Together, the analysis of total maternal serum, serum lipoprotein fractions and maternal liver indicate that lack of maternal LDL alters the distribution of beta-carotene within circulating lipoproteins with beta-carotene levels in the apoB- and apoE-containing lipoproteins showing a trend to be higher in LDLr-/- *versus* LDLr+/- dams. Furthermore, these data suggest that hepatic clearance of beta-carotene containing lipoproteins may be reduced in the absence of LDLr.

**Table 8. Distribution of cholesterol, triglycerides and beta-carotene in the serum lipoprotein fractions of LDLr+/- and LDLr-/- pregnant mice supplemented with beta-carotene**

| Maternal genotype/ treatment | Fraction collected | TC (mg/dL) | TG (mg/dL) | bc (µg/dL) | n | Serum TC | Serum TG |
|---|---|---|---|---|---|---|---|
| LDLr+/- bc | VLDL+Chyl | 11.2 ± 1.3 | 11.7 ± 0.6 | 9.2 ± 5.5 | 3 | 157.0 ± 15.6 | 25.6 ± 7.1 |
| | LDL+IDL | 58.2 ± 16.1 | 12.0 ± 2.3 | 81.5 ± 65.6 | | | |
| | HDL | 89.7 ± 9.1 | 8.9 ± 0.8 | 427.7 ± 74.5 | | | |
| LDLr-/- bc | VLDL+Chyl | 33.0 ± 10.3* | 17.9 ± 4.8 | 37.0 ± 23.5* | 6 | 207.9 ± 23.0* | 28.9 ± 5.5 |
| | LDL+IDL | 90.5 ± 13.1* | 12.4 ± 1.8 | 340.1 ± 248.5* | | | |
| | HDL | 85.8 ± 42.4 | 13.5 ± 2.2 | 240.9 ± 179.3 | | | |

n=4-6 females per genotype; Values expressed as mean ± standard deviation; Total cholesterol, (TC); Total serum triglycerides, (TG); VLDL and chylomicron fraction, (VLDL+Chyl); LDL and IDL fraction, (LDL+IDL); HDL fraction, (HDL); *, $p<0.05$ vs. respective lipoprotein fraction in LDLr+/- bc group; Non-parametric tests, Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc.

**Table 9. Retinyl ester (RE) concentrations in the serum of LDLr+/- and LDLr-/- pregnant mice with or without beta-carotene (bc) supplementation**

| Maternal genotype/treatment | n | bc (ug/dL) | RE (ug/dL) |
|---|---|---|---|
| LDLr+/- veh | 4 | n.d. | 17.2 ± 9.8 |
| LDLr+/- b-c | 6 | 480.2 ± 178.5 | 4.8 ± 1.0 |
| LDLr-/- veh | 3 | n.d. | 47.3 ± 26.1* |
| LDLr-/- b-c | 5 | 828.8 ± 446.0 | 16.3 ± 6.2*§ |

Values expressed as mean ± SD; n.d., not detected; *, $p<0.05$ vs. LDLr+/- similar treatment; §, $p<0.05$ vs. LDLr-/- veh; Non-parametric tests, Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc

**Table 10. Beta-carotene (bc), retinol (ROH) and retinyl ester (RE) concentrations in the liver of LDLr+/- and LDLr-/- pregnant mice with or without beta-carotene supplementation (bc or veh, respectively)**

| Maternal genotype/treatment | n | bc (ug/g) | ROH (ug/g) | RE (ug/g) |
|---|---|---|---|---|
| LDLr+/- veh | 4 | n.d. | 6.6 ± 1.3 | 447.9 ± 169.1 |
| LDLr+/- bc | 6 | 86.4 ± 18.9 | 6.3 ± 1.0 | 721.0 ± 285.3 |
| LDLr-/- veh | 3 | n.d. | 5.0 ± 1.3 | 597.8 ± 63.7 |
| LDLr-/- bc | 5 | 39.9 ± 25.0* | 5.7 ± 1.3 | 668.9 ± 309.2 |

Values expressed as mean ± SD; n.d., not detected; *, $p<0.05$ vs. group with respective treatment; Student's t-test, SPSS Inc.

### *3-3.3. Effects of the lack of LDLr on placental beta-carotene and retinoid content and metabolism*

In order to investigate whether the increased beta-carotene levels in maternal serum apoB- and apoE-containing lipoproteins would affect placental beta-carotene uptake, we first measured by HPLC beta-carotene and retinoid levels in placenta of both LDLr-/- and LDLr+/- dams with or without beta-carotene supplementation (total 4 groups of dams). The placenta is an organ that develops during embryonic development to facilitate exchange of gas and nutrients between mother and fetus (Desforges and Sibley, 2010; Duttaroy, 2009; Jones and Parer, 1983). Cells of both maternal and embryonic origin contribute to its structure (Watson and Cross, 2005). Thus, depending upon their developmental origin, different layers of cells within the placenta may have different genotypes, if maternal and embryonic genotypes are different. The layer called syncytiotrophoblast constitutes the site where maternal and fetal circulation come in contact and constitute the most important “barrier” component. The syncytiotrophoblast layer is of embryonic origin (Watson and Cross, 2005), hence its genotype is the same as that of the embryo. For the purpose of this study, since LDLr is expressed in the placental syncytiotrophoblast (Albrecht et al., 1995; Desoye et al., 2011; Desoye et al., 1994), we will refer to the placental genotype as the genotype of the embryos. In summary, under this experimental scheme, a total of 8 groups of placentae were available for the analysis (2 placenta genotypes X 2 maternal genotypes X 2 maternal treatments).

Dams supplemented with vehicle did not show detectable beta-carotene in the serum or any of the tissues, as expected (Kim et al., 2011; Wassef et al., 2012) (data not shown). Among the beta-carotene supplemented groups, placenta that expressed LDLr showed elevated beta-carotene levels as compared to the ones lacking LDLr, but only in the case of LDLr-/- dams (Figure 14). Placental beta-carotene levels were similar in the LDLr+/- dams irrespective of the placental genotype (Figure 14). These data suggest a potential role of placental LDLr in beta-carotene uptake by this organ, at least under certain circumstances. However to exclude that the genotype-driven difference in placental beta-carotene levels in the case of the LDLr-/- dams was due to different mRNA expression levels of the two beta-carotene cleavage enzymes, we performed qPCR analysis. As shown in Figure 15, CMOI and CMOI mRNA levels were not different between the two groups of placenta.

We also measured placental retinoid levels by HPLC. Interestingly, while placental retinol levels were not different between the groups, irrespective of either placental or maternal genotype or treatments (Table 11), placental retinyl esters levels were elevated in LDLr-/- dams supplemented with beta-carotene in both the placental genotypes (Table 11). These data suggested that lack of maternal LDLr might influence placental retinoid levels.

