

# Transfer of maternal cholesterol to embryo and fetus in pregnant mice

Shumi Yoshida<sup>1,\*</sup> and Yoshinao Wada<sup>\*,†</sup>

Graduate School of Medicine,<sup>\*</sup> Osaka University, Suita 565-0871, Japan; and Department of Molecular Medicine,<sup>†</sup> Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi 594-1101, Japan

**Abstract** Cholesterol is essential for antenatal development. However, the transport of maternal cholesterol to the embryo has not been sufficiently studied, and that to the fetus is still controversial. To this end, a 1 mg dose of [3,4-<sup>13</sup>C<sub>2</sub>]cholesterol was injected daily into pregnant mice and the labeled cholesterol was measured by gas chromatography-mass spectrometry. After venous injections from days 10 to 17 of gestation, [<sup>13</sup>C]cholesterol levels in total (<sup>12</sup>C and <sup>13</sup>C) cholesterol were increased to 5.1% and 2.8% in maternal and fetal plasma, respectively. Labeled cholesterol was identified in the liver, kidneys, and intestines, but not in the brain, of the fetus. After injections from days 1 to 8, [<sup>13</sup>C]cholesterol levels were increased to 12.4% and 8.0% of total cholesterol in maternal plasma and the embryo, respectively. The level of 11.5% in the yolk sac was higher than that in the embryo. **Intrauterine transfer of maternal cholesterol to the embryo as well as the fetus was evident in mice, and both the placenta and the yolk sac appear to be sites of intermediate passage in murine pregnancy.**—Yoshida, S., and Y. Wada. **Transfer of maternal cholesterol to embryo and fetus in pregnant mice.** *J. Lipid Res.* 2005. 46: 2168–2174.

**Supplementary key words** placenta • fetus • yolk sac

Cholesterol is a lipid molecule with a characteristic four-ring steroid structure. It is a metabolic precursor of bile acids and steroid hormones, and it is an important component of plasma membranes, in which cholesterol renders the lipid bilayer more rigid, thereby decreasing permeability. Cholesterol has recently been reported to play a role in development (1–3). For example, cholesterol modulates the function of Sonic hedgehog (Shh), a group of proteins essential for morphogenesis, by binding a functional Shh fragment and thereby restricting the distribution and activity of the Shh signal on the cell membrane (4). In this context, congenital disorders of cholesterol synthesis can readily be envisaged as causing developmen-

tal defects. In fact, Smith-Lemli-Opitz syndrome, an autosomal recessive disorder caused by a deficiency of 7-dehydrosterol reductase, which catalyzes the conversion of 7-dehydrocholesterol to cholesterol, is characterized by craniofacial anomalies, renal agenesis, mental retardation, and behavioral abnormalities (5, 6).

To understand the antenatal physiology of cholesterol and the pathogenesis of relevant disorders, the dynamics of this vital molecule in the intrauterine life of mammals warrants investigation. However, only limited data are available on two possible sources, de novo synthesis and maternal supply, of fetal and embryonic cholesterol. The rat fetus synthesizes enough cholesterol for normal development and receives very little cholesterol from the mother (7, 8). In another report, fetal cholesterol was suggested to be composed of maternal cholesterol in the rat (9). In hamsters, maternal LDL and HDL are taken up by the fetal membrane, whereas a majority of cholesterol in fetal tissues is of fetal origin (10). The data on the maternal supply, obtained using radiolabeled cholesterol, range from 0% to 50% maternal origin for fetal cholesterol in mid and late gestations when the placenta transports nutrients (11–14). Recently, Schmid et al. (15) demonstrated the movement of cholesterol from the apical (maternal circulation) to the basolateral (fetal tissue) side of BeWo choriocarcinoma cells in vitro, suggesting cholesterol transport across the trophoblast layer in the placenta. Furthermore, considering that both apolipoprotein B (apoB) and microsomal triglyceride transfer protein are expressed by the human placenta, it is quite plausible that apoB-100-containing lipoproteins secreted by the placenta participate in lipid transport (16). Thus, it is very likely that fetal cholesterol is derived from the maternal circulation. Consistently, cholesterol levels have been shown to correlate strongly in human maternal plasma and fetal tissues, but only before the 6th month of gestation, never thereafter (17, 18). This suggests that placental transfer of

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<sup>1</sup> To whom correspondence should be addressed.  
e-mail: s-yoshida@mch.pref.osaka.jp

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maternal cholesterol to the fetus is dependent on gestational age. On the other hand, only a limited number of studies have focused on cholesterol transport in the earlier preplacental stage. ApoB expression is abundant in the yolk sac membrane in rodents (19–21) and humans (22). In rodents, the yolk sac synthesizes apoB-containing lipoproteins, and apoB has been suggested to deliver lipid nutrients to the developing fetus (23). These observations suggested that maternal cholesterol can enter at the embryonic side via the yolk sac, although no direct evidence has been reported.

