Developmental abnormalities in mouse embryos lacking the HDL receptor SR-BI

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Received September 27, 2012; Revised November 20, 2012; Accepted November 28, 2012

The srbi gene encodes a lipoprotein receptor with high affinity for high density lipoprotein that is mainly expressed in the liver and in steroidogenic tissues. Disruption of this gene in mice and mutations in humans lead to alterations in lipoprotein metabolism and/or fertility. During murine development, scavenger receptor class B member I (SR-BI) is present in the yolk sac and the placenta and is only expressed in the embryo itself late in gestation. In humans, it has been detected in trophoblast cells and placenta. Although the proportion of mice carrying a null mutation in SR-BI obtained from heterozygous intercrosses is lower than the expected by the Mendelian ratio, suggesting the involvement of this receptor in intrauterine development, the cause of this demise has remained unknown. In this work, we show that embryos lacking SR-BI exhibit a high prevalence of exencephaly with a sex bias toward females. Immunolocalization studies confirmed that SR-BI is not expressed in the embryo at early stages of development and allowed a more detailed description of its localization in the cells that mediate maternal–fetal transport of nutrients. SR-BI-null embryos contain less cholesterol than their wild-type littermates, suggesting the involvement of SR-BI in materno-fetal cholesterol transport. Newborn SR-BI-deficient pups exhibit intrauterine growth restriction, suggesting that this receptor is also important for fetal growth. Altogether, the results of our work suggest that the presence of SR-BI in extraembryonic tissues is involved in the maternal–fetal transport of cholesterol and/or other lipids with a role during neural tube closure and fetal growth.

INTRODUCTION

The scavenger receptor class B member I (SR-BI) is a multi-ligand protein that binds lipoproteins, glycoproteins and anionic phospholipids. It binds high density lipoprotein (HDL) with high affinity allowing bidirectional transport of liposoluble molecules to and from lipoprotein particles and is a key regulator of plasma cholesterol levels in the adult mouse (1–3). In the liver, SR-BI mediates the uptake of cholesteryl esters from cholesterol-rich HDL particles for its excretion to the bile, and in steroidogenic tissues, this receptor provides cholesterol for steroid hormones synthesis. Mice carrying a null mutation in the srbi locus exhibit an accumulation of cholesterol in abnormal HDL particles and different phenotypes related to the defective lipoprotein metabolism, including high susceptibility to atherosclerosis, adrenal insufficiency and female infertility (3). No loss-of-function mutations have been reported for the gene encoding SR-BI in humans, in contrast to the gene for the low density lipoprotein LDL receptor (LDLR), where mutations induce hypercholesterolemia due to impaired LDL endocytosis. However, recent studies have shown that an abnormal SR-BI expression and/or function can also affect human lipoprotein metabolism and health (4,5). An interesting observation provided by the genetic inactivation of SR-BI in mice was that the proportion of knockout animals weaned from heterozygous intercrosses was lower than expected according to the Mendelian ratio, leading researchers to postulate for years that the lack of

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SR-BI could hinder embryo development (2). In mice, SR-BI is dynamically expressed in the maternal–fetal interfaces during pregnancy (6). Interestingly, SR-BI+/− E16.5 fetuses have been shown to take up less radioactive cholesterol than their wild-type and heterozygous littermates (7). In humans, SR-BI is expressed in term placenta, and its protein levels are reduced in intrauterine growth restriction (IUGR) when compared with normal pregnancies (8). Furthermore, human fetal placental endothelial cells express SR-BI and are able to efflux cholesterol to HDL particles (9).

Several lines of evidence show that cholesterol is necessary for normal embryo development in mammals. In humans, the most common inborn defect in cholesterol metabolism is the Smith–Lemli–Opitz (SLO) syndrome, caused by mutations in 7-dehydrocholesterol reductase (7-DHCR), the enzyme that catalyzes the last step in cholesterol synthesis. This disease exhibits several clinical manifestations, ranging from mental retardation to the lethal cranial malformation holoprosencephaly (10,11). Interestingly, SLO infants with null mutations in 7-DHCR still show measurable amounts of cholesterol indicating that there is provision of this lipid from maternal sources (12). Although the relative contribution of cholesterol from endogenous synthesis versus maternal source for embryonic/fetal development is still controversial, the direct uptake of maternal cholesterol by fetuses has been demonstrated in different species, including mice and humans (13,14).

