A novel function for apolipoprotein B: lipoprotein synthesis in the yolk sac is critical for maternal–fetal lipid transport in mice

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Abstract Apolipoprotein (apo) B, the principal structural component necessary for the synthesis and secretion of triglyceride-rich lipoproteins by the intestine and liver, is highly expressed in the yolk sac visceral endoderm of mammals, although its function in this tissue has been hitherto unclear. Disruption of the apoB gene in mice results in embryonic lethality (~9.5–10.5 d). Here we demonstrate that apoB is normally expressed at early time points in embryonic development in yolk sac visceral endodermal cells, and that this expression is associated with the synthesis and secretion of apoB-containing lipoproteins. The lack of apoB in the visceral endoderm resulted in an accumulation of intracellular lipid droplets, an absence of lipoproteins from the secretory pathway, and reduced concentrations of cholesterol and α-tocopherol in tissues of *apoB*$^{-/-}$ embryos. Visceral endoderm of *apoB*$^{+/-}$ embryos exhibited an intermediate phenotype. Our results suggest that apoB plays an essential role in the transport of lipid nutrients to the developing mouse embryo via the yolk sac-mediated synthesis and secretion of apoB-containing lipoproteins.–**Farese, R. V., Jr., S. Cases, S. L. Ruland, H. J. Kayden, J. S. Wong, S. G. Young, and R. L. Hamilton.** A novel function for apolipoprotein B: lipoprotein synthesis in the yolk sac is critical for maternal–fetal lipid transport in mice. *J. Lipid Res.* 1996. **37:** 347–360.

Supplementary key words cholesterol • development • gene targeting • lipoproteins • tocopherol • yolk sac

The principal function of the B apolipoproteins, apolipoprotein (apo) B-100 and apoB-48, is to provide a structural framework for packaging neutral lipids, such as triglycerides and cholesteryl esters, into lipoproteins for their transportation in an aqueous circulation (for reviews, see refs. 1–3). Apolipoprotein B is highly expressed in mammalian liver and intestine, where it is required for the synthesis and secretion of very low density lipoproteins (VLDL) and chylomicrons, respectively. When apoB is virtually absent, as in the human syndromes of abetalipoproteinemia and homozygous familial hypobetalipoproteinemia (4), affected individuals have extremely low plasma levels of triglycerides and cholesterol and have impaired intestinal absorption of lipids and fat-soluble vitamins (e.g., vitamin E). Impaired vitamin E absorption can result in degenerative neurologic disease, unless the individual is treated with high doses of vitamin E (4).

We recently generated mice containing a disruption of the 5′ region of the apoB gene by using gene targeting in embryonic stem (ES) cells (5) and found that apoB was essential for mouse embryonic development: apoB knockout mice were not viable and most *apoB*$^{-/-}$ embryos underwent resorption before gestational day 11.5. A few embryos survived until later stages of gestation, although most of these exhibited exencephalus. In another study using gene targeting, Homanics et al. (6) showed that mice homozygous for a mutation that results in the production of low levels of a truncated apoB, apoB-70, were viable but frequently exhibited hydrocephalus or exencephalus. Because developmental abnormalities have not been previously reported to be associated with apoB deficiency in humans, the results of these studies were somewhat unexpected and have subsequently focused attention on a poorly understood area of biology: the role of apoB and lipoproteins in embryonic development.

A role for apoB in development was first suggested about a decade ago, when several groups demonstrated that apoB was expressed at high levels in the yolk sac

Abbreviations: ES, embryonic stem; RT-PCR, reverse transcriptase-polymerase chain reaction; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ER, endoplasmic reticulum.

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membranes of rodents (7–9) and humans (10); the physiologic purpose for the yolk sac expression of apoB, however, was unclear. In mice (and rats), the yolk sac develops soon after implantation (days 6–8) and is said to be "inverted," with its absorptive surface oriented towards the maternal vascular and decidual tissues (11). Here the yolk sac and its associated vitelline circulation serve as the primary interface between the embryo and maternal tissues and are thought to play a major nutritive role for the mouse embryo during the early gestational period, at least until a functioning chorioallantoic placenta is established (days 9–10). The mouse yolk sac comprises two layers: a thin, noncontiguous outer layer of parietal endodermal cells that adhere to Reichert's membrane, and an inner layer of columnar visceral endodermal cells that overlie the extraembryonic mesoderm and vitelline vessels. In human yolk sacs, the expression of apoB mRNA has been localized to the yolk sac visceral endodermal cells by in situ hybridization (12). In mice, the apo-B protein has been detected in yolk sac membranes as early as gestational day 10.5 (5, 7, 8). However, whether apoB is expressed at earlier time points in development or whether apoB expression at these early time points is associated with the production of apoB-containing lipoproteins has not been investigated.

In this study, we sought to better understand the functional role of apoB in mouse embryonic development and to investigate the mechanisms underlying the embryonic lethality when apoB is absent. Specifically, using wild-type and apoB knockout embryos, we sought to: *1)* define the normal apoB expression pattern in early mouse development, *2)* determine whether mouse yolk sac visceral endodermal cells synthesize and secrete lipoproteins during the early stages of development, and *3)* investigate the functional consequences when the yolk sac expression of apoB is absent. Our results indicate that the yolk sac visceral endoderm synthesizes and secretes apoB-containing lipoproteins during the early stages of mouse development and suggest that this expression of apoB plays an essential role in the delivery of lipid nutrients to the developing mouse embryo.

MATERIALS AND METHODS

Mice

Mice heterozygous for an apoB gene disruption were generated by homologous recombination in mouse ES cells as described (5). All mice used in this study were hybrids of genetic strains C57BL/6 and 129/Sv. Mice were housed in a pathogen-free transgenic barrier facility with a 12-h light and 12-h dark cycle and allowed free access to water and rodent chow (Picolab Mouse Chow 20, no. 5053, Purina). Embryos were harvested from timed matings of wild-type mice or apoB knockout heterozygous intercrosses as described (13). For genotyping, genomic DNA was prepared from portions of yolk sac membranes or embryonic tissues and analyzed by Southern blotting as described (5).