In the present study, the transport of maternal cholesterol to the embryo and fetus was examined at different murine gestational stages using stable isotope-labeled cholesterol. The passage of maternal cholesterol was evident, and the yolk sac appears to be involved in the mechanism.

## MATERIALS AND METHODS

### Animals and diets

Animal protocols were approved by the institutional animal care and use committee at Osaka Medical Center and Research Institute for Maternal and Child Health.

BDF1 male and ICR female mice were purchased from SLC (Shizuoka, Japan). The caged mice were subjected to alternating 12 h periods of light and darkness. The room was temperature- and humidity-controlled. Male mice were fed normal chow, and

female mice were fed the standard diet containing 0.05% (w/w) cholesterol (CLEA Japan, Tokyo, Japan) for at least 1 week before the experiments, with ad libitum access to water. After this diet-conditioning period, the animals were mated. The day the plug was identified was regarded as day 1 of pregnancy.

### Dose of [3,4-<sup>13</sup>C]cholesterol

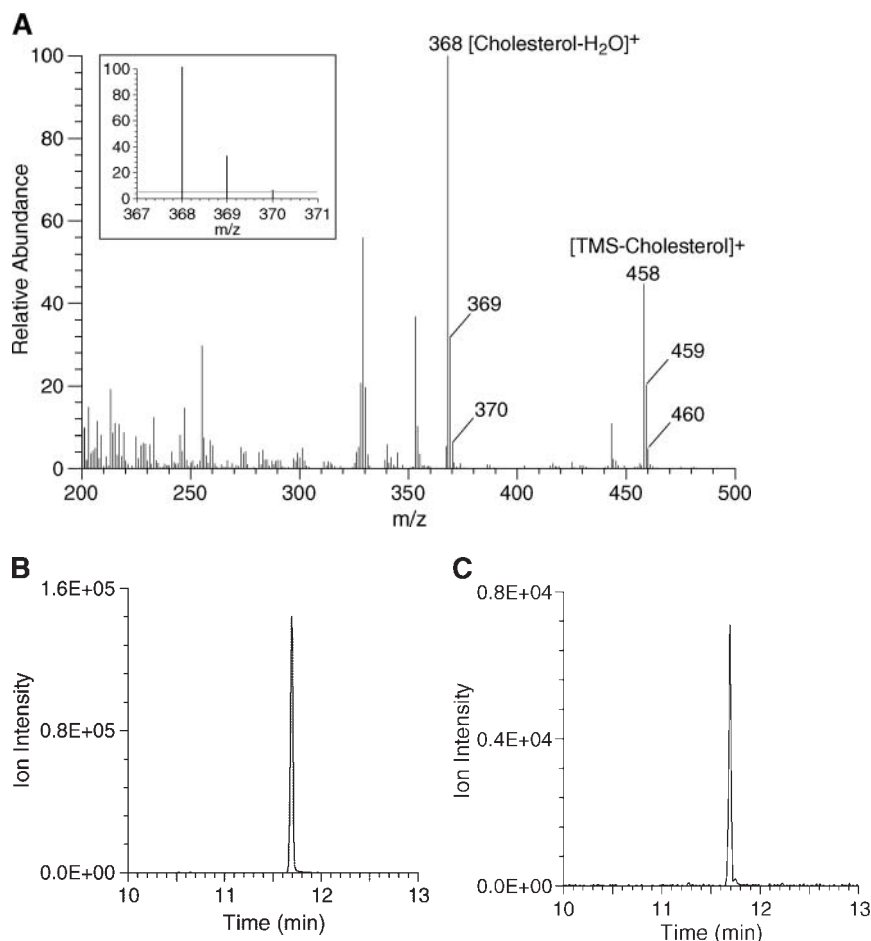
This study was conducted according to three different protocols, depending on the gestational period during which the labeled cholesterol was administered. Injections were repeated daily from 4 days before mating until term for protocol 1, and from day 10 of gestation until term for protocol 2, and from the day 1 until day 8 of gestation for protocol 3.