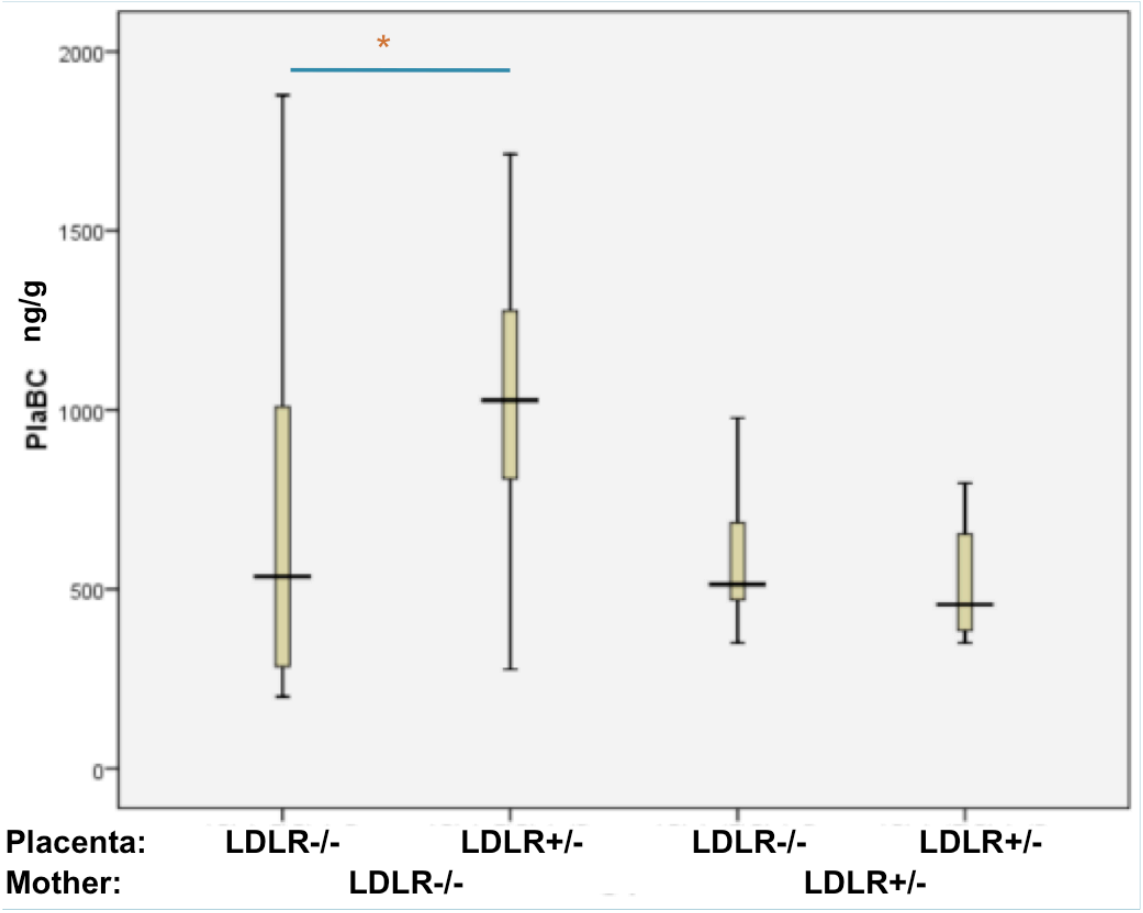



**Figure 14. Beta-carotene levels analyzed by HPLC in LDLr-/- and LDLr+/- placenta from LDLr-/- and LDLr+/- dams supplemented with beta-carotene at mid-gestation.** Analysis by HPLC. n=14-23 placentae per group; *, $p<0.05$ vs. LDLr-/- placenta from LDLr-/- dams; Non-parametric tests, Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc.

**Table 11. Retinol (ROH) and retinyl ester (RE) concentrations in the placenta of LDLr heterozygous (LDLr+/-) and LDLr knockout (LDLr-/-) mice supplemented with either vehicle (veh) or beta-carotene (bc) at mid-gestation**

| Maternal genotype/treatment | Placenta genotype | n | ROH (ng/g) | RE (ng/g) |
|---|---|---|---|---|
| LDLr+/- veh | LDLr+/- | 6 | 91.8 ± 18.9 | 41.6 ± 6.0 |
| | LDLr-/- | 3 | 98.6 ± 27.9 | 54.4 ± 23.7 |
| LDLr+/- bc | LDLr+/- | 23 | 96.9 ± 23.2 | 45.7 ± 16.9 |
| | LDLr-/- | 14 | 99.0 ± 25.1 | 50.9 ± 17.4 |
| LDLr-/- veh | LDLr+/- | 8 | 98.5 ± 18.1 | 50.9 ± 17.7 |
| | LDLr-/- | 7 | 90.1 ± 14.1 | 44.9 ± 9.8 |
| LDLr-/- bc | LDLr+/- | 21 | 101.4 ± 24.2 | 73.3 ± 40.9*# |
| | LDLr-/- | 14 | 85.8 ± 26.8 | 84.7 ± 22.3*# |

Values expressed as mean ± SD; *, $p<0.05$ vs. LDLr+/- similar treatment; #, $p<0.05$ vs. LDLr-/- veh; Non-parametric tests, Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc.