ApoB mRNA analyses

Embryos were dissected from maternal decidual tissue into magnesium- and calcium-free phosphate-buffered saline (PBS) (Life Technologies, Inc.) and washed several times in PBS at 4°C. RNA was prepared from individual or pooled embryos using an RNeasy Total RNA kit (Qiagen). For embryos of gestational age 9.5 d and older, RNA preparations were made from yolk sac membranes that were dissected away from the embryos; for 7.5–8.5-day-old embryos, total embryonic and extraembryonic tissues were used. First-strand cDNA was synthesized from ~4.5 μg of RNA using a Stratagene First Strand Synthesis Kit (#200420, La Jolla, CA). Samples of cDNA (~1/10 of the first-strand reaction) were amplified in separate reactions using primers specific for mouse apoB or glyceraldehyde-3-phosphate dehydrogenase (G3PDH). For mouse apoB, the sense primer is located in exon 25 (5′-TGGGATTCCATCTGCCATCTCGAG-3′), and the antisense primer is located in exon 26 (5′-GTAGAGATCCATCACAGGACAATG-3′); the amplification product for apoB cDNA is 303 bp in length. For G3PDH, the sense (5′-GTCGGTGTGAACGGATT-3′) and antisense (5′-CATGTAGGCCATGAGGT-3′) primers yield a 978-bp amplification product for mouse cDNA. For apoB, amplifications were carried out for 30 cycles each of 30 s at 96°C, 1 min at 60°C, and 2 min at 72°C; for G3PDH, amplifications were carried out for 35 cycles each of 30 s at 96°C, 1 min at 55°C, and 2 min at 72°C. PCR reaction volumes were 100 μl and contained 100 ng of each primer, 2.5 units of Taq polymerase, and 2.0 mM $MgCl_2$. The enzymatically amplified DNA was analyzed by electrophoresis on 1% agarose gels.

In situ hybridizations were performed on paraffin-embedded sections of mouse embryos (obtained from Novagen; embryonic tissues isolated from NIH Swiss mice) using a nonradioactive system from Boehringer-Mannheim that uses digoxigenin-labeled riboprobes (DIG RNA Labeling Kit [SP6/T7], Cat. No. 1175025) and an alkaline phosphatase detection system (DIG Nucleic Acid Detection Kit, Boehringer-Mannheim, Cat. No. 1175041). Embryo sections were dewaxed, rehydrated in PBS, and treated with proteinase K (20 μg/ml) for 15 min at room temperature. Proteinase K was inactivated by washing the sections with 0.2% glycine in PBS for 10 min at room temperature, followed by washes of the embryo sections in PBS and refixation in

4% paraformaldehyde in PBS. After another wash in PBS, sections were treated with 0.25% acetic anhydride containing 0.1 M triethylethanolamine for 10 min at room temperature, washed again in PBS, dehydrated in ethanol, and air-dried for 1 h. Prehybridization was performed using a solution containing 50% formamide, 1 × Denhardt's solution, 2 × SSC, 250 μg/ml salmon sperm DNA, and 0.5% SDS for 45 min at 37°C in a humidified chamber. For hybridizations, the same solution containing 10 μg/ml of digoxigenin-labeled riboprobe was used, and incubations were performed overnight at 50°C. For mouse apoB, a 245-bp XbaI–MscI fragment from the 5′ region of exon 26 was used to synthesize both sense and antisense riboprobes. A 156-bp SacI–ClaI fragment of the mouse tubulin cDNA was used as a control for RNA integrity. Sections were washed three times in 4 × SSC for 30 min at 50°C; once in 50% formamide in 2 × SSC for 30 min at 50°C; once in a buffer containing RNase T1 (1 unit/ml), 0.5 M NaCl, 1 mM EDTA, and 10 mM Tris (pH 8.0) for 30 min at

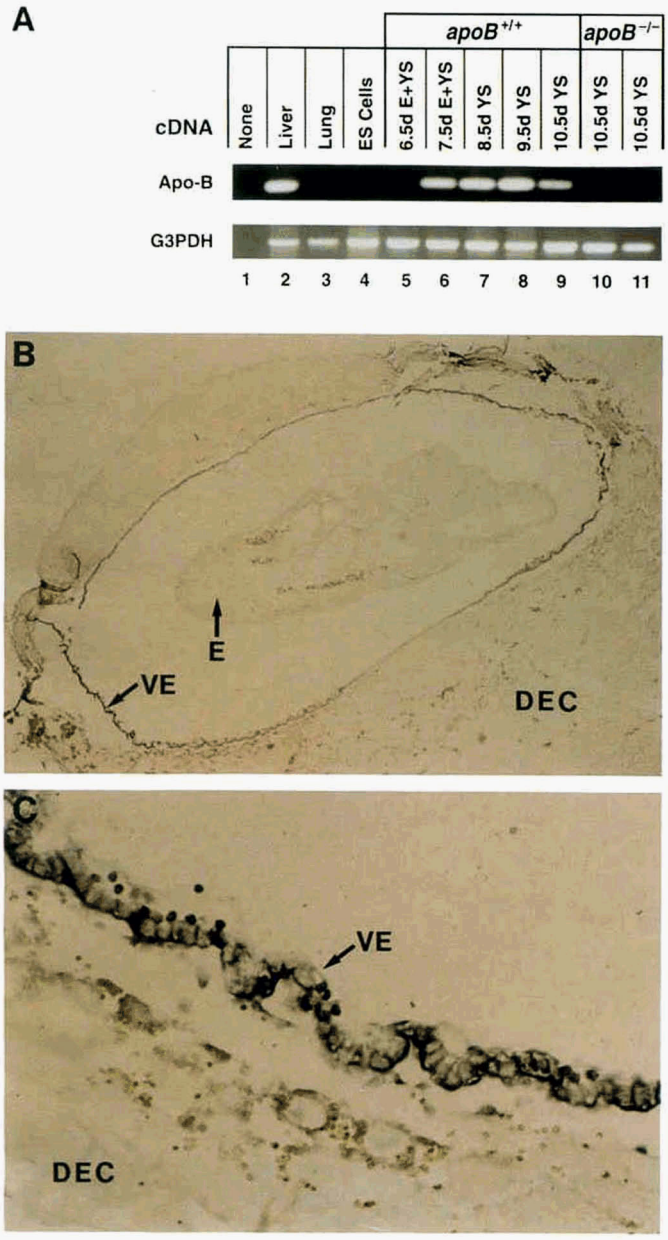


Fig. 1. Apolipoprotein B mRNA expression in normal mouse embryos. (A) Reverse transcriptase-PCR assay. Total RNA was prepared from various adult and embryonic tissues, and ~4 μg of RNA was used for first-strand cDNA synthesis using reverse transcriptase. Apolipoprotein B mRNA was amplified from cDNA samples using a sense primer in exon 25 and an antisense primer in exon 26 that yield a 303-bp product from apoB cDNA. As a control for RNA integrity, a separate reaction was performed with primers specific for a 978-bp G3PDH cDNA amplification product. (B and C) In situ hybridization analysis of normal 9-day-old mouse embryos for apoB mRNA. Paraffin-embedded sections of mouse embryos were hybridized with digoxigenin-labeled antisense riboprobes for apoB as described in Materials and Methods. Hybridization products were detected using an alkaline phosphatase detection system. Staining of the yolk sac visceral endoderm (VE) can be seen at low (B, 10 ×) and high (C, 50 ×) magnification. E, embryo; DEC, decidua.