One milligram of [3,4-<sup>13</sup>C]cholesterol (Cambridge Isotope Laboratories, Andover, MA) dissolved in 40  $\mu$ l of ethanol was mixed with 10% Intralipid (Otsuka Pharmaceutical, Tokyo, Japan) (1:1, v/v) just before administration, and the lipid solution was injected into a tail vein using a plastic syringe fitted with a 26 gauge needle. The injection was done slowly over 45 s to prevent any back-bleeding after withdrawal of the needle. Animals without Intralipid injection served as controls.

The number of fetuses per mouse and the fetal weight did not differ between the noninjection control and injection groups.

### Blood and tissue sampling

Blood and tissues were sampled 24 h after injection. Blood was drawn by cutting the medial plantar vein and collected in heparinized capillary tubes. Blood sampling was repeated every few days to monitor the stable isotope enrichment in plasma cholesterol. Plasma was collected by centrifugation at 800 g and then stored at  $-80^{\circ}\text{C}$  until use.



**Fig. 1.** GC-MS of trimethylsilylated cholesterol (TMS-cholesterol) from the plasma of a control mouse. A: Mass spectrum of TMS-cholesterol. The dehydrated molecular ion of cholesterol is observed. B, C: Selected ion monitoring chromatograms for the ions at  $m/z$  368 (B) and  $m/z$  370 (C).

For protocols 1 and 2, the animals were euthanized at term (day 18) and the fetuses were isolated from the uterus by abdominal section. Fetal blood was collected by decapitation. Fetal plasma was pooled from four littermates. Fetal organs, liver, kidney, intestine, and brain, were isolated immediately after the fetuses were taken from the uterus, and the blood on these organs was removed by extensive washing with PBS. For protocol 3, animals were euthanized on day 9. The embryos, and the yolk sac with allantoic membrane, were removed under microscopic observation. To avoid contamination with maternal tissues or blood, these specimens were washed three times with PBS. The collected tissues were homogenized in an organic solution of hexane and isopropanol (3:2, v/v), and the homogenate was then centrifuged at 15,000 *g*. The resulting supernatant was collected and stored at  $-80^{\circ}\text{C}$  until use.

### Sample preparation

Plasma and tissue homogenates were saponified with KOH and then subjected to lipid extraction by Folch's method. The extract was dried at  $55^{\circ}\text{C}$  under nitrogen, and the residue was dissolved in pyridine. Trimethylsilylated cholesterol (TMS-cholesterol) was prepared by incubating the extract with a solution of *N,O*-bis[trimethylsilyl]trifluoroacetamide (Pierce, Rockford, IL) containing 1% trimethylchlorosilane (Pierce) at  $60^{\circ}\text{C}$  for 1 h.

### Gas chromatography-mass spectrometry

Cholesterol was measured by GC-MS according to the method of Linnet (24) with some modifications. GC-MS was carried out with a GCQ mass spectrometer with an Xcalibur data-processing system (ThermoFinnigan, San Jose, CA). The system was equipped with an autosampler, COMBI PAL cycle composer (CTC Analy-

tics AG, Zwingen, Switzerland), and a Chrompack fused silica capillary column 5CB (30 m, 0.25 mm inner diameter, 0.25  $\mu\text{m}$  film thickness; Varian, Palo Alto, CA). The carrier gas was helium, and the flow rate was 1 ml/min. One microliter of TMS-cholesterol solution was applied to GC-MS by splitless injection at an injector temperature of  $290^{\circ}\text{C}$ . The column temperature was increased as follows:  $10^{\circ}\text{C}/\text{min}$  from 180 to  $250^{\circ}\text{C}$ ,  $20^{\circ}\text{C}/\text{min}$  from 250 to  $320^{\circ}\text{C}$ , and  $320^{\circ}\text{C}$  for 10 min. The ionizing energy for electron ionization was 70 eV. Selected ion monitoring was performed on the ions at *m/z* 368 and 370.