The extraembryonic tissues responsible for the absorption of lipids from the maternal bloodstream express numerous proteins involved in lipoprotein metabolism, including very low density lipoprotein receptor, LDLR, ApoER1/2, LDL receptor-related protein (LRP1/2), cubulin, ABCA1 and, as mentioned previously, SR-BI (6,15–17). Moreover, the placenta and yolk sac in mice as well as the human placenta mediate the regulated transport of lipoprotein lipids during post implantation development (12,13,18). Mouse mutants deficient in different proteins involved in lipoprotein metabolism, i.e. cubulin (19), LRP1 (20), LRP2/Megalin (21), ApoB and microsomal triglyceride transfer protein (22–24) have shown developmental abnormalities. At present, no embryonic defects have been associated with SR-BI inactivation.

Based on previous evidence showing that SR-BI is expressed in tissues from the maternal–fetal interfaces and its deficiency is associated with the demise of SR-BI−/− mice, we hypothesized that the lack of SR-BI-mediated cholesterol transport hindered an essential developmental process that affected the viability of SR-BI-deficient mouse embryos. The aim of this study was to evaluate the existence of possible phenotype(s) leading to lethality in SR-BI−/− mouse embryos and to analyze the participation of SR-BI in materno-fetal cholesterol transport using this model. First, homozygous null (SR-BI−/−) mice born from heterozygous (SR-BI+/−) progenitors were retrieved at three stages of gestation and at birth and were then analyzed to evaluate possible lethality-inducing abnormalities. Next, the presence of SR-BI in embryos and extraembryonic tissues at those developmental stages was determined by immunohistochemistry. Finally, whole body cholesterol content was compared between SR-BI−/− and their littermates to analyze whether the lack of SR-BI affected cholesterol content in embryos/fetuses.

RESULTS

Neural tube closure defect (NTD) and exencephaly explain the deviation from the Mendelian ratio found in SR-BI-deficient mice obtained from heterozygous intercrossing

Conceptuses in SR-BI+/- females after mating with SR-BI+/− males were examined at embryonic days 9.5 (E9.5), 12.5 (E12.5) and 16.5 (E16.5). At the three stages analyzed, resorptions were sporadic (0.8 ± 0.2 resorptions per female), and the total number of living embryos was normal (8.7 ± 0.3 embryos per female), when compared with those found in uteri from wild-type intercrosses (0.75 ± 0.25 resorptions and 7.8 ± 0.4 embryos per female). Individual genotyping of developing embryos/fetuses showed that the proportions of wild-type (SR-BI+/-), heterozygous and knock out individuals were similar to those expected from the Mendelian ratio (Table 1), suggesting that SR-BI−/− embryos underwent implantation and developed at least until E16.5. However, the morphologic analyses showed that a proportion of embryos/fetuses at the different gestational ages exhibited neural tube closure defects (NTDs) and exencephaly, a brain defect that leads to perinatal death (Fig. 1). Individual genotyping of embryos and fetuses revealed that this phenotype was almost exclusive in SR-BI−/− individuals (34 versus 6% in SR-BI+/- and 3% in SR-BI+/+ littermates) (Fig. 1 and Table 1). Histologic analyses of brains from exencephalic SR-BI−/− embryos showed multiple defects consistent with an abnormal neural tube closure. When compared with brains from E12.5 SR-BI+/- embryos, in which ventricles were evident and lined by neuroepithelium (NE), brains from E12.5 SR-BI−/− exencephalic embryos exhibited collapsed lateral ventricles with portions of NE exposed to the amniotic fluid (Fig. 2A, B). E16.5, brains from SR-BI+/- fetuses showed expanded ventricles exhibiting two structures expected at this stage of development: transitory bulges known as ganglionic eminences (GE) and cerebrospinal fluid-secreting choroid plexi (Fig. 2C). A cartilaginous skull primordium and epidermis covering the brain were also evident in these embryos (Fig. 2E). In exencephalic SR-BI−/− embryos, the lateral ventricles were collapsed and the GE were not evident (Fig. 2D). Moreover, the third and fourth ventricles were absent and choroid plexi were abnormally localized, exposed to the amniotic fluid. In SR-BI−/− exencephalic fetuses, no epidermal or cartilaginous tissue separated the developing brain from the amniotic fluid (Fig. 2F).