37°C; and once in 2 × SSC for 15 min at 37°C. Two high-stringency washes were then performed using 0.5 × SSC for 15 min at 37°C. Immunologic detection was performed as recommended by the manufacturer; a 1/2,000 dilution of the detection antibody was used, and the color detection reaction was allowed to develop overnight.

Histology and electron microscopy

Embryos were dissected into PBS, fixed in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned using standard techniques. Sections were stained with hematoxylin and eosin.

For electron microscopy, embryos were excised into normal saline at 4°C. A portion of the yolk sac membrane was removed for genotyping, and the embryos were then transferred into a fixation buffer containing 1.5% glutaraldehyde, 4% PVP, 0.05% calcium chloride, 0.1 M sodium cacodylate, pH 7.4. Fixed yolk sac membranes were separated from the embryos and stained for lipids using an imidazole-buffered osmium tetroxide procedure as described (14). Tissue was block-stained in 2% aqueous uranyl acetate for 1 h at 4°C prior to its embedding in Epon. Ultrathin sections were stained for 5 min with 0.8% lead citrate. Sections from *apoB*$^{+/+}$ (n = 5), *apoB*$^{+/-}$ (n = 4), and *apoB*$^{-/-}$ (n = 3) embryos were

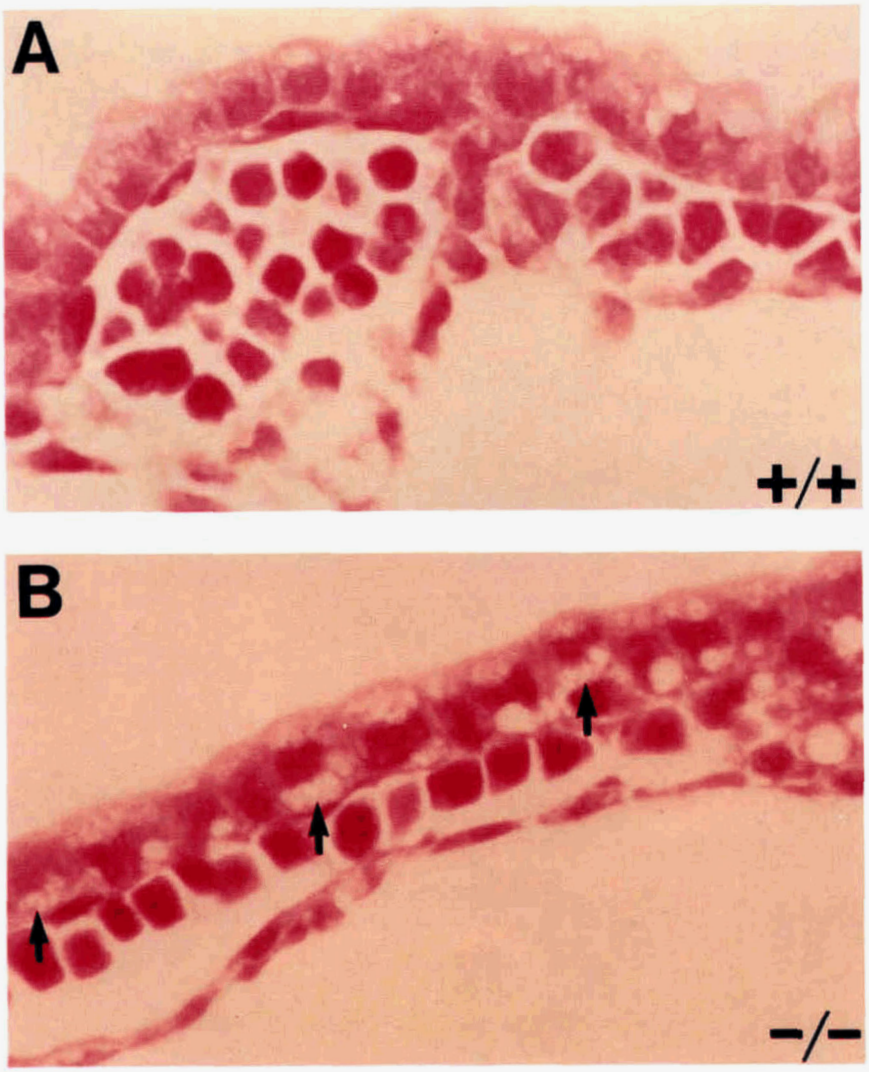


Fig. 2. Histologic sections of yolk sac visceral endoderm from *apoB*$^{+/+}$ and *apoB*$^{-/-}$ embryos. Embryos were dissected in PBS. Portions of the yolk sac membranes were removed for genotype analysis by Southern blotting, and the remainder was fixed in 4% paraformaldehyde. Fixed embryos were embedded in paraffin and sectioned using standard techniques; sections were stained with hematoxylin and eosin. Sections from *apoB*$^{+/+}$ (A) and *apoB*$^{-/-}$ (B) yolk sacs are shown. A layer of yolk sac visceral endodermal cells is visible overlying the embryonic mesoderm and blood islands, with the apical surface of these cells facing the maternal tissues. The arrows indicate vacuoles at the basal aspect of the visceral endodermal cells in the *apoB*$^{-/-}$ yolk sacs. Magnification, 200 ×.