### Plasma cholesterol levels

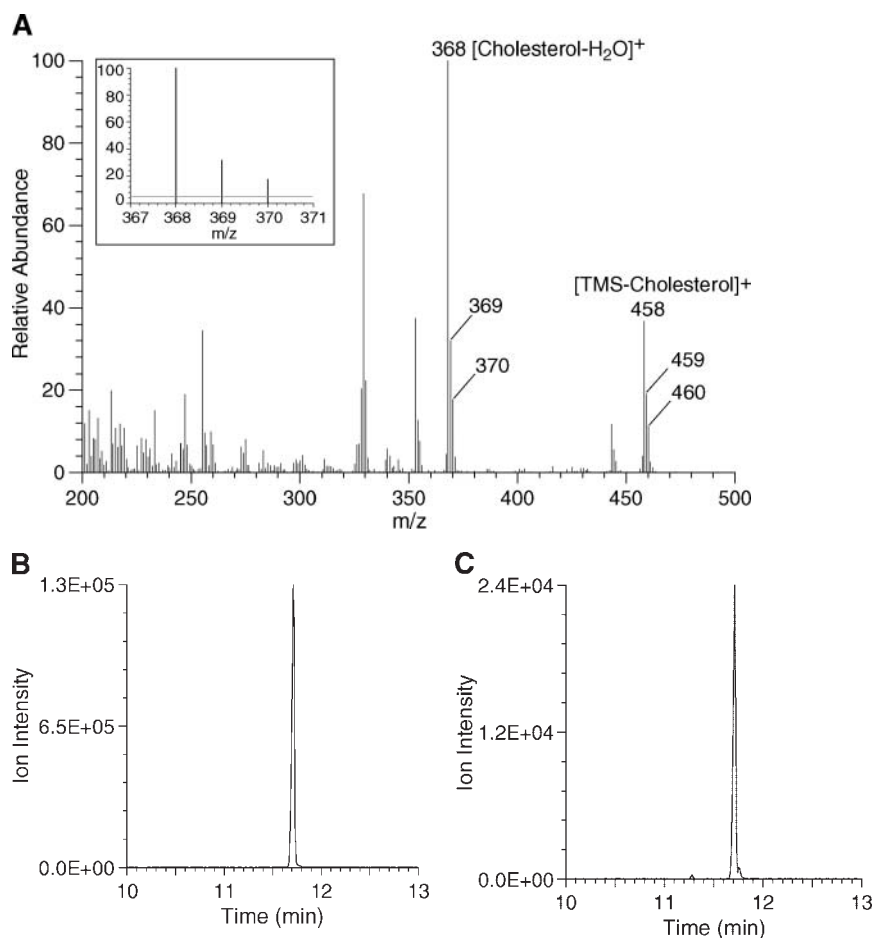
The plasma cholesterol concentration was measured using a Cholesterol E-test kit (Wako Pure Chemical, Osaka, Japan) based on the cholesterol oxidase reaction (25).

### Statistical analysis

Kruskal-Wallis and Scheffe multiple comparison tests were used for statistical analysis, except for the time course of maternal plasma cholesterol levels, for which the unpaired *t*-test was applied.

## RESULTS

The passage of cholesterol from the maternal circulation to fetuses or embryos was examined by injecting stable isotope-labeled [ $3,4\text{-}^{13}\text{C}$ ]cholesterol into tail veins of pregnant mice. **Figures 1A, 2A** show the mass spectra of TMS-cholesterol from the plasma of noninjection control



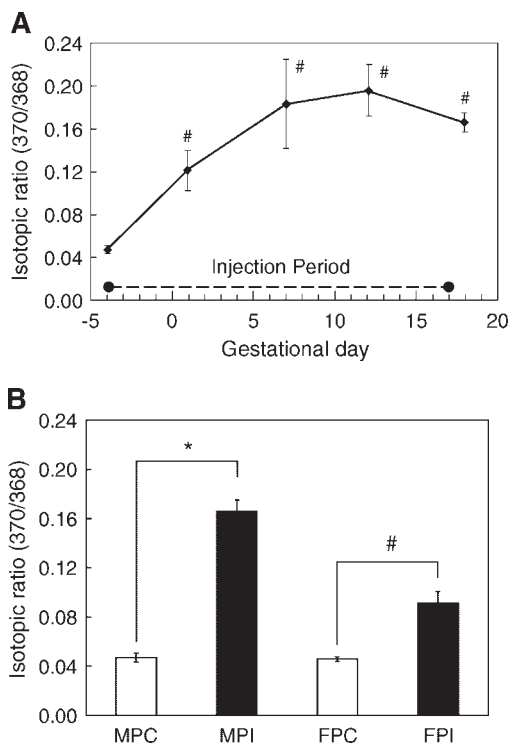
**Fig. 2.** GC-MS of TMS-cholesterol from the plasma of a [ $^{13}\text{C}_2$ ]cholesterol-injected mouse. **A:** Mass spectrum of TMS-cholesterol. **B, C:** Selected ion monitoring chromatograms for the ions at *m/z* 368 (**B**) and *m/z* 370 (**C**).