Neural tube closure involves a complex sequence of events, including the elevation, bending and fusion of the neural folds that require the coordinate occurrence of cell proliferation and cell death. Immunohistochemical analyses of cranial embryo sections at E9.5 showed that the percentage of proliferative cells (Ki67-positive) were not different in the neural tubes from SR-BI+/- and SR-BI−/− embryos with or without NTD (data not shown). Apoptotic cells, identified as cells showing nuclear condensation and/or blebbing and positive staining for active caspase-3, were sporadic in neural tubes of embryos from both genotypes (data not shown).
Other defects in facial development in SR-BI-deficient exencephalic fetuses

The analysis of SR-BI-deficient fetuses at E16.5 revealed additional defects in facial features. First, exencephalic individuals had an open mouth with a protruding tongue, a finding that was never observed in wild-type fetuses (Fig. 3A, B). Histologic analysis of tongue sections from exencephalic fetuses showed that the myogenic fibers were disorganized in comparison to those from normal embryos, where one central fiber was arranged in the middle of several parallel horizontal myogenic fibers (Fig. 3C, D). In addition, only two out of five exencephalic fetuses analyzed had completed the fusion of the eyelids, a process that is normally completed at E16.5 and observed in 100% of SR-BI+/− fetuses. Histologic examination of eye sections in those individuals confirmed that the eyelids were short and never reached the fusion area and also revealed multiple defects in the organization of the tissues within the eye (Fig. 3E, F). As both the neural tube and the eyelids constitute rostral structures that require tissue extension and fusion, we analyzed palatal shelf fusion, a developmental step involving similar processes, in SR-BI−/− fetuses. All fetuses analyzed had successfully completed the fusion of the palatal shelves at E16.5, as evidenced by the presence of a normally structured nasopharyngeal cavity (Fig. 3G, H). Beyond the defects described above, SR-BI−/− exencephalic embryos were apparently normal.

Non-exencephalic SR-BI−/− embryos, corresponding to 66% of the total number of SR-BI−/− individuals analyzed, did not show detectable developmental abnormalities in the brain or in other vital organs, i.e. liver and heart.

Table 1. Number of embryos/fetuses mice from each genotype recovered from heterozygous intercrosses

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotypea</th>
<th>SR-BI+/+ Normal</th>
<th>NTD</th>
<th>SR-BI+/− Normal</th>
<th>NTD</th>
<th>SR-BI−/− Normal</th>
<th>NTD</th>
<th>Total</th>
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<tr>
<td>E9.5</td>
<td>48 (96%)</td>
<td>2 (4%)</td>
<td></td>
<td>93 (89%)</td>
<td>12 (11%)</td>
<td>41 (70%)</td>
<td>18 (30%)</td>
<td>214</td>
</tr>
<tr>
<td>E12.5</td>
<td>31 (97%)</td>
<td>1 (3%)</td>
<td></td>
<td>61 (100%)</td>
<td>0 (0%)</td>
<td>18 (67%)</td>
<td>9 (33%)</td>
<td>120</td>
</tr>
<tr>
<td>E16.5</td>
<td>11 (100%)</td>
<td>0 (0%)</td>
<td></td>
<td>24 (100%)</td>
<td>0 (0%)</td>
<td>3 (37%)</td>
<td>5 (63%)</td>
<td>43</td>
</tr>
<tr>
<td>E(9.5+12.5+16.5)</td>
<td>90 (97%)</td>
<td>3 (3%)</td>
<td></td>
<td>178 (94%)</td>
<td>12 (6%)</td>
<td>62 (66%)</td>
<td>32 (34%)</td>
<td>377</td>
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<tr>
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<td>202b</td>
<td></td>
<td>40b</td>
<td></td>
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Table 1 indicates postnatal age of 30 days.

aNumber of individuals (percentage of total).
bχ² P < 0.0001 versus Mendelian ratio.

Figure 1. Detection of abnormal neural tube closure and exencephaly in embryos/fetuses obtained from SR-BI+/− intercrosses. Embryos and fetuses obtained at E9.5 (A, D), E12.5 (B, E) and E16.5 (C, F) were photographed under a dissecting microscope. Arrows indicate an open neural tube at E9.5 (D) or exposed brain tissue at E12.5 and E16.5 (E and F). Forceps used to keep the embryo in position are visible in (A). Bar: A, D: 500 μm; B–F: 3 mm.
The frequency of exencephaly is higher in SR-BI-deficient females

As reported previously (2) and confirmed in this study, the proportion of SR-BI$^{-/-}$ mice weaned from our colony was lower than expected by the Mendelian ratio. An interesting additional observation, however, was that the proportion of 30-day-old SR-BI$^{-/-}$ females weaned was even lower than the proportion of males and accounted for only one-third of total number of SR-BI$^{-/-}$ mice (Table 2). Previous human and mouse studies have shown that females are more susceptible than males to the development of NTDs (25,26). To determine if there is an increased susceptibility of SR-BI$^{-/-}$ females to exencephaly, sex ratios were determined for NTD and normal embryos of each genotype. Sex determination was performed by discrimination of $smeC$ and $smeY$ alleles by PCR amplification from yolk sacs or embryo tails. Results showed that female embryos accounted for 72% of NTD SR-BI$^{-/-}$ individuals, as exencephaly was significantly higher in female than in male embryos (44% in females versus 20% in males) (Table 2), explaining the reduced number of female mice obtained at weaning and supporting the idea that the underlying cause of SR-BI$^{-/-}$ mice demise is indeed NTD.