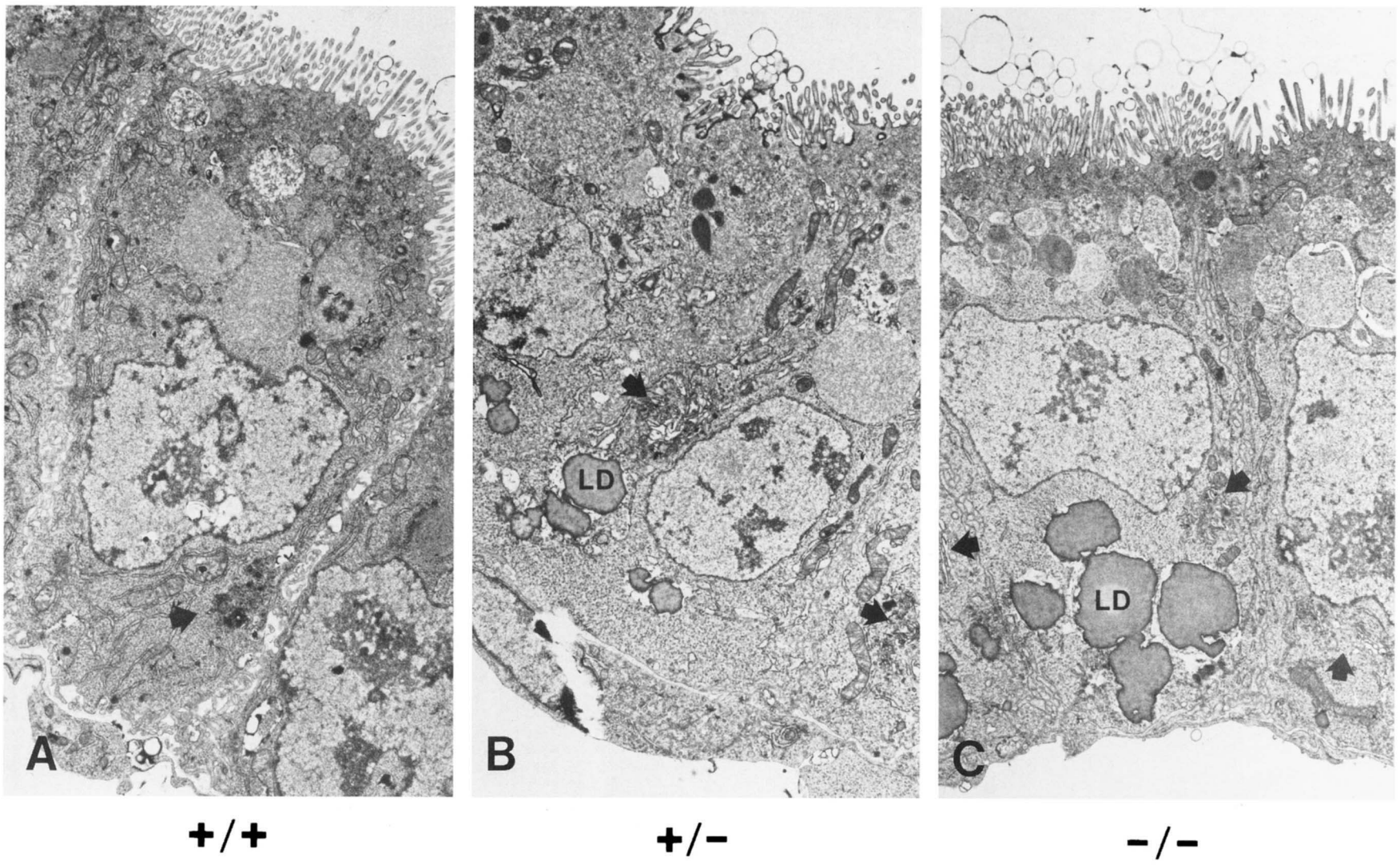


Fig. 3. Electron micrographs of yolk sac visceral endodermal cells from wild-type and apoB knockout embryos. Embryos were dissected in saline. Portions of the yolk sac membranes were removed for genotype analysis by Southern blotting, and the remainder were transferred to a fixation buffer. Sections were stained for lipids using imidazole-buffered osmium tetroxide (14). Sections from $apoB^{+/+}$ (A), $apoB^{+/-}$ (B), and $apoB^{-/-}$ (C) yolk sacs are shown. Intracellular cytosolic lipid droplets (LD) located at the basal aspect of the visceral endodermal cells are rarely observed in wild-type cells but are evident in increasing amounts in $apoB^{+/-}$ and $apoB^{-/-}$ cells. Arrows point to Golgi apparatus regions, which stain for lipids intensely in $apoB^{+/+}$ cells, much less intensely in $apoB^{+/-}$ cells, and not at all in $apoB^{-/-}$ cells. Magnification, 5,600 ×.

Fig. 4. Electron micrographs of rough endoplasmic reticulum (A) and Golgi apparatus (B and C) of visceral yolk sac endoderm of *apoB*$^{+/+}$ (A and B) and *apoB*$^{-/-}$ (C) embryos at 9.5 d. In A and B, arrowheads point to individual lipoprotein particles, occurring singly and in rows, within the lumen of the rough endoplasmic reticulum. Most of these particles measure 300–800 Å in diameter, the size range of VLDL. Arrows point to individual lipoproteins (also 300–800 Å in diameter) in forming secretory vesicles of the Golgi apparatus (B) in *apoB*$^{+/+}$ epithelium. Both the rough endoplasmic reticulum and the Golgi apparatus of *apoB*$^{-/-}$ embryos completely lacked lipid staining particles of VLDL size as detected by lipid staining (C). In addition, the Golgi apparatus of *apoB*$^{-/-}$ visceral endodermal cells often appeared to be diminished in amount owing to the absence of lipoprotein particle content (compare B and C). Magnification, 48,000 ×.

analyzed and photographed using a Siemens 101 electron microscope.

α-Tocopherol and cholesterol measurements

Embryos were dissected into PBS, the yolk sac membranes were removed for genotyping, and the embryos were immediately frozen at -70°C. Of the 72 embryos dissected from heterozygous intercrosses, we were able to assign a genotype to 70 from Southern analysis. At the time of analysis, embryos were defrosted and weighed; 29 embryos had tissue samples that were too small for accurate weighing and were thus not included in the analysis. For the remaining 41 embryos, α-tocopherol and cholesterol concentrations were determined using HPLC and GC methods, respectively, with comparison to internal standards as described (15, 16). Cholesterol and α-tocopherol concentrations for different genotypes are reported as mean ± SD; groups were compared using analysis of variance and Student-Newman-Keuls tests.

RESULTS

Because apoB deficiency results in embryonic lethality and because the temporal expression pattern of apoB mRNA in mouse development had not been studied previously, we investigated the temporal expression of apoB mRNA in mouse embryos using an RT-PCR assay (**Fig. 1A**). Apolipoprotein B mRNA was readily detectable in embryos or isolated yolk sacs from gestational day 7.5 and after at a level similar to that found in adult mouse liver, a positive control. The results from 6.5–7.5-day-old embryos reflect whole embryos (intraembryonic and extraembryonic tissues excluding the ectoplacental cone); the results from days 8.5–10.5 reflect isolated yolk sac membranes. Trace amounts of amplification product were detected in mRNA from adult lung, 6.5-day-old embryos, and the yolk sac of one of the 10.5-day-old *apoB*$^{-/-}$ embryos. In the latter sample, the faint amplification product could theoretically result from very low levels of intrachromosomal recombination at the targeted locus (resulting in very low levels of intact apoB mRNA), or from low levels of a nonfunctional apoB mRNA containing the 11-kb gene knockout vector. Regardless of the mechanism, we have previously shown that the apoB protein is not detectable in 10.5-day-old *apoB*$^{-/-}$ yolk sacs by immunoblotting (5). G3PDH mRNA, a positive control, was detected in every sample, demonstrating the integrity of the RNA used to generate cDNA.