and injection pregnant mice, respectively. The signal at  $m/z$  368 in either mass spectrum is the  $^{12}\text{C}$ -monoisotopic ion for a fragment of cholesterol. The ion at  $m/z$  370 in Fig. 1A was derived from the native cholesterol containing two atoms of natural  $^{13}\text{C}$ , whereas that in Fig. 2A represents a mixture of this native cholesterol species and the  $^{13}\text{C}_2$ -labeled cholesterol. The levels of the labeled cholesterol in fetal plasma can be represented by the ratio of the peak area for  $m/z$  370 (Figs. 1C, 2C) to that for  $m/z$  368 (Figs. 1B, 2B) in the mass chromatogram and are designated "isotopic ratio" (IR) in this study. The percentage of  $^{13}\text{C}$ cholesterol to total cholesterol was calculated as follows:

$$\% \text{ labeled cholesterol} = 100 (\text{IR} - \text{C}) [1 + (\text{IR} - \text{C})]^{-1}$$

where C, which represents the IR value of the natural cholesterol, is 0.046 based on the natural abundance, 1.11%, of  $^{13}\text{C}$ .

Three protocols were conducted to examine transfer in different stages of gestation. Maternal plasma cholesterol was  $112 \pm 9$  mg/dl in early gestation and  $70 \pm 5$  mg/dl at term, and the fetal plasma level was  $63 \pm 8$  mg/dl at term. These levels were unaffected by the administration of cholesterol.



**Fig. 3.** Isotopic ratio (IR) for protocol 1. A: Time course of IR after  $^{13}\text{C}_2$ cholesterol administration. Injection was started 4 days before mating. Each value represents the mean  $\pm$  SD for seven animals. # Significant difference versus preinjection control ( $P < 0.002$ ). B: IR values of maternal and fetal plasma at term. MPC, maternal plasma of the noninjection control group ( $n = 7$ ); MPI, maternal plasma of the injection group ( $n = 5$ ); FPC, fetal plasma of the noninjection control group ( $n = 5$ ); FPI, fetal plasma of the injection group ( $n = 7$ ). Each value represents the mean  $\pm$  SD. \*  $P < 0.001$ ; #  $P < 0.002$ .

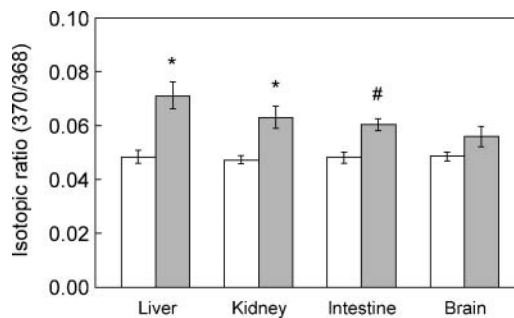
### Maternal-to-fetal transport of cholesterol during gestation

First, a daily dose of 1 mg of labeled cholesterol was injected from 4 days before mating until term. The IR of plasma cholesterol in pregnant mice increased significantly to  $0.125 \pm 0.018$  at the first day of gestation, and was  $0.169 \pm 0.008$  at day 18 (Fig. 3A). This IR value at day 18 indicated 11.0% of total cholesterol to be  $^{13}\text{C}$ cholesterol, based on the equation presented above. IR for the fetuses at day 18 was  $0.088 \pm 0.009$ , corresponding to 4.0%  $^{13}\text{C}$ cholesterol, and was significantly increased compared with the value ( $0.048 \pm 0.003$ ;  $P < 0.002$ ) of noninjection fetuses (Fig. 3B).

Next, the levels in various fetal organs were measured. The IR values for the liver, kidneys, and intestines in fetuses were  $0.070 \pm 0.005$ ,  $0.063 \pm 0.004$ , and  $0.060 \pm 0.002$ , respectively. They were significantly increased compared with their corresponding control values,  $0.048 \pm 0.002$ ,  $0.047 \pm 0.002$ , and  $0.049 \pm 0.002$ , respectively, but were lower than that of fetal plasma described above (Fig. 4). The brain level was slightly but not significantly increased at  $0.056 \pm 0.004$  ( $0.049 \pm 0.002$  for control). Considering that these organs developed rapidly after placental formation, it is likely that maternal plasma cholesterol was transported to fetuses via the placenta or the yolk sac and deposited in all fetal tissues except the brain. Placental tissue was contaminated with maternal and fetal blood, and the IR value was  $0.130 \pm 0.012$  at day 18.