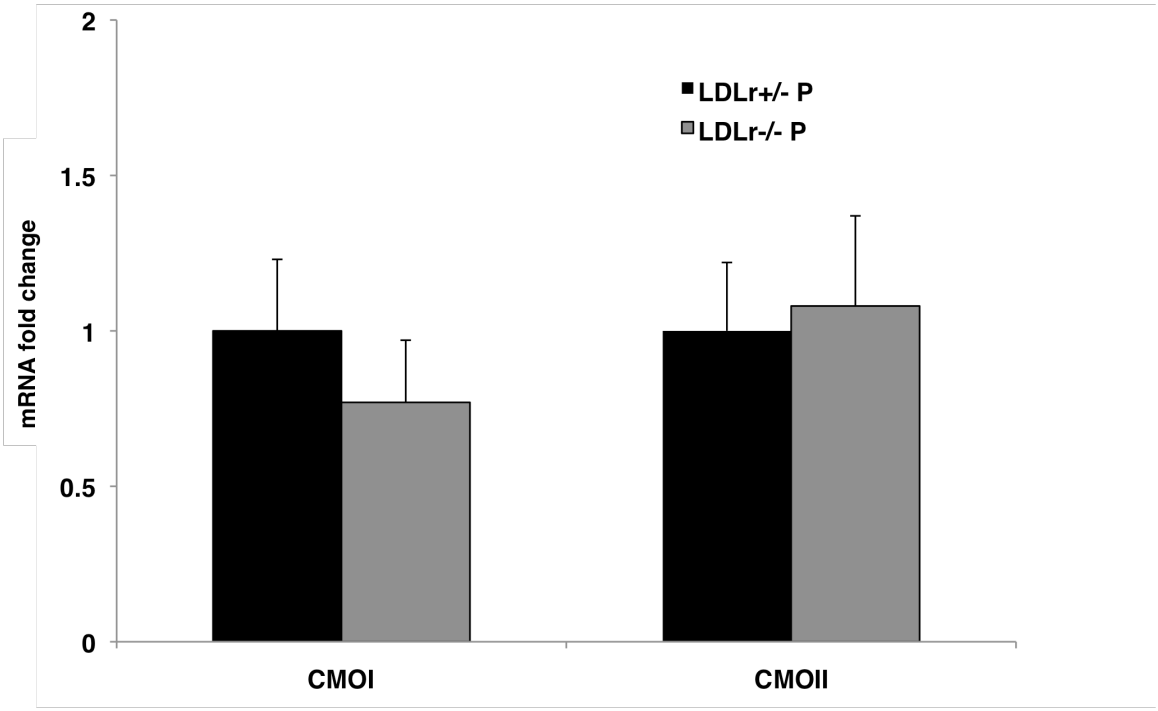



**Figure 15. mRNA expression levels of beta-carotene cleavage enzymes CMOI and CMOII in the placenta from LDLr-/- dams supplemented with beta-carotene at mid-gestation.** Analysis by qPCR. Black bars, LDLr+/- placenta from LDLr-/- dams + bc; Grey bars, LDLr-/- placenta from LDLr-/- dams + bc; Values expressed as fold change of LDLr+/- placenta; n=6 placenta per group; Student's t-test, SPSS Inc.

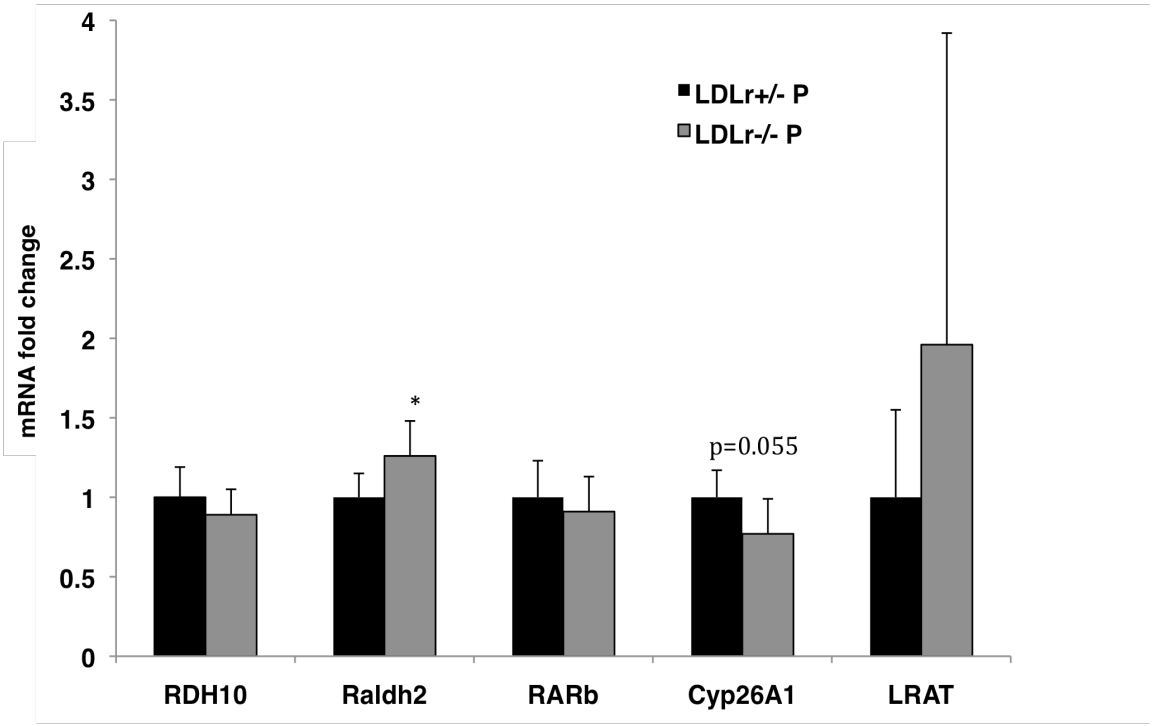



**Figure 16. mRNA expression levels of key regulators involved in retinoid homeostasis in the placenta from LDLr-/- dams supplemented with beta-carotene at mid-gestation.** Analysis by qPCR**.** Black bars, LDLr+/- placenta from LDLr-/- dams + bc; Grey bars, LDLr-/- placenta from LDLr-/- dams + bc; Values expressed as fold change of LDLr+/- placenta; n = 6 placenta per group; *, $p<0.05$; Student's t-test, Non-parametric tests Kruskal-Wallis test followed by Mann-Whitney test, SPSS Inc

### *3-3.4. Effects of the lack of LDLr on beta-carotene and retinoid homeostasis*

Since the placenta mediates the transfer of nutrients from mother to fetus (Burke et al., 2009; Desforges and Sibley, 2010; Duttaroy, 2009; Herrera and Amusquivar, 2000; Marceau et al., 2007), we next wondered whether the higher levels of beta-carotene that seemed to be taken up by the LDLr+/- placenta in LDLr-/- dams would result in increased levels of beta-carotene in the corresponding embryos. However, this did not seem to be the case. Indeed, by HPLC analysis, we showed that embryos from all the beta-carotene supplemented dams displayed detectable levels of beta-carotene (Table 12). Nevertheless, these levels did not differ based on either maternal or embryonic genotype (Table 12). In addition, embryonic retinoids did not show any difference irrespective of maternal or embryonic genotype or treatment (Table 12).

**Table 12. Beta-carotene (bc), retinol (ROH) and retinyl ester (RE) concentrations in the embryos of LDLr heterozygous (LDLr+/-) and LDLr knockout (LDLr-/-) pregnant mice supplemented with beta-carotene**

| Maternal genotype/treatment | Embryo genotype | n | bc (ng/g) | ROH (ng/g) | RE (ng/g) |
|---|---|---|---|---|---|
| LDLr+/- bc | LDLr+/- | 24 | 13.8 ± 4.2 | 90.2 ± 7.2 | 209.5 ± 45.0 |
| | LDLr-/- | 15 | 17.5 ± 7.4 | 87.2 ± 7.5 | 225.6 ± 34.2 |
| LDLr-/- bc | LDLr+/- | 21 | 16.4 ± 5.1 | 95.5 ± 9.0 | 196.2 ± 28.8 |
| | LDLr-/- | 13 | 18.8 ± 8.1 | 85.1 ± 14.2 | 205.3 ± 36.0 |

Values expressed as mean ± SD; $p<0.05$, Student's t-test, SPSS Inc.