To determine which embryonic cell type expressed apoB, we performed in situ hybridization in sections from normal embryos using an antisense RNA probe to detect mouse apoB mRNA. In 9-day-old embryos, apoB mRNA expression was localized to the yolk sac visceral endodermal cells (Fig. 1B and C). Hybridization of the apoB probe was not observed in any other embryonic or maternal tissues at this gestational timepoint. No hybridization was observed using a sense probe for mouse apoB (not shown).

We next examined stained histological sections of the yolk sac visceral endoderm from wild-type and *apoB*$^{-/-}$ embryos. The yolk sac visceral endodermal cells from 9.5-day-old wild-type embryos (**Fig. 2A**) comprised a single layer of columnar epithelium resting on the extraembryonic mesoderm, which included vitelline vessels and hematopoietic blood islands. The visceral endodermal cells were characterized by an apical microvillus surface oriented toward the maternal tissues, numerous apical vacuoles containing eosinophilic material, and basophilic nuclei located near the basal surface. In contrast to the wild-type cells, the yolk sac visceral endodermal cells of *apoB*$^{-/-}$ embryos consistently contained numerous small vacuoles at their basal surface, which often had a foamy appearance (Fig. 2B). Basal vacuoles were rarely observed in cells of wild-type embryos.

We hypothesized that the foamy vacuoles observed in the yolk sac visceral endodermal cells of *apoB*$^{-/-}$ embryos might represent lipids that accumulated because of the inability of these cells to package lipids into lipoproteins for secretion. To examine this possibility, we performed electron microscopy on the yolk sac visceral endodermal cells of 9.5-day-old *apoB*$^{+/+}$, *apoB*$^{+/-}$, and *apoB*$^{-/-}$ embryos, employing a stain for lipids (**Fig. 3**). Whereas the wild-type cells contained only rare intracytosolic lipid droplets (Fig. 3A), the *apoB*$^{+/-}$ and *apoB*$^{-/-}$ cells were characterized by a gene–dose-related accumulation of lipid droplets in the cytosol at their basal aspect: increased amounts of lipid were observed in the *apoB*$^{+/-}$ embryos (Fig. 3B), and extensive accumulation was observed in the *apoB*$^{-/-}$ embryos (Fig. 3C). At low magnification, the wild-type cells (Fig. 3A) exhibited characteristic features

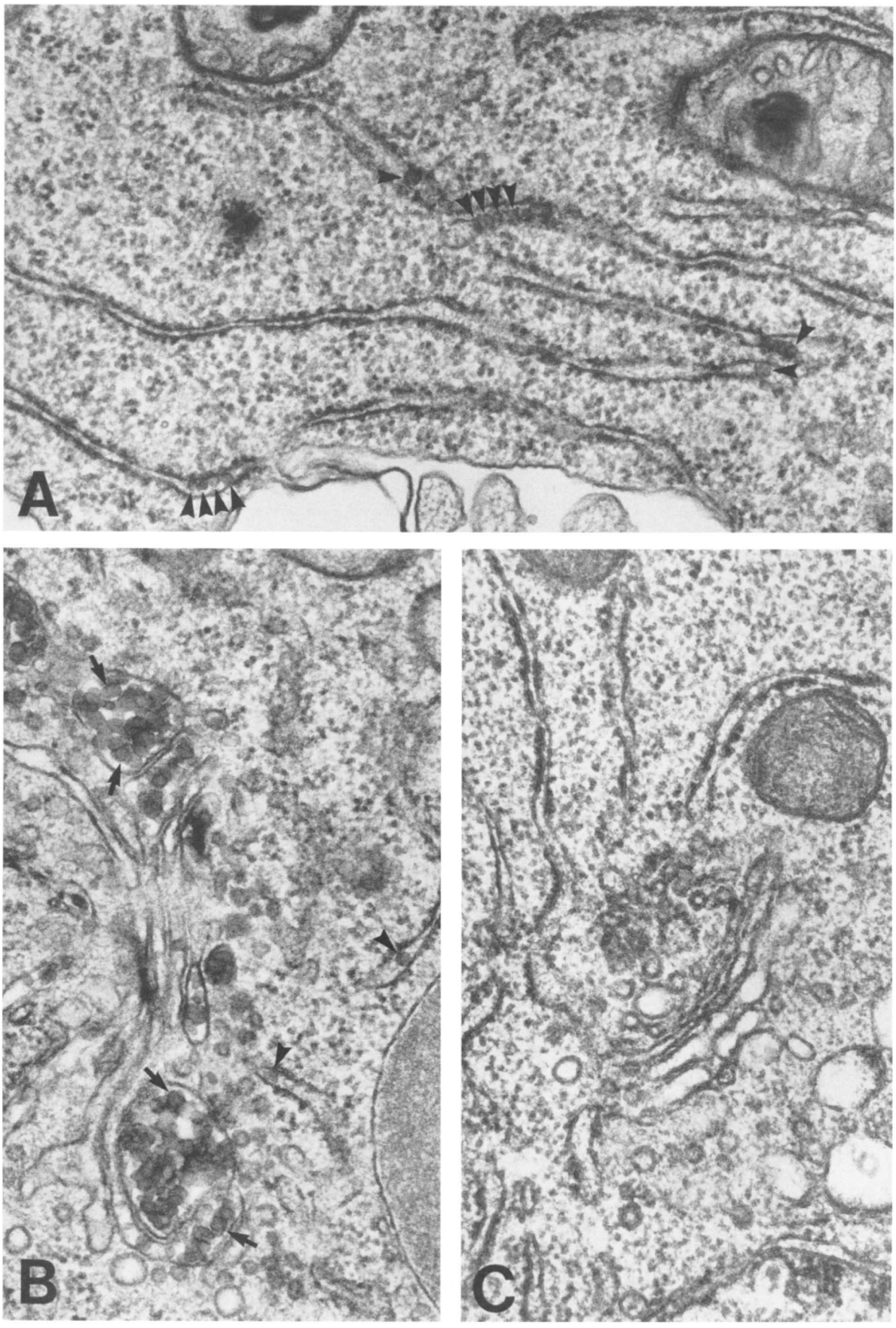


Fig. 5. Electron micrographs of the apical and basal surfaces of yolk sac visceral endoderm cells from wild-type embryos. Extensive microvilli on the apical surface (A) of the visceral endodermal cells often showed maternal lipoproteins (arrowheads) adherent to the microvillar surface membrane. Intensely dark-stained areas (coated pit regions) are visible at the basal aspects of several of the microvilli, suggesting imidazole staining of lipid-rich substances, which may represent active sites of lipid hydrolysis (open arrow). At the basal surface of these cells (B), numerous lipoproteins are visible in secretory vesicles (SV) near the surface. Individual particles and aggregates (arrowheads) in the extracellular space adjacent to the mesoderm and vitelline vessels are consistent with the secretion of lipoproteins from the basal surface. Magnification, 35,380 ×.