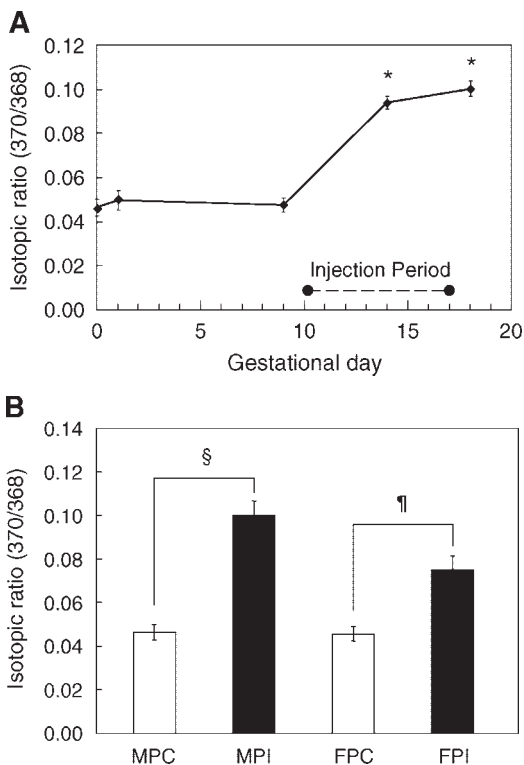
### Placental transport of cholesterol

To confirm placental transport, administration of the labeled cholesterol was initiated at day 10, when the placenta as a fetomaternal interface begins to transport nutrients. The IR of maternal plasma cholesterol was significantly increased after daily injections for 4 days and reached  $0.100 \pm 0.003$  at day 18 (Fig. 5A). The fetal plasma IR of  $0.075 \pm 0.007$  at day 18 in the injection group was significantly increased compared with the value,  $0.048 \pm 0.002$ , for the control fetuses ( $P < 0.05$ ) (Fig. 5B).  $^{13}\text{C}$ cholesterol levels at day 18 were 5.1% and 2.8% for maternal and fetal plasma, respectively. The levels were increased in fetal organs except for the brain (Fig. 6). These results indicate maternal cholesterol transport to fetuses



**Fig. 4.** IR values in fetal organs for protocol 1. Each value represents the mean  $\pm$  SD. Tissues from the noninjection control group ( $n = 20$  for liver;  $n = 5$  for other tissues) are indicated by white bars, and those from the injection group ( $n = 28$ ) are indicated by hatched bars. \*  $P < 0.001$ ; #  $P < 0.002$ .



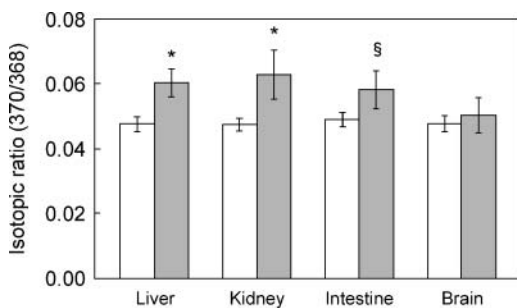


**Fig. 5.** IR for protocol 2. A: Time course of IR after [ $^{13}\text{C}_2$ ]cholesterol administration. Injection was started on day 10. Each value represents the mean  $\pm$  SD of seven animals. \* Significant difference versus preinjection control ( $P < 0.001$ ). B: IR values of maternal and fetal plasma at term. MPC, maternal plasma of the noninjection control group ( $n = 5$ ); MPI, maternal plasma of the injection group ( $n = 5$ ); FPC, fetal plasma of the noninjection control group ( $n = 5$ ); FPI, fetal plasma of the injection group ( $n = 5$ ). Each value represents the mean  $\pm$  SD. §  $P < 0.005$ ; ¶  $P < 0.05$ .

after establishment of the placental circulation. The IR of the placenta at day 18 was  $0.080 \pm 0.003$ .