### *3-3.5. Uptake and metabolism of beta-carotene and retinoids in the embryos is not LDLr dependent*

In order to gain insights on the mechanism by which LDLR+/- and LDLr-/- embryos from LDLr-/- dams maintained homeostatic beta-carotene and retinoid levels despite the different beta-carotene levels in their respective placenta, we examined the ability of the embryo itself to regulate its-own supply and metabolism of the provitamin A carotenoid. Firstly, we analyzed by qPCR the mRNA levels of the genes responsible for cleavage and subsequent metabolism of beta-carotene in the above-mentioned embryos. As shown in Figure 17, mRNA expression levels of *CMOI* and *CMOII* were not different between the two embryonic genotypes thus excluding a differential cleavage of beta-carotene by the embryos from these groups. Similarly, mRNA expression levels of key genes controlling tissue retinoid homeostasis, such as *LRAT, RDH10, Raldh2, and Cyp26A1* (Kim et al., 2008b) were not different between the two groups analyzed (Figure 17).

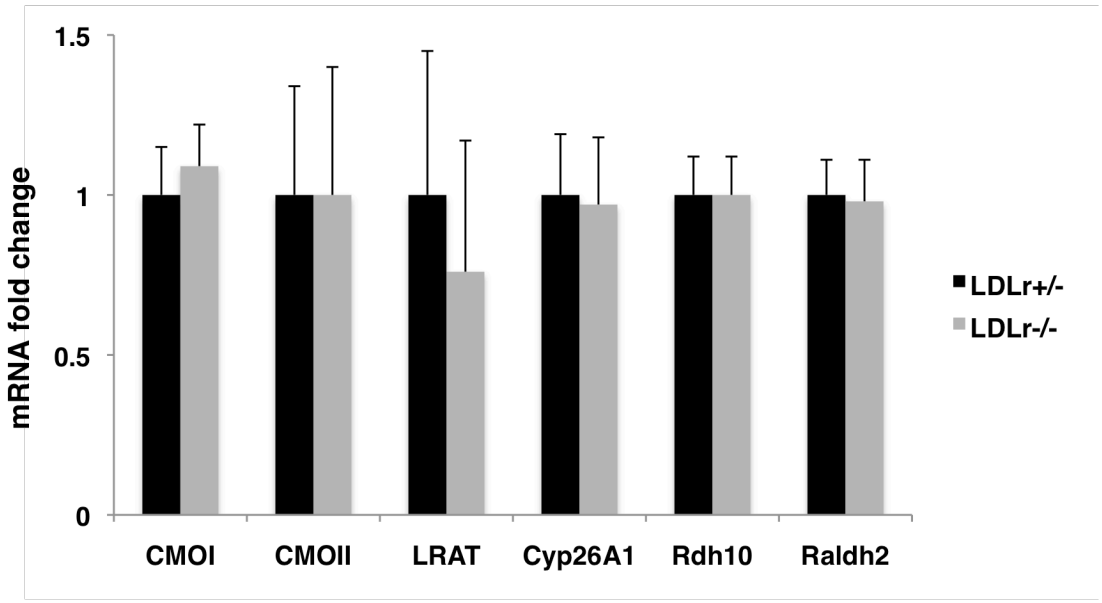



**Figure 17. mRNA expression levels of beta-carotene cleavage enzymes and other key enzymes involved in retinoid homeostasis in the embryos from LDLr-/- dams supplemented with beta-carotene at mid-gestation.** Analysis by qPCR. Black bars, LDLr+/- embryos from LDLr-/- dams + bc; Grey bars, LDLr-/- embryos from LDLr-/- dams + bc; Values expressed as fold change of LDLr+/- embryos; n=6 embryos per group; $p<0.05$, Student's t-test, SPSS Inc.

We next asked whether the similar beta-carotene levels observed in LDLr+/- and LDLr-/- embryos from LDLr-/- dams embryos could be ascribed to a differential uptake of beta-carotene. We therefore measure by qPCR analysis the mRNA expression levels of the main receptors and enzymes involved in the uptake of lipoproteins that transport beta-carotene. Specifically, we analyzed mRNA expressions levels of LDL-receptor related protein 1 (*LRP1*, the receptor for chylomicron remnants) (Go and Mani, 2012; Martins et al., 2000), VLDL-receptor (*VLDLr*) (Nakajima et al., 2011; Nilsson-Ehle et al., 1980), Scavenger receptor type B-1 (*SRB-1*, the HDL receptor) (Babitt et al., 1997; Nguyen et al., 2009), *ApoER2* (Go and Mani, 2012) also known as LRP8, lipoprotein lipase, the enzyme responsible for hydrolysis of lipoproteins (Duttaroy, 2009; Higgins and Fielding, 1975), and the receptor for fatty acid uptake *CD36* (Drover et al., 2008; Duttaroy, 2009). None of the above mentioned-genes showed different mRNA levels between the two above-mentioned groups of the embryos, except for a small but statistically significant reduction (5%) in *LRP1* and *ApoER2* mRNA levels in LDLr-/- embryos compared to LDLr+/- littermates (Figure 18). This difference may not be physiologically significant due to the small magnitude of the change and is not completely understood at this moment.

Overall, these data indicate that metabolism and uptake of beta-carotene in LDLr+/- and LDLr-/- embryos from LDLr-/- dams is not LDLr dependent.

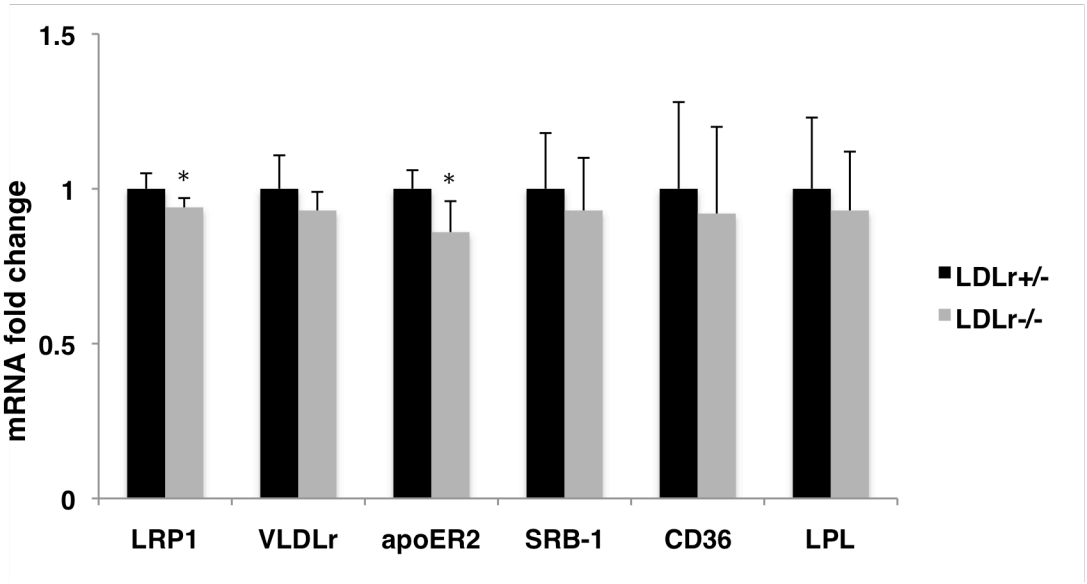



**Figure 18. mRNA expression levels of lipoprotein receptors in the embryos from LDLr-/- dams supplemented with beta-carotene at mid-gestation**. Analysis by qPCR. Black bars, LDLr+/- embryos from LDLr-/- dams + bc; Grey bars, LDLr-/- embryos from LDLr-/- dams + bc; Values expressed as fold change of LDLr+/- embryos; n = 6 embryos per group; *, $p<0.05$, Student's t-test, SPSS Inc.