of visceral endodermal cells: an extensive apical microvillus membrane, numerous apical vacuoles containing material of assorted electron densities, rough endoplasmic reticulum (ER) located near the basal side of the nucleus, and a perinuclear Golgi apparatus.

We next sought to determine whether apoB expression in the yolk sac visceral endodermal cells was associated with lipoprotein synthesis and secretion at this early developmental time point. Higher magnification electron microscopy of the Golgi apparatus of wild-type cells demonstrated numerous lipoprotein particles in the lumen of the Golgi apparatus and in secretory vesicles (**Fig. 4A and B**). The particles were mostly VLDL-sized, ranging from 300 to 800 Å; few particles measuring < 250 Å were observed. Lipoprotein particles were also observed in the cisternae of the rough ER. The visceral endodermal cells were devoid of smooth ER.

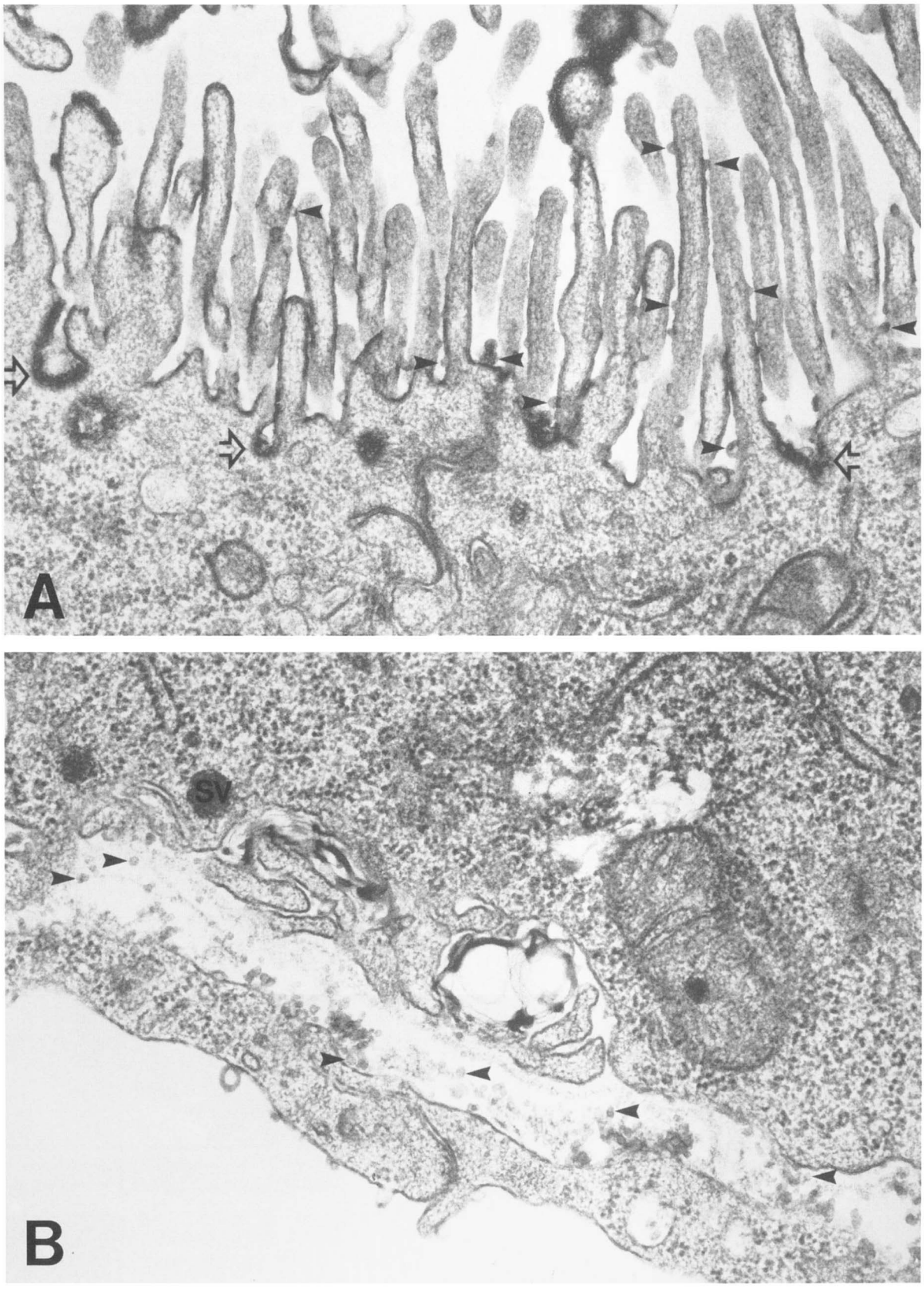
In contrast to the abundant lipoproteins observed in wild-type yolk sac visceral endodermal cells (Fig. 4A and B), cells from *apoB*$^{+/-}$ embryos had consistently reduced amounts of lipoproteins in their Golgi apparatus (see cover figure), and cells from *apoB*$^{-/-}$ embryos contained no lipoproteins in their secretory pathway (Fig. 4C). These findings were consistently observed in many sections from three to five embryos of each genotype.