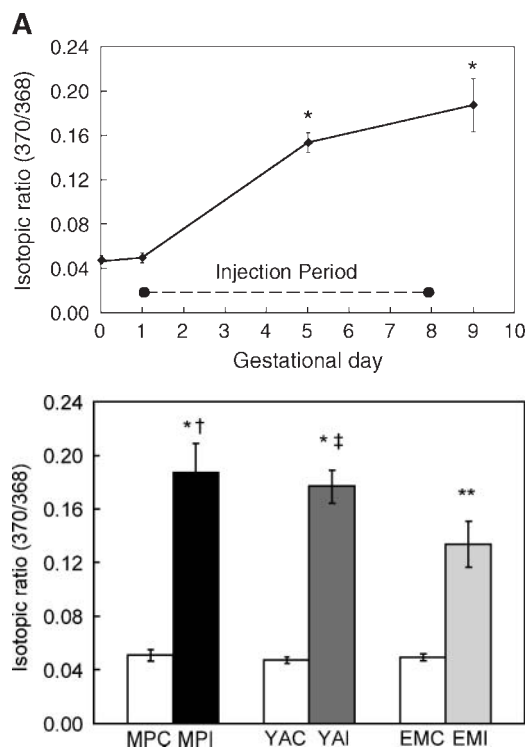
#### Transport of cholesterol in early pregnancy

In rodents, the yolk sac plays a role in incorporating maternal substances into embryos (26). In fact, maternal lipoproteins undergo either hydrolysis or receptor-mediated internalization at the apical surface of visceral endodermal cells. The visceral endodermal cells then repack-



**Fig. 6.** IR values in fetal organs for protocol 1. Each value represents the mean  $\pm$  SD. Tissues from the noninjection control group ( $n = 20$  for liver;  $n = 5$  for other tissues) are indicated by white bars, and those from the injection group ( $n = 28$  for each tissue) are indicated by hatched bars. \*  $P < 0.001$ ; §  $P < 0.002$ .

age maternally derived or endogenously synthesized lipids into apoB-containing lipoproteins for secretion at the basal membrane and subsequent transport via the vitelline circulation to the embryo. Thus, it is plausible that cholesterol of apoB-containing lipoproteins in the maternal circulation is transported to embryos during the first half of gestation. To test this hypothesis, we injected labeled cholesterol into mice from days 1–8, and the mice were euthanized on day 9. As shown in Fig. 7A, the IR for maternal plasma was evident on day 5 and thereafter. When the IR of maternal plasma was  $0.187 \pm 0.021$ , that of embryonic tissues was significantly higher at  $0.133 \pm 0.017$  (Fig. 7B). [ $^{13}\text{C}$ ]cholesterol was calculated to be 12.4% and 8.0% of total cholesterol for the former and latter, respectively. The IR for the yolk sac with allantoic membrane in the injection group was  $0.176 \pm 0.012$  (11.5% [ $^{13}\text{C}$ ]cholesterol), significantly higher than the  $0.046 \pm 0.001$  for the same materials from the noninjection control group ( $P < 0.05$ ) (Fig. 7B). The yolk sac levels were as high as the maternal level and even higher



**Fig. 7.** IR for protocol 3. A: Time course of IR after [ $^{13}\text{C}_2$ ]cholesterol administration. Injection was started on day 1. Each value represents the mean  $\pm$  SD of five animals. \* Significant differences versus preinjection control ( $P < 0.001$ ). B: IR values of maternal plasma, embryo, and yolk sac with allantoic membrane at day 9. MPC, maternal plasma of the noninjection control group ( $n = 5$ ); MPI, maternal plasma of the injection group ( $n = 5$ ); YAC, yolk sac and allantoic membrane of the noninjection control group ( $n = 16$ ); YAI, yolk sac and allantoic membrane of the injection group ( $n = 16$ ); EMC, embryos of the noninjection control group ( $n = 16$ ); EMI, embryos of the injection group ( $n = 16$ ). Each value represents the mean  $\pm$  SD. Asterisks indicate significant differences versus the noninjection control group (\*  $P < 0.001$ , \*\*  $P < 0.05$ ). Daggers indicate significant differences versus the injection group embryos (†  $P < 0.01$ , ‡  $P < 0.05$ ).

than those of embryonic tissues. These results indicate that maternal cholesterol is indeed transported to embryos in early pregnancy in mice.

## DISCUSSION

In the present study, serum cholesterol levels were not increased by intravenous injection of labeled cholesterol. The labeled cholesterol per se must have been transported to fetuses or embryos, because it cannot be reproduced from any other compounds such as bile acids or steroid hormones derived from cholesterol (27). The major pathway of cholesterol catabolism in mammals is bile acid formation and subsequent excretion in the bile. Most of the secreted bile acids are then recycled in the enterohepatic circulation, whereas a small escaped amount is metabolized by microorganisms in the large intestine and then excreted. Cholesterol is never broken down into acetyl-CoA, the precursor of cholesterol.