### *3-3.6. Is placental beta-carotene secretion LDLr-dependent?*

The data shown above, strongly suggested that the placenta may regulate the amount of beta-carotene secreted towards the fetus thus resulting in similar levels of beta-carotene in LDLr+/- and LDLr-/- embryos from LDLr-/- dams despite the different beta-carotene levels in their respective placenta. It is not known how placenta secretes beta-carotene into the fetal circulation. Since placenta is known to assemble and secrete lipoproteins in order to transport various classes of lipids (Madsen et al., 2004; Rindler et al., 1991), we postulated that beta-carotene could also be carried in the above-mentioned lipoproteins for its transfer to the embryo. We thus performed qPCR analysis of genes involved in the assembly of lipoproteins in LDLr+/- and LDLr-/- placenta from LDLr-/- dams. While mRNA levels of apolipoproteins, such as apoE, apoB and apoA-I, were not different between the two groups, mRNA levels of microsomal triglyceride transfer protein (*MTTP*) was upregulated by ~30% in LDLr-/- placenta (Figure 19). MTTP acts as a chaperon to transfer endogenous lipids to nascent lipoproteins and is a rate-limiting protein in the assembly of apoB lipoproteins (Hussain et al., 2012). Therefore, these data suggest that assembly of apoB-containing lipoproteins could take place at an increased rate in the placenta of embryos lacking LDLr from LDLr-/- dams.

To confirm this hypothesis, secretion of beta-carotene from the LDLr+/- and LDLr-/- placenta from both heterozygous and knockout dams supplemented with beta-carotene was measured *ex vivo*. As previously described, dams were

mated and supplemented with beta-carotene at 13.5 days of gestation. They were sacrificed 3-4 hours following beta-carotene supplementation and fresh placentae from these dams were immediately cultured in DMEM medium (with 0.5% penicillin and streptomycin) for up to 48 hours. The medium was collected at various time points and subjected to HPLC analysis to determine the amount of beta-carotene secreted in the medium from the placenta over time. As shown in Figure 20, no significant differences were observed in the net amount of beta-carotene in the medium for these cultures, regardless of maternal or placenta genotype. However, beta-carotene secretion at 48 hours was seen in only one group where both, the placenta and the dams lacked LDLr (i.e. LDLr-/- placenta from LDLr-/- dams). In all other groups, beta-carotene secreted was close to zero 24 hr after the plating. This experiment suggested that lack of placental and maternal LDLr may lead to extended placental secretion of beta-carotene incorporated in the lipoproteins. However, this experiment presented a few limitations since the number of placentae analyzed was small. In addition, this experiment does not provide any information as to whether the beta-carotene was secreted from apical or basolateral side of the placenta leading to beta-carotene release in either maternal or embryonic circulation.

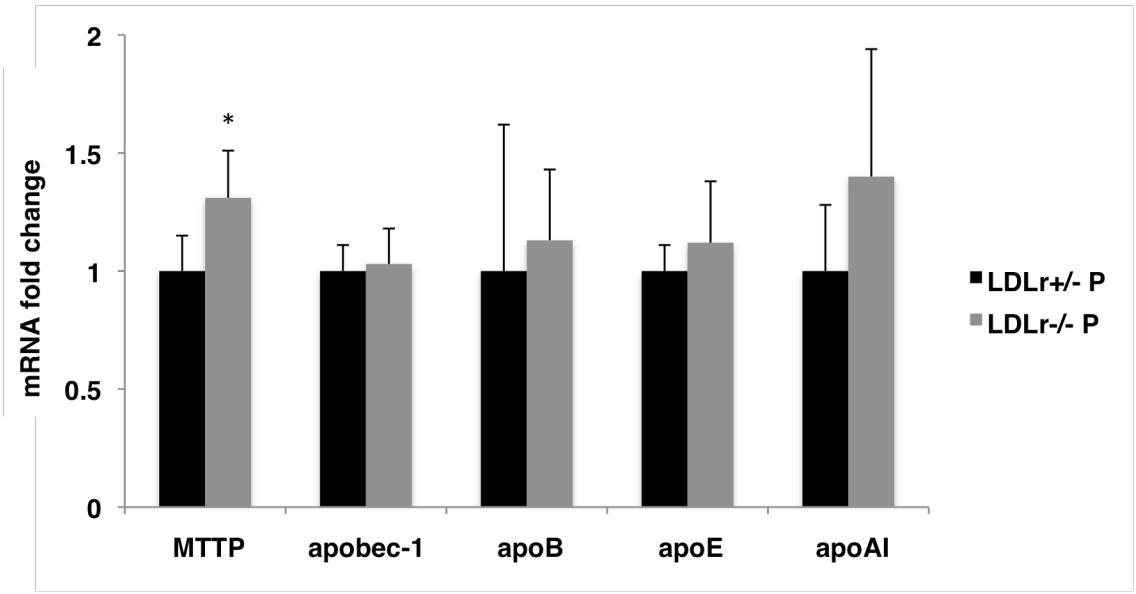



**Figure 19. mRNA expression levels of genes involved in the assembly of various lipoproteins in the placenta from LDLr-/- dams supplemented with beta-carotene at mid-gestation.** Analysis by qPCR. Black bars, LDLr+/- placenta from LDLr-/- dams + bc; Grey bars, LDLr-/- placenta from LDLr-/- dams + bc; Values expressed as fold change of LDLr+/- placenta; n = 6 placenta per group; *, $p<0.05$, Student's t-test, SPSS Inc.