Electron microscopy of the apical surface of wild-type yolk sac visceral endodermal cells revealed numerous lipid-staining particles of lipoprotein size adherent to the microvilli (**Fig. 5A**). It is probable that these particles represent maternal lipoproteins that had crossed Reichert's membrane to deliver lipids to the yolk sac. Coated pit regions that stained intensely for lipids were frequently observed at the bases of microvilli. Less often, the apices of individual microvilli also stained intensely for lipids (Fig. 5A). The thickness of these lipid-stained areas (~300–800 Å) was consistent with the diameter of plasma VLDL particles, suggesting that maternal TG-rich lipoproteins were highly concentrated at these locations. Interestingly, intense lipid-stained compartments were not observed deeper in the cytoplasm of the visceral endodermal cells. Additionally, endocytic compartments such as multivesicular bodies were not observed in these cells, consistent with previous observations in rat yolk sac visceral endoderm (17); these results suggest that extensive lipid hydrolysis of maternal lipoproteins may occur at or near the plasma membrane rather than within endosomal or lysosomal organelles, as documented for many other cell types (3). At the basolateral aspect of these cells (Fig. 5B), lipoproteins were observed in secretory vesicles and in the pericellular space both beneath the basal cell surface and between cells. It is likely that these extracellular lipoproteins represent newly secreted lipoproteins destined for the embryo via the vitelline circulation.

Because our findings indicated that the lack of apoB in the yolk sac visceral endodermal cells was associated with impaired lipoprotein synthesis and a resultant defect in lipid export from these cells, we examined whether apoB deficiency was associated with reduced cholesterol or α-tocopherol concentrations in intraembryonic tissues of *apoB*$^{+/+}$, *apoB*$^{+/-}$, and *apoB*$^{-/-}$ embryos (**Fig. 6**). At 9.5–10.5 days of age, cholesterol concentrations were reduced in *apoB*$^{+/-}$ embryos (0.75 ± 0.23 μg/mg tissue vs. 0.93 ± 0.22 μg/mg tissue for *apoB*$^{+/+}$ embryos, $P < 0.05$) and in *apoB*$^{-/-}$ embryos (0.59 ± 0.16 μg/mg tissue, $P < 0.01$ vs. *apoB*$^{+/+}$ embryos). The α-tocopherol concentrations were not significantly different in *apoB*$^{+/+}$ and *apoB*$^{+/-}$ embryos (5.69 ± 3.43 ng/mg tissue and 4.28 ± 2.28 ng/mg tissue, respectively), but were undetectable in the *apoB*$^{-/-}$ embryos ($P < 0.01$ vs. *apoB*$^{+/+}$ or *apoB*$^{+/-}$ embryos).

DISCUSSION

Previously, we used gene targeting techniques in mice to demonstrate that apoB is essential for mouse development: *apoB*$^{-/-}$ embryos die early in development (around gestational day 9.5–10.5) and the embryonic lethality was associated with an absence of apoB in the yolk sac (5). In this study, we sought to better understand the functional role of apoB in mouse embryonic development and to investigate the mechanisms underlying the embryonic lethality by analyzing wild-type and apoB knockout embryos. Our results indicate that apoB expression begins early in mouse embryonic development, shortly after the yolk sac is formed (~day 6–7), and that apoB expression at this developmental stage is accompanied by the production of lipoproteins by the yolk sac visceral endodermal cells. Further, we show that when apoB is lacking in the yolk sac, these cells exhibit an apparent lipid-export defect characterized by an accu-



mulation of cytosolic lipid droplets at their basal surface and an absence of lipoprotein particles in their Golgi apparatus. As a result, the tissues of the developing embryo contain reduced concentrations of at least two substances normally transported by lipoproteins, cholesterol and α-tocopherol. These findings suggest that impaired nutrition of the developing embryo may underlie the embryonic lethality when apoB is lacking.

In several species, including rodents, the yolk sac is thought to play an important nutritive role in early embryonic development. In these species, the yolk sac is said to be "inverted" (i.e., the absorptive surface faces the maternal tissues rather than surrounding a yolk), where it absorbs nutrients and transports them into the vitelline circulation to the embryo (reviewed in ref. 11). In the mouse, this process is thought to provide nutrients to the embryo until at least gestational day 10–11 (of 20–21 total days), when the chorioallantoic placental circulation is established. The yolk sac visceral endodermal cells express a wide range of proteins similar to those expressed by the adult liver (7). Among these is apoB, which is expressed at high levels in the yolk sacs of mice (5, 7, 8), rats (9), and humans (10) at various gestational timepoints. The earliest timepoint previously examined in mouse embryos was gestational day 10.5. Because the embryonic lethality in the apoB knockout embryos occurs by 9.5–10.5 gestational days, we examined the developmental time course of apoB mRNA expression using a sensitive RT-PCR assay and found that significant expression was detectable by day 7.5 in normal embryos. In situ hybridization techniques localized the expression of apoB mRNA to the yolk sac visceral endodermal cells of 9-day-old embryos. It is likely that the yolk sac endoderm represents the primary site of apoB expression in the embryo until at least gestational day 10, when the embryonic liver and intestine begin to form.

Because a major function of apoB is to serve as a structural protein for the formation of triglyceride-rich lipoproteins, it was likely that the yolk sac expression was associated with the production of lipoprotein particles. This possibility was first suggested by Shi and Heath (8) who were able to immunoprecipitate apolipoproteins from the media of 10.5-day-old cultured mouse yolk sacs. More recently, Plonné et al. (17) and Franke et al. (18) have used electron microscopy and immunohistochemical methods to demonstrate that the visceral yolk sac from near-term rat embryos synthesizes and secretes apoB-containing lipoproteins that are mostly of LDL size and density. The synthesis and secretion of lipoproteins by the yolk sac endoderm at early stages of mouse development, however, had not been directly demonstrated. Our electron microscopy findings clearly demonstrate that lipoproteins are synthesized and secreted by the yolk sac visceral endoderm early in development. The lipoproteins were mostly VLDL-sized and were readily detectable in the cisternae of rough ER, in the Golgi apparatus, in secretory vesicles in and distant from the Golgi areas, and in the extracellular space at the basal aspect of the cells. The VLDL-sized particles we observed