SR-BI is present in different tissues of the maternal–fetal interface

We next analyzed the localization of SR-BI in embryos and extraembryonic tissues from wild-type mice by immunohistochemistry using anti-SR-BI as a primary antibody. At E9.5, SR-BI was localized in the surface of antimesometrial trophoblast giant cells (TGCs) that correspond to the outermost cells surrounding the early conceptus (Fig. 4B). Despite the association between SR-BI deficiency and NTD, this receptor was not detected in the embryo itself at E9.5 (Fig. 4C), suggesting an indirect effect of TGCs on the initial differentiation of the neural tissues. At E12.5, SR-BI was detected in the placental labyrinth, specifically in one of the two layers of syncytiotrophoblast that separates maternal and fetal circulations. This syncytiotrophoblast layer number III is localized between the fetal endothelial cells and the cytokeratin-positive syncytiotrophoblast layer II that lines maternal sinuses (MS) (27) (Fig. 4D, E). At E12.5, SR-BI was also present in the apical region of visceral endoderm (VE) cells of the yolk sac (Fig. 4F). The specificity of the antibody used for these experiments was assessed by immunohistochemistry (Fig. 4B, inset) and western blot using extraembryonic tissues from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ E9.5 embryos (Fig. 4G).

Despite the high expression of SR-BI in the placental labyrinth, no gross structural abnormalities were found by histologic analysis in placentas lacking this receptor (data not shown). Both the area of the placenta and of the labyrinth measured in sagittal sections through the midline of SR-BI$^{+/+}$ and SR-BI$^{-/-}$ placentas were similar (3.7 ± 0.2 mm² versus 3.3 ± 0.2 mm² and 2.1 ± 0.2 mm² versus 1.9 ± 0.2 mm² for total area and labyrinth area from SR-BI$^{+/+}$ and BI$^{-/-}$ placentas, respectively).

**Figure 2.** Histologic confirmation of exencephaly in SR-BI$^{-/-}$ abnormal fetuses. Transversal cranial sections were stained with hematoxylin and eosin. Representative brain section from E12.5 SR-BI$^{+/+}$ embryo showing lateral (LV), third (3V) and fourth (4V) ventricles lined by NE (enclosed by dotted lines) (A) when compared with a brain section from E12.5 SR-BI$^{-/-}$ exencephalic embryo exhibiting portions of neuroepithelium exposed to the amniotic fluid (indicated by arrows) and collapsed lateral ventricles (B). Representative brain section from E16.5 SR-BI$^{+/+}$ fetus showing expanded ventricles with evident GE and choroid plexi and visible epidermal (E) and cartilaginous (Cg) tissue in skull primordium (C) when compared with brain from SR-BI$^{-/-}$ exencephalic fetus with collapsed lateral ventricles, absent third ventricle, open fourth ventricle and choroid plexus exposed to the amniotic fluid (D). Coronal sections from E16.5 fetuses immunostained with an antibody against the epithelial marker cytokeratin comparing the presence (E, black arrowhead) and absence (F, white arrowhead) of epidermis surrounding the developing brain in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ exencephalic fetuses, respectively. Bar: A, B: 1 mm. C, D: 2 mm. E, F: 200 μm.
Figure 3. Identification of defective tongue and eye structures in exencephalic SR-BI−/− fetuses. Exencephalic SR-BI−/− E16.5 fetuses exhibited open mouths with protruding tongues and open eyelids (B, arrow), abnormal features that were not observed in SR-BI+/+ fetuses at this stage (A, B). Coronal sections of tongues from exencephalic SR-BI−/− fetuses showed larger tongues with disorganized muscle fibers when compared with tongues from SR-BI+/+ fetuses, where a central vertical fiber and several horizontal, parallel myogenic fibers are observed (C, D). Histologic examination of sagittal sections indicated that exencephalic fetuses showed abnormal eye differentiation and defective eyelid fusion (E, F). The fusion of the palatal shelves (Pal) was not affected in the exencephalic fetuses when compared with SR-BI+/+ fetuses, as evidenced by the presence of a normal nasopharyngeal cavity (G, H). Bar: A, B: 1 mm. C−F: 200 μm. G, H: 500 μm.
SR-BI-deficient embryos show a moderate decrease in their body cholesterol content