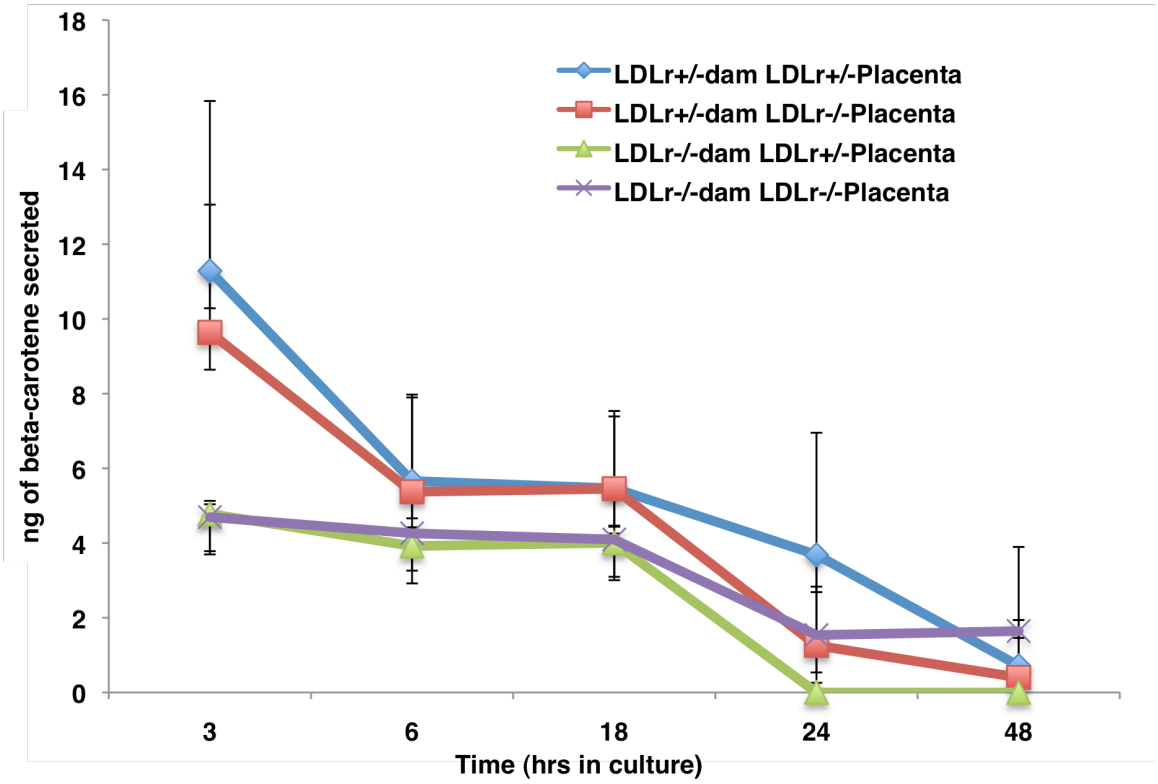



**Figure 20. Beta-carotene secreted by placenta of LDLr+/- and LDLr-/- dams supplemented with beta-carotene at 13.5 dpc and maintained in *ex vivo* culture up to 48 hours after dissection.** The placenta were cultured in DMEM medium incubated at 37°C. Media were collected at various time points and beta-carotene secreted by the placenta in the media were determined by HPLC analysis; n = 3-6 placenta per group; Values expressed as mean ± SD; ANOVA, SPSS Inc.

### *3-4. Discussion*

The goal of our study was to examine *in vivo* the role of LDLr in mediating the transfer of maternal circulating beta-carotene to the developing tissues. Mice lacking LDLr have been generated a few decades ago (Ishibashi et al., 1993) but they have never been utilized as a model to address the above-mentioned question. Our study confirmed that LDLr-/- mice have reduced clearance of serum cholesterol (Ishibashi et al., 1993; Ishibashi et al., 1996). Indeed, dams lacking LDLr showed elevated total serum cholesterol and LDL-cholesterol as well as elevated serum retinyl esters, when compared to dams heterozygous for the knockout allele (LDLr+/-). We would also like to point out that total serum cholesterol and LDL+IDL fraction cholesterol were higher in LDLr+/- mice when compared to WT mice with the same genetic background (data not shown), as previously reported (Goldstein, 2001; Rader et al., 2003) confirming that even the absence of a single copy of the LDLr gene affects the lipoprotein profile.

Our data also confirmed that beta-carotene circulates in the bloodstream in association with all the various classes of lipoproteins, as reported in humans (Auletta and Gulbrandsen, 1974; Bjornson et al., 1976; Erdman et al., 1993; Johnson and Russell, 1992). This indicates that our model of maternal beta-carotene supplementation mimics well the human physiological status. Interestingly, we found that the distribution of beta-carotene within the various lipoproteins seemed to be similar to that of cholesterol, with the lowest concentration in VLDL+Chyl and the highest concentration in HDL. The

mechanisms of beta-carotene incorporation into lipoproteins are not known, but our data suggest that such incorporation could be driven by the cholesterol content, which would likely provide a favorable hydrophobic moiety that would stabilize beta-carotene within the lipoprotein particles. The placental uptake and secretion of cholesterol has been extensively studied as reviewed by Woollett (Woollett, 2008, 2011). A receptor mediated as well as receptor-independent uptake of LDL-cholesterol in the placenta has been reported (Wyne and Woollett, 1998). Cholesterol can thren exit the placenta in association with lipoprotein particles (Madsen et al., 2004; Woollett, 2005) to be released into the fetal circulation. A mechanism of LDLr mediated uptake of cholesterol in the placenta has been reported (Burke et al., 2009; Wyne and Woollett, 1998). Since our data suggested that beta-carotene shares similar mechanistic pathways to be incorporated into the lipoproteins, it is likely that the placenta uptake, transfer and secretion of beta-carotene could follow a similar pathway as that of cholesterol.

In humans, beta-carotene is mainly associated with LDL (Bjornson et al., 1976; Johnson and Russell, 1992; Traber et al., 1994). Our data, however, indicated beta-carotene content of the HDL to be the highest. We interpret this difference as likely due to the fact that, in mice, HDL contributes to the majority of the lipoproteins in the circulation (Clevidence and Bieri, 1993). HPLC analysis of serum VLDL+chylomicrons, LDL+IDL and HDL fractions showed that beta-carotene was distributed differently among various lipoproteins in heterozygous and knockout dams, with elevated beta-carotene levels in VLDL+chylomicron

fractions and a trend towards increased beta-carotene levels in LDL+IDL fraction of the knockout dams. Like for serum cholesterol levels, this altered distribution of beta-carotene in the serum lipoproteins seems to be related to a reduced hepatic clearance of beta-carotene. Indeed, we observed a dramatic effect of the lack of LDLr in the maternal liver on beta-carotene accumulation in maternal liver, strongly suggesting that LDLr may mediate hepatic uptake of beta-carotene. The possibility that the reduced beta-carotene levels in the liver of the LDLr-/- dams may be due to enhanced metabolism of the provitamin A carotenoid in this strain seems to be unlikely, but cannot be completely ruled out at the moment. Analysis of the expression levels of key regulators of retinoid and carotenoid metabolism needs to be performed to fully exclude this hypothesis. Overall, a reduced hepatic and peripheral uptake of apoB-containing lipoproteins VLDL, chylomicrons, LDL and IDL, all of which can be internalized by LDL receptor *via* endocytosis (Go and Mani, 2012; Ishibashi et al., 1994; Martins et al., 2000), seems to be responsible for the altered distribution of beta-carotene in the lipoproteins of the LDLr-/- dams. In general, apoB-lipoproteins of dams lacking LDLr had higher concentrations of beta-carotene as compared to the ones of LDLr heterozygous dams.

We therefore wondered whether the altered maternal serum beta-carotene distribution of the LDLr-/- dams and/or LDLr expressed in the placenta had any effect on the beta-carotene uptake and utilization by the developing tissues. The placenta is an organ formed during embryogenesis that constitutes the barrier

between maternal blood and embryonic circulation and develops from cells of both maternal and embryonic origin (Watson and Cross, 2005). LDLr is expressed in the placental syncytiotrophoblast layer, the main site of contact between maternal and fetal circulation, which is of embryonic origin (Desoye et al., 2011). Thus, embryonic LDLr genotype is essentially the same as placental genotype for the purpose of our study.