### Yolk sac transport

De novo synthesis was previously demonstrated in a rat embryo culture (28). It is intriguing that the embryo receives maternal cholesterol as well, as demonstrated in our study. The yolk sac is formed at day 6 of gestation in mice, and the labeled cholesterol was administered during days 1–8. The level of labeled cholesterol in the yolk sac was similar to that in the maternal circulation and was higher than those of embryonic plasma and organs. This result suggests the yolk sac to play a key role in the transport of cholesterol to embryos in early gestation. The yolk sac expresses scavenger receptor class B type I, which is involved in HDL-cholesterol uptake in rodents (29), and also expresses apoA-I, which is the major protein constituent of HDL (20). The yolk sac expresses gp330, which is involved in apoB-containing LDL uptake (30–33), and also expresses apoB (23). It is thus likely that the yolk sac uses these carrier molecules (HDL and LDL) to incorporate cholesterol from the maternal circulation and to transfer it to the embryonic side. In rodents, the expression of the multifunctional endocytic receptors cubilin and megalin, which internalize apoA-I-containing HDL, begins earlier, in the blastocyst stage (34), and these endocytic receptors may be responsible for nutrient intake before the yolk sac becomes available. In the present study, however, we did not specifically investigate cholesterol transport in the pre-yolk sac stage.

### Placental transport

To date, the placental transport of cholesterol remains controversial (11–14), whereas various nutrients, including amino acids, glucose, and fatty acids, are known to cross the placenta (35, 36). We have demonstrated here that maternal cholesterol was transported to the fetus in late pregnancy, when the placenta, as the fetomaternal interface, supplies nutrients to meet the high demand of the developing fetus. In the present study, labeled cholesterol in fetal plasma was increased after long-term injection,


but it was still lower than the maternal level (Fig. 3B). This result indicates that fetal cholesterol is derived from the maternal circulation as well as from de novo synthesis by the fetus.

The placenta was reported to incorporate maternal lipoproteins via LDL receptors located on the microvillous membranes (37, 38). HDLs are unlikely to be internalized, but cholesteryl esters in HDL are selectively taken up by trophoblasts of placental microvilli (39, 40). In either case, it is necessary for the incorporated cholesterol to subsequently be reconstituted in lipoproteins for secretion. A recent study has indicated that both apoB and microsomal triglyceride transfer protein, which in concert participate in de novo lipoprotein formation (41), are expressed by the human placenta, which secretes apoB-100-containing lipoproteins (16). Taking these findings into account, it is most likely that placental trophoblasts import maternal cholesterol via lipoprotein receptors and then secrete it in the form of newly synthesized apoB-containing lipoproteins.

### Implications for metabolic diseases

Accumulation of the transferred cholesterol differed among fetal organs. The level of labeled cholesterol was remarkably increased in the liver and, to a lesser extent, in the kidneys and intestines (Fig. 4). The slight increase in fetal brain might be attributable to contamination with fetal peripheral blood. Alternatively, maternal cholesterol may have entered the brain very early, but the amount detected is not significant because of the abundance of newly synthesized cholesterol. In either case, de novo synthesis is the major source of fetal brain cholesterol, consistent with earlier reports (42–44).

Our results may also be applicable to “fetal programming,” the notion that maternal conditions during pregnancy affect postnatal and even adult morbidity, including disorders such as type 2 diabetes, hypertension, and coronary heart disease, in offspring (45, 46). This hypothesis involves some adult diseases originating antenatally via developmental plasticity and fetal adaptations against a deranged maternal nutrient supply. Palinski and Napoli (47) have presented supporting data showing maternal hypercholesterolemia to be associated with fatty streak formation in fetal arteries and with accelerated progression of atherosclerosis in childhood and probably in later life. Given that maternal cholesterol is shown to be transported to the fetus/embryo and deposited within fetal/embryonic tissues in humans as well, maternal serum cholesterol levels during pregnancy should be managed to prevent atherosclerosis of fetal origin.

In conclusion, maternal cholesterol is transported to murine embryos and fetuses. The yolk sac accumulates maternal cholesterol and probably transfers it to the embryo, just as the placenta does later in pregnancy. 

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