The main function of SR-BI is to mediate the uptake of esterified cholesterol and other lipids (e.g. vitamin E) from HDL lipoproteins to cells (1,2). The relevance of SR-BI for embryo brain development and the localization of this receptor in cells that constitute the maternal–fetal interfaces at E9.5 and E12.5 suggest that SR-BI may be involved in the transport of lipoprotein cholesterol across these barriers during early development. To analyze this possibility, whole body cholesterol content was compared in SR-BI+/+, SR-BI−/− and SR-BI−/− embryos at E9.5 and E12.5. A modest, but not statistically significant, decrease in the cholesterol content of E9.5 SR-BI−/− embryos when compared with their SR-BI+/+ littersmates was detected (Fig. 5A). At this stage, cholesterol levels were highly variable among embryos in the different genotypes. The cholesterol content of SR-BI−/− embryos showing NTD was similar to the one found in SR-BI−/− embryos without NTD. At E12.5, a significant decrease was observed in the cholesterol content of SR-BI deficient embryos, both SR-BI−/− and SR-BI+/+, when compared with their SR-BI+/+ littersmates (Fig. 5B). Again, we observed no difference in the cholesterol content of SR-BI−/− embryos with or without NTD.

SR-BI-deficient embryos without NTD show IUGR

It has been reported that the protein levels of SR-BI in human term placenta are lower in pregnancies complicated by IUGR when compared with normal pregnancies (8). To determine the relevance of SR-BI for fetal growth in mice, we analyzed the weight of SR-BI−/− embryos not exhibiting NTD at different stages of gestation. At E12.5, when intrauterine development starts being almost fully sustained by a mature placenta, SR-BI−/− embryos were slightly lighter than their SR-BI+/+ littersmates, but this difference was not statistically significant (results not shown). However, at E16.5, SR-BI−/− fetuses were already smaller that their littersmates (data not shown), and this defect was maintained until the day of birth when SR-BI−/− pups showed a small, but significant reduction in their mean weight when compared with SR-BI+/+ littersmates (Fig. 6). Analyzing the weight of pups individually indicated that 62% of SR-BI−/− and 16% of SR-BI+/+ newborns were below the 10th percentile of the weight from SR-BI+/+ littersmates, demonstrating a high prevalence of IUGR associated with the SR-BI null genotype.

DISCUSSION

Previous studies reporting abnormally low numbers of SR-BI−/− mice weaned from SR-BI+/− intercrosses suggested the involvement of SR-BI in intrauterine development (2). The main findings of our study were as follows: (i) a proportion of SR-BI deficient embryos exhibit exencephaly, a neural tube defect that leads to perinatal lethality, with more prevalence in females, (ii) SR-BI is localized in specific cell types involved in materno-fetal nutrient transport, (iii) the lack of SR-BI results in a modest reduction in embryonic cholesterol levels and (iv) SR-BI−/− newborns with normal brain development are smaller than their littersmates.

The existence of more than 200 mutations in different genes leading to exencephaly in mice illustrates the complexity of the genetic basis underlying this abnormal condition (28). As in most mouse models of NTD (28), SR-BI−/− embryos showed a defect specifically at the cranial region, as spina bifida aperta was never observed in these embryos. Two additional developmental abnormalities were detected in rostral structures from exencephalic SR-BI−/− embryos: abnormal tongue morphogenesis and defective eyelid closure associated with disorganization of eye structures, suggesting that the differentiation of the neural tube, the eyes and the tongue might involve a common pathogenic pathway controlled by SR-BI. Other NTD mouse models with defective eye development have been identified, although the connection between neural tube closure and eye differentiation is not clear (28,29). Interestingly, some common morphogenetic pathways, i.e. BMP signaling, have been found in neural tube closure as well as eye and tongue development (30–33).

The human condition analogous to mouse exencephaly is anencephaly, characterized by the lack of the skull vault and brain tissue at birth because of the degeneration of the developing brain in utero induced by the exposure to the amniotic fluid. Both epidemiologic and experimental studies in humans and mice have found that anencephaly and exencephaly are more prevalent in female individuals (26). Sexing of SR-BI−/− developing embryos showed that the proportion of exencephalic females doubled the proportion of males with this defect. Although the mechanism explaining the high prevalence of NTD in females is unknown, the current hypothesis suggests that male and female embryos differ in some specific aspect(s) of the neurulation process that increases the susceptibility of females to the development of exencephaly (25). It has been suggested that this may be related to the high levels of methylation required to inactivate one of the X-chromosomes (28), although this hypothesis has not yet been fully validated.