Based on our HPLC analysis, elevated beta-carotene levels in the placenta expressing one copy of the LDLr gene suggested the involvement of LDLr in regulating placental beta-carotene uptake. However, since this effect was observed only in the dams lacking LDLr, we propose that both maternal and placental LDLr determine the amount of beta-carotene that is taken up by the placenta. In LDLr-/- dams, elevated levels of beta-carotene-rich, apoB-containing lipoproteins are available for placental uptake and only under this circumstance placental LDLr seems to facilitate the uptake of beta-carotene carried by these lipoproteins (Figure 21).


**Figure 21. Proposed model of mechanism of placental uptake of beta-carotene rich, apoB-containing lipoproteins in mice lacking LDLr.** LDLr-/- dams have elevated levels of apoB-containing lipoproteins that carry beta-carotene. Only under these circumstances, placental LDLr enhances the amount of beta-carotene that is taken up by the placenta.

It is remarkable that, despite the difference in placental beta-carotene levels discussed above, the respective embryos showed similar concentrations of beta-carotene and retinoids, regardless also of the maternal genotype. Our data seem to rule out that the lack difference in embryonic beta-carotene levels could be due to differential uptake, cleavage and metabolism of beta-carotene by embryos with and without LDLr. Indeed, embryonic mRNA levels of the beta-carotene cleavage enzymes and those of the key players involved in embryonic retinoid homeostasis were similar, irrespective of the embryonic genotype. Likewise, except for a small downregulation in *LRP1* and *ApoER2* mRNA in embryos lacking LDLr (which may not be physiological meaningful), lack of changes in expression levels of other receptors and enzyme involved in lipoprotein uptake between LDLr-/- and LDLr+/- embryos makes unlikely that the embryo itself could control the amount of beta-carotene that is taken up once released from the placenta. In contrast, our results seem to suggest that beta-carotene could be secreted at a different rate from LDLr-/- and LDLr+/- placenta towards the respective embryos. It is not known how the beta-carotene is transferred from the placenta into the fetal circulation. It is not unlikely, though, that the provitamin A will follow the same route of cholesterol that has been shown to exit the placenta towards the fetal circulation in association with lipoprotein particles (Madsen et al., 2004; Woollett, 2005). Specifically the placenta has been reported to secrete cholesterol-containing apoB-100-lipoproteins (mainly LDL) (Madsen et al., 2004). The most remarkable change

observed between placenta LDLr-/- and LDLr+/- from supplemented LDLr-/- dams was the upregulation (~30%) of *Mttp,* a gene encoding a protein that binds and chaperones lipids to nascent apoB, preventing its degradation by proteosomes and assisting in the intracellular assembly of apoB-containing lipoproteins (Hussain et al., 2012; Raabe et al., 1999). MTTP is rate-limiting protein in apoB assembly and thus controls transport of dietary and endogenous lipids *via* lipoproteins. It is generally regarded that mRNA expression, protein expression and activity of MTTP are in agreement and that regulation of MTTP occurs predominantly at the transcriptional level (Dai and Hussain, 2012; Hussain et al., 2011). Thus, these data seem to suggest that an increased secretion of beta-carotene containing lipoproteins could take place from the LDLr-/- placenta (compared to LDLr+/-). Thus, the lower beta-carotene content of the LDLr-/- placenta coupled with an increased lipoprotein secretion (compared to placenta LDLr+/-) could result in similar amount of beta-carotene transferred to their respective embryos. This interpretation of our data would need to be confirmed with additional experiments aimed at investigating placental beta-carotene secretion rate *ex vivo* and *in vivo*.

It is not clear at the moment how the lack of LDLr in placenta and/or the beta-carotene supplementation of LDLr-/- placenta may influence lipoprotein secretion. It is interesting that transcription of Raldh2, the enzyme responsible for oxidation of retinaldehyde into retinoic acid (Clagett-Dame and DeLuca, 2002; Niederreither et al., 1999) was upregulated in see a change in transcriptional

levels of retinoic acid responsive genes such as *RARb.* However, this is not the case. This is indeed puzzling and we are not sure about the meaning of these data at this point. Direct measurement of retinoic acid in the placenta of the dams lacking LDLr and supplemented with beta-carotene by liquid chromatography-mass spectrometry (LC-MS) would be of great interest to answer this question. Whether *MTTP* is a retinoic-acid target gene is not clear. However, It has been reported that the *MTTP* promoter region contains a direct repeat 1 (DR1) element which recruits homo- and hereto-dimers of nuclear receptors such as RAR/RXR, RXR/RXR and PPAR/RXR (Hussain et al., 2011). Thus future studies investigating mechanisms of regulation of *MTTP* at the transcriptional level and whether such regulation is retinoic-acid mediated will be of great interest.

Overall, our results show that besides being a major regulator of lipid transport to the cells, LDLr is an important mediator of beta-carotene uptake in the maternal liver as well as in the placenta of the dams in which lipoprotein metabolism is altered due to lack of LDLr. Since our data indicated that maternal as well as the developing tissues accumulated significant amounts of beta-carotene upon supplementation, even in the absence of LDLr, they most likely point towards the existence of a functional redundancy of the LDLr with other lipoprotein receptors. Other members of LDLr family such as LRP1 and VLDLr Other members of LDLr family such as LRP1 and VLDLr could be mediating the uptake of chylomicron remnants and VLDL carrying beta-carotene. Even SRB-1 could be participating in the uptake of beta-carotene transported in HDL. Thus

contribution of LDLr towards regulating the beta-carotene uptake in the maternal liver and the developing tissues seems to be only partial and in the absence of LDLr, other lipoprotein receptors could facilitate the uptake of this provitamin A carotenoid in the maternal as well as the developing tissues.

In addition, our data also suggests that LDLr exerts its role in the uptake of beta-carotene in an autosomal recessive fashion i.e. an LDLr-facilitated uptake of beta-carotene in the placenta is observed only in the homozygous females (LDLr-/-). In humans, several mutations (over 1000) in the *LDLr* gene have been identified. Although severity of these mutations in causing hypercholesterolemia varies greatly, many of these lead to several-fold elevation in the serum cholesterol (Bertolini et al., 2013; Lye et al., 2013; Raal and Santos, 2012). We speculate that in the cases of severe hypercholesterolemia, which mimics a situation that is observed in the case of LDLr knockout animals in our studies, LDLr may play a crucial role in the uptake of carotenoids incorporated into lipoproteins at least in the liver and the placenta. In the case of vitamin A deficiency, such populations may be at a greater need of effective beta-carotene supplementation strategies, which may be different from the ones utilized in the case of populations with normal lipid metabolism. We believe that this knowledge would help in designing more effective beta-carotene supplementation methods in populations with altered lipoprotein profiles, especially during pregnancy.