In most of the genetic mouse models showing NTD, the mechanism of failure of neural tube closure is attributed to the absence of that gene function either in the neural folds per se or in the adjacent embryonic tissues (28). The scenario seems to be different for SR-BI, as the existing evidence shows that this receptor is not expressed in the embryo itself before E14.5 (our results and 6). Alternatively, the pattern of SR-BI expression in cells from extraembryonic tissues of the interface between mother and embryo/fetus at different stages of development suggests the involvement of this receptor in nutrient materno-fetal transport. In early gestation,
Figure 5. Moderate decrease in body cholesterol content in SR-BI−/− embryos. The determination of total cholesterol in embryos showed that at E9.5, the cholesterol content in embryos from each genotype was not significantly different (A), whereas at E12.5, the SR-BI-deficient embryos contained less cholesterol than their SR-BI+/+ littermates (B). Different lettering indicates statistically significant differences (P < 0.05).

SR-BI is localized exclusively in parietal TGCs, cells with important endocrine and paracrine functions, secreting different hormones as well as numerous angiogenesis- and hematopoiesis-related factors (34). Their function is to facilitate implantation, to initiate decidual differentiation and maternal vascularization of the placenta and to avoid embryo rejection. Our data showing that the percentage of resorptions and the total number of SR-BI−/− embryos obtained from SR-BI+/− intercrosses are normal suggest that implantation or initial placentogenesis is not affected by the lack of SR-BI. Interestingly, cholesterol synthesis is insignificant in mouse embryos before E10 (35), so other materno-fetal cholesterol transport mechanisms are probably compensating for the supply of this lipid in SR-BI−/− embryos during neural tube closure. On the other hand, our results linking NTD to the lack of SR-BI in TGCs during neurulation offer a new perspective on the role of this cell type during early gestation.

During early development, visceral endoderm cells of the yolk sac are functional and express lipoprotein endocytic receptors, such as cubilin and megalin, that actively take up lipids, proteins, ions and vitamins (19,36). Nutrient transport across the yolk sac is important during neurulation, as several mouse mutants deficient in proteins expressed specifically in the VE exhibit NTD (18). Unexpectedly, we and others have not been able to detect SR-BI in the yolk sac before E10.5, suggesting that SR-BI might have a specific function in TGCs and not in the yolk sac during neurulation. The fact that SR-BI−/− embryos show a reduction in whole embryo cholesterol content suggests that TGCs might be involved in the regulation of cholesterol uptake into the embryo either directly or by exerting a paracrine or endocrine effect on other tissues, i.e. the yolk sac. After E10.5, SR-BI shows a polarized localization on the apical region of visceral endoderm cells facing the maternal side of the conceptus (our results and 6).

Hatziopoulos et al. (6) had previously reported that SR-BI was present in syncytiotrophoblasts cells lining MS. Although our studies also localized SR-BI to syncytiotrophoblasts, SR-BI was detected specifically in syncytiotrophoblast layer III,

Figure 4. Localization of SR-BI in extraembryonic tissues. In (A), schematic representations of the organization of extraembryonic tissues at E9.5 and E12.5 are shown. Upper panel, E9.5 tissues: TGCs, parietal endoderm (PE), yolk sac (YS) and amnion. Lower left panel, layers of the E12.5 placenta: decidua (Dec), spongiotrophoblast (SpT), labyrinth (Lab). Lower right panel, details of trophoblast layers (I, II and III) at the interface of the maternal and fetal circulations in the labyrinth at E12.5. Sections from E9.5 conceptuses immunostained with anti-SR-BI antiserum showed specific staining for SR-BI in antimesometrial TGCs (negative control in inset) (B) and the absence of staining in the embryo proper (NE, neuroepithelium; Am, amnion) (C). In E12.5 placentas, staining for SR-BI was observed in syncytiotrophoblast layer III, adjacent to fetal vessels (endothelial cell nuclei is indicated by an arrowhead) containing immature red blood cells (asterisks) (D), whereas cytokeratin staining was detected in syncytiotrophoblast layer II, localized surrounding MS containing mature red blood cells (arrows) (E). Specific staining for SR-BI was also observed in the VE of the yolk sac at E12.5 (F). Western blot analysis showed a specific band for SR-BI in SR-BI+/+, but not −/− E9.5 extraembryonic tissues (α-COP was used as a loading control) (G). Bar: B, F: 25 μm. C: 100 μm. D, E: 10 μm.

Figure 6. Reduced weight in SR-BI−/− pups at birth. Weights from pups obtained from heterozygous intercrosses were expressed as the relative pup weight (percentage of the mean weight determined in SR-BI+/+ pups from the same litter). Different lettering above each group indicates statistically significant differences (ANOVA; P < 0.001). The dotted line indicates the 10th percentile of SR-BI+/+ pups. **P < 0.01 Fisher’s exact test versus SR-BI+/+. n: SR-BI+/+ = 20; SR-BI+/− = 31; SR-BI−/− = 13.
lining fetal vessels and separated from MS by cytokeratin-positive syncytiotrophoblasts layer II (27). Our results support the idea that, despite their structural similarity, the two layers of syncytiotrophoblasts separating maternal and fetal circulations could have different molecular features probably underlying distinct functions.

An interesting observation from our localization studies is that whereas in some cell types, such as the visceral endoderm of the yolk sac, SR-BI exhibits a polarized cell surface localization, in other cells such as TGC and syncytiotrophoblasts, this receptor does not seem to be polarized. It has been shown that in polarized cells, SR-BI resides in caveolae and mediates cholesterol transport across the cells via transcytosis regulated by the cholesterol content of the cells (37,38). Regardless of its subcellular localization, the presence of SR-BI in one of the three layers of trophoblasts in the placenta is in agreement with its function as a materno-fetal transporter.

Given the importance of cholesterol for brain development, and the well-known function of SR-BI in the uptake of this lipid in placental cells, we hypothesized that SR-BI-deficient embryos could have reduced cholesterol levels. Surprisingly, the reduction found in the whole body cholesterol content was significant but modest, and there was no difference between exencephalic and non-exencephalic SR-BI−/− individuals. Besides its role as a cholesterol transporter, SR-BI has also been shown to be involved in the cellular uptake of liposoluble vitamins (i.e. vitamin E and vitamin D) in the intestine and other tissues (39–41). Thus, it cannot be ruled out that the lack of SR-BI also affects the uptake of vitamins in extraembryonic tissues that are required for neural tube closure. For this reason, further studies will involve feeding pregnant SR-BI+/− females with diets supplemented with vitamin E or other antioxidants and analyzing the prevention of NTD in SR-BI−/− embryos.

One of the most striking functions of cholesterol is the modulation of the activity of Sonic hedgehog (Shh), a morphogen involved in neural tube closure, among many developmental processes (42–45). An interesting hypothesis to explain NTD in SR-BI−/− embryos is that the decrease in cholesterol levels may negatively affect Shh activation. In a preliminary study, we have not found differences in the mRNA levels of five genes regulated by Shh (Gli1, Gli2, Patched1, Pax6 and Nkx2.1) using quantitative RT-PCR in SR-BI+/+ versus SR-BI−/− embryos, suggesting that Shh signaling during development is not highly affected by the lack of SR-BI. The transport of several morphogens in lipoproteins has been shown to be crucial to establish their concentration gradients (46–48). Thus, other signaling pathways might be dysregulated in embryos lacking SR-BI because of deficient morphogen transport by abnormal lipoproteins.

Studies in humans have found reduced levels of SR-BI protein in term placenta from newborns showing IUGR (8). Interestingly, a significant proportion of SR-BI−/− newborn mice that undergo normal neural tube closure exhibit lower birth weights than their wild-type littermates. Although the difference in weight detected in SR-BI−/− pups might seem small, it has been shown in humans that a weight at birth below the 10th percentile seriously affects the health of newborns and increases the prevalence of disease in adulthood (49). In fact, two-thirds of SR-BI−/− individuals were below the 10th percentile, indicating that in fetuses not exhibiting NTD, the lack of SR-BI is associated with a high prevalence of IUGR. As IUGR is associated with high perinatal mortality and morbidity (50), this phenotype might also contribute to the low number of weaned SR-BI−/− embryos. Given the importance of the placenta for the transport of nutrients between the mother and the fetus, our results offer an attractive model to study the impact of placental lipoprotein metabolism in fetal growth (49). These results are also interesting from a clinical perspective, as IUGR children are prone to cardiovascular disease, obesity and type 2 diabetes in adulthood (49). The design of mouse models lacking SR-BI specifically in the embryo and not in extraembryonic tissues could be useful to understand the role of SR-BI during fetal growth and the impact of this receptor in early life on adult metabolism and disease conditions.