# Chapter 4: General Conclusions and Future directions

Vitamin A deficiency (VAD) is a major public health concern not only in the developing countries but also in the western world, with pregnant women (and the developing fetus) being among the categories at major risk (Grune et al., 2010). In developing countries dietary intervention studies with beta-carotene supplementation of pregnant women have showed promising results in ameliorating the detrimental effects of vitamin A-deficiency on the maternal and offspring health (Dijkhuizen et al., 2004; West et al., 1999). Recently, studies from our laboratory have provided an unequivocal proof of molecular mechanisms by which the developing embryo can utilize beta-carotene as a local source of vitamin A (Kim et al., 2011). Despite this evidence, it is not known how the developing tissues acquire beta-carotene and which are the key regulators of this process. It has also not been investigated whether the maternal vitamin A status and dietary intake affects beta-carotene uptake by the developing tissues. Nevertheless, understanding these mechanisms is crucial in order to develop more effective beta-carotene supplementation strategies in populations with different vitamin A status.

Studies described in this thesis aimed at addressing the above-mentioned open questions in the field of carotenoid research. Firstly, we investigated how maternal vitamin A status i.e. the ability to store and mobilize vitamin A affects the uptake of beta-carotene by the developing tissues. We demonstrated that a marginal vitamin A deficient status of the mothers led to an increased placental uptake of beta-carotene and also influenced its cleavage in the developing

tissues. This is in agreement with the previous reports which suggested that conversion of beta-carotene to retinol in human placenta is dependent on the nutritional status of the mother, being particularly efficient in a more depleted state (Dimenstein et al., 1996). These findings could impact the recommended dose of beta-carotene during pregnancy especially in the case of a vitamin A deficient status of the mother. Perhaps, even lower doses of beta-carotene (compared to the ones currently employed) could be very effective in improving the vitamin A status of severely vitamin A deficient populations.

On the other hand, total intakes of preformed vitamin A that exceed the tolerable upper intake levels (UL = 10,000 IU in women) (NIH, 2013) and use of synthetic retinoids for topical applications can lead to retinoid toxicity and cause congenital birth defects (Ross, 2010b; Ross, 2005). In such populations, whether the uptake of beta-carotene in the developing tissues is altered has not been previously studied. Here, we showed that an excessive maternal dietary vitamin A intake led to negligible accumulation of beta-carotene in the embryos. We also provided transcriptional evidence that this effect is due to a regulatory mechanism by the placenta rather than the embryo itself. In our mouse model of excessive vitamin A intake there were no signs of toxicity for the mother and/or the embryo, however a rather dramatic effect was observed in regards to the total amount of beta-carotene that reached the developing embryo. These data suggest the existence of very effective mechanisms to protect the fetus from the potential detrimental effect not only of retinoids but also of their precursor beta-

carotene. The limits of these protective mechanisms still remain to be established.

Furthermore, we also demonstrated, for the first time, that placental lipoprotein receptors LRP1 and VLDLr could be the main mediators of beta-carotene uptake in this organ in the case of mothers under the vitamin A sufficient conditions. Since beta-carotene availability resulted in the placental downregulation of these receptors, it would be of great interest to investigate whether retinoic acid formed by the cleavage and subsequent metabolism of retinoids dictates the transcriptional regulation of LRP1 and VLDLr. Although retinoic acid is considered as the main transcriptionally active form of vitamin A, recent evidence has shown that retinaldehyde could act as a transcriptional regulator of the metabolic response under certain conditions such as high-fat diet (Ziouzenkova et al., 2007). We cannot rule out the involvement of retinaldehyde in the transcriptional regulation of these receptors. In order to identify which of these vitamin A metabolite is responsible for downregulation of these receptors, placental transcriptional response upon direct supplementation of mice with either retinoic acid or retinaldehyde could be measured.

This evidence of potential placental regulation of LRP1 and VLDLr by beta-carotene invites rather important area of further investigations. Intrauterine growth environment and transplacental delivery of lipids to the embryo epigenetically programs the lipid metabolic pathways in the offspring (Woollett, 2011) and by influencing fetal growth may decide its vulnerability to develop

disorders such as obesity and metabolic syndrome in the adult life (Jones et al., 2009; Rebholz et al., 2011). It would be very interesting to investigate a possible role of beta-carotene in attenuating fatty acid transport to the fetus by placental regulation of lipoprotein receptors. An understanding of such mechanism will help in developing *in utero* intervention strategies of beta-carotene supplementation to alleviate detrimental effects of excessive lipid uptake by the developing tissues. Towards this end, future studies specifically investigating whether beta-carotene supplementation of the dams fed a high-fat diet attenuates the fat transport to the embryo will be of great interest. In addition, to develop better supplementation strategies, it would be important to understand how a chronic *versus* acute dose of beta-carotene influences the lipid uptake in the developing tissues.

With the studies presented in this thesis, we demonstrated a role for LDLr in facilitating placental beta-carotene uptake even though under the specific condition of mothers with elevated levels of circulating lipoproteins (like the LDLr knockout dams). In humans, mutations in *LDLr* gene are not uncommon and over 1000 such mutations have been identified, many of which result in several-fold elevation of serum lipoprotein cholesterol (Bertolini et al., 2013; Lye et al., 2013; Raal and Santos, 2012). Our findings suggested that under such circumstances, placental beta-carotene uptake could be mediated by LDLr. Notably, our data also show that efficient mechanisms to protect the developing tissues from potentially toxic levels of carotenoid-derived retinoids do exist and take place specifically in the placenta, confirming the primary role of this organ as regulator

of nutrient intake. It remains to be established what is the upper “threshold” of serum lipoprotein levels that when exceeded, either due to genetics or the diet, influences the uptake of this provitamin A by the developing tissues. Future studies employing mouse models with elevated serum cholesterol either due to disrupted lipoprotein metabolism or due to consumption of high-cholesterol diet would be of interest in order to understand beta-carotene uptake across the developing tissues.

Interestingly, our work has provided novel preliminary evidence that the placenta could regulate the amount of beta-carotene transported to the embryo by regulating the assembly of beta-carotene-containing lipoproteins. Similar mechanism regulating the transport of cholesterol and fatty acids from maternal circulation to the embryo has been shown to exist in the placenta (Woollett, 2011). A few studies have provided a proof that placenta can secret lipoproteins (Madsen et al., 2004) and that the lipoprotein transport could be controlled by receptor-dependent and -independent pathways (Willnow et al., 2007; Woollett, 2005). Although our data suggests beta-carotene could follow similar mechanistic pathways, further studies analyzing *in vitro* and *in vivo* the mechanisms of placental beta-carotene secretion, the rate of such secretion under various physiological and pathological conditions and its directionality (apical vs. basolateral) are needed to unequivocally confirm this hypothesis.

To conclude, the studies presented here have ultimately expanded our knowledge in regards to the effects of maternal status and nutrition on the beta-

carotene transport to the embryo and provide new insights into designing more effective beta-carotene supplementation strategies during pregnancy.

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