Altogether, the results of the present work suggest that the presence of SR-BI in extraembryonic tissues is important for the materno-fetal transport of cholesterol and/or other lipid molecule(s) required for neural tube closure, eye and tongue differentiation and fetal growth.

**MATERIALS AND METHODS**

**Animals**

Mice with a targeted deletion of the SR-BI gene on a mixed C57BL/6 × 129 background (B6;129S2-Scarb1tm1Kri/J) (2) were used in these studies. Animals were housed in a temperature- and light-controlled room and were allowed to consume standard chow (Prolab RMH3000, Labdiet) and water ad libitum.

**Sampling of embryos and pups from heterozygous intercrosses**

Two- to three-month-old virgin SR-BI+/− females were housed with SR-BI+/− males, and the presence of vaginal plugs was checked every morning as an indication of mating. The day when the vaginal plug was detected was recorded as embryonic day 0.5 (E0.5). Protocols were conducted in agreement with the National Research Council (NRC) publication Guide for Care and Use of Laboratory Animals (copyright 1996, National Academy of Science). The studies conducted were approved by the Ethics Committee for Animal Welfare from the School of Medicine of the Pontificia Universidad Católica de Chile. Pregnant dams were sacrificed at 9.5, 12.5 or 16.5 days post coitum after anesthesia with a mix of ketamine:xylazine (0.18 mg:0.012 mg per gram of mouse). The embryos and extraembryonic membranes were retrieved using a dissecting microscope whenever necessary, and the morphology of each embryo was registered before fixation in Bouin’s fixative or flash freezing in liquid nitrogen. Yolk sacs were used for genotyping by PCR as described previously (2). Alternatively, some dams were allowed to get to term and undergo vaginal delivery, and the pups were weighed and genotyped using tail tips.

**Histologic and immunohistochemical analyses**

Bouin-fixed tissues were dehydrated, cleared and embedded in paraffin. Tissue sections (8 μm) were stained with hematoxylin and eosin or used for immunohistochemical procedures. For immunolocalization, sections were subjected to antigen
retrieval using hot citrate and then incubated with a primary antibody against SR-BI developed in rabbit (1:200,6) or commercial rabbit antibodies against cytokeratin (1:200, Dako), Ki67 (1:200, Abcam) or cleaved caspase 3 (1:200, Promega). After extensive washing, sections were incubated with secondary antibodies coupled to peroxidase (1:800, Sigma) and revealed with diaminobenzidine (Sigma). Sections from two tissues known to express SR-BI, ovary and liver were used as positive controls. Negative controls included slides where the primary antibody was replaced by non-immune rabbit serum, and sections where SR-BI+/− tissues were incubated with anti-SR-BI antibody.

Sex determination of embryos by PCR

Individual sexing of embryos was performed by PCR as described elsewhere (51). The following primers F: 5′-CCGC TGCCAAATTTTTGG-3′ and R: 5′-TGAAGCTTTTGCT TTGAG-3′ were used to amplify bands of different sizes corresponding to the smcx/y gene in the X and Y chromosomes.

Immunoblotting

Tissues from E9.5 conceptuses and E12.5 embryos were analyzed by immunoblot as described elsewhere (2).

Cholesterol determinations

Whole embryos were homogenized in lysis buffer [25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA pH 8, 1% IGEPAL (Sigma), 0.01% SDS and 0.35 mg/ml PMSF], and lipids were extracted in methanol/chloroform 1:2 following standard procedures. The cholesterol content in E12.5 embryos was determined using an enzymatic colorimetric assay previously described (Sigma), 0.01% SDS and 0.35 mg/ml PMSF], and lipids from two tissues known to express SR-BI, ovary and liver were used as positive controls. Negative controls included sections where the primary antibody was replaced by non-immune rabbit serum, and sections where SR-BI+/− tissues were incubated with anti-SR-BI antibody.

ACKNOWLEDGEMENTS

We would like to thank Dr Monty Krieger and Dr Richard Haynes for useful discussions. We also acknowledge Jenny Corthorn and Ludwig Amigo for their technical assistance in performing the histologic analyses and cholesterol determinations, respectively.

Conflict of Interest statement: The authors do not have any conflict of interest.

FUNDING

This work was supported by the Chilean National Council for Scientific and Technological Research (CONICYT) programs Inserción de Investigadores Jóvenes en Academia (79090028 to D.B.) and Fondo Nacional del Desarrollo Científico y Tecnológico (11090064 to D.B. and 1110712 to A.R.) and by the Summer Research Program for Medical Students from the School of Medicine, Pontificia Universidad Católica de Chile (to I.P.).

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