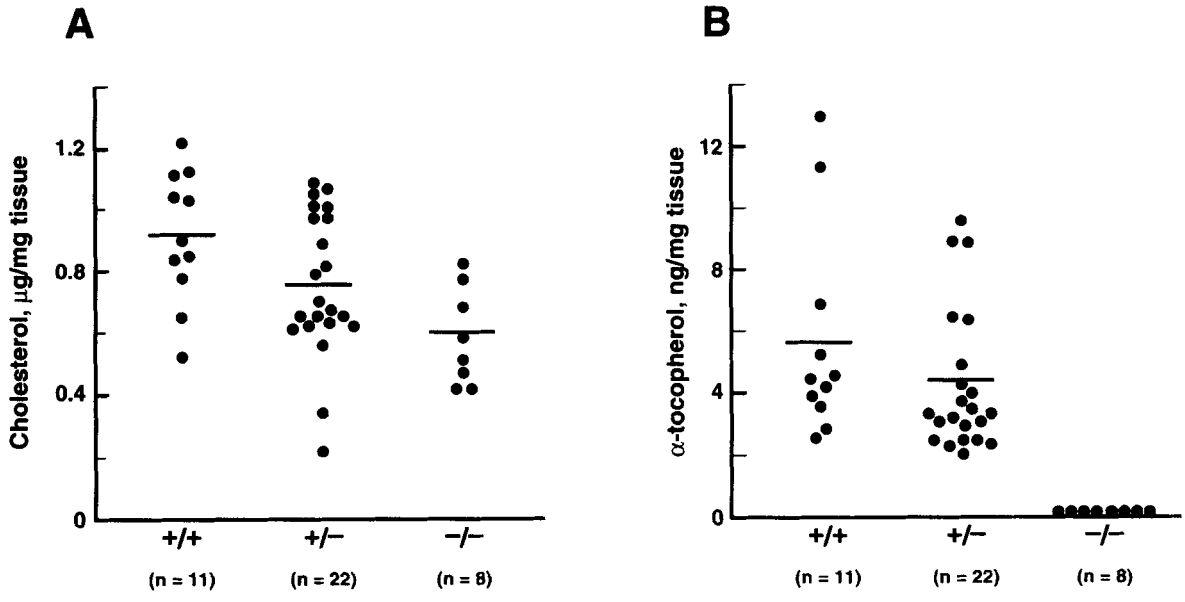


Fig. 6. Cholesterol and α-tocopherol concentrations of wild-type and apoB knockout embryos. Embryos were dissected in PBS. Yolk sac membranes were removed for genotype analysis by Southern blotting, and the embryonic tissues were assayed for cholesterol (A) and α-tocopherol (B) as described in Materials and Methods. The horizontal lines indicate means for each group.

contrast with the predominantly LDL-sized particles observed by Plonné et al. (17) and Franke et al. (18) in cultured yolk sacs of near-term rats; the reason for this difference is not clear. Interestingly, in these cells, we found no evidence of smooth ER, an organelle that has been postulated to be important in the synthesis and secretion of triglyceride-rich lipoproteins from rat hepatocytes (19) and human enterocytes (20). We also found no morphologic evidence of transcytosis of maternal apoB-containing lipoproteins; in particular, we observed no vesicles containing lipoproteins in the yolk sacs of *apoB*$^{-/-}$ embryos (see below), which one would expect if transcytosis of lipoproteins from the *apoB*$^{+/-}$ mother was occurring.

The major finding of this study is that when apoB is lacking, there is an apparent defect in lipid export from the yolk sac visceral endodermal cells. Lipid droplets accumulated in the cytosol at the basal aspect of these cells, and the normally present lipoproteins were absent from the Golgi apparatus and secretory vesicles of visceral endodermal cells. Both of these effects appeared to be related to gene dosage, as heterozygous embryos exhibited an intermediate phenotype for these findings. This result suggests that the ability of the yolk sac visceral endodermal cells to synthesize and secrete apoB-containing lipoproteins is closely correlated to, and dependent on, the level of apoB expression. That the lipid transport problems are associated with functional consequences to the embryos was demonstrated by the findings of diminished cholesterol and α-tocopherol concentrations in the tissues of *apoB*$^{-/-}$ embryos. The inability to detect α-tocopherol in these embryos was intriguing in that vitamin E deficiency in pregnant rats results in a phenotype similar to that of apoB-deficient mice (21–23). Recent studies by Homanics et al. (15) using a mouse model of apoB deficiency (apoB-70 mice), however, question whether vitamin E is the sole deficient nutrient. These investigators fed high doses of vitamin E to apoB-70 heterozygous pregnant mice and found that although α-tocopherol concentrations could be raised in the homozygous embryos, a significant decrease in the incidence of neural tube-related developmental defects was not observed (there was, however, a trend in this direction). Thus, it is presently unclear whether vitamin E deficiency is an associative or causative finding for the developmental defects observed when apoB is deficient.

Cholesterol deficiency in the embryo can also be considered as a possible causative factor. In the chick embryo, the yolk sac endoderm plays an important nutritive role in embryonic development, secreting lipoproteins into the fetal circulation and apparently providing large amounts of cholesteryl esters to the embryonic liver and other tissues (24). Although rodent embryonic tissues are capable of synthesizing cholesterol during development (25, 26), it is not clear at what developmental stage embryonic synthesis of cholesterol begins and whether the embryo might be more dependent on maternal cholesterol in the very early stages. Finally, it is possible that the observed embryonic lethality may result from a deficiency in another lipid, such as triglycerides or phospholipids, or from a more general nutritional defect.

Our findings lend new insights into the role of apoB and lipoproteins in the provision of lipid nutrients to the developing embryo, and suggest a hypothetical model that can be tested further (**Fig. 7**). It is probable that maternal lipoproteins cross Reichert's membrane and bind to receptors on the apical microvilli of the yolk sac visceral endodermal cells. The morphologic findings suggest that maternal lipoproteins undergo hydrolysis at or near the plasma membrane surface, rather than after receptor-mediated uptake and lysosomal catabolism. Our findings suggest that these maternally derived lipid nutrients, as well as lipids synthesized by the yolk sac endoderm, such as cholesterol (25), are then repackaged into apoB-containing lipoproteins, which are subsequently secreted from the basal surfaces of these cells into the vitelline circulation (or possibly into the exocoelom at very early stages) for transportation to the embryo. One could speculate from this model that any species that is dependent on a yolk sac for nutrition, whether via an inverted yolk sac absorbing nutrients from the mother as in rodents or a yolk sac enclosing yolk as in chickens, might be dependent on apoB to facilitate this process. Indeed, the yolk sac visceral endoderm of the chicken embryo expresses apoB mRNA by embryonic day 3 of development (27), and the morphology of this tissue is remarkably similar to that we have observed in the mouse (28). Additionally, one could speculate from this model that the microsomal triglyceride transfer protein (MTP), which is necessary for triglyceride-rich lipoprotein formation, is likely to be present in mouse visceral endoderm. Indeed, in preliminary experiments, we have detected MTP in mouse yolk sac endoderm by immunoblotting (R. Farese and D. Gordon, unpublished data). Furthermore, the absence of MTP, which causes abetalipoproteinemia in humans (29), might also prove lethal for mouse embryos. This latter hypothesis will await testing by the generation of an MTP knockout mouse.

The present findings clearly demonstrate that the yolk sac visceral endoderm, in addition to the liver and the intestine, is a lipoprotein-secreting tissue with physiologic relevance in mice. It is at present unclear whether this physiologic need for apoB-containing lipoproteins during embryonic development extends to humans. Certainly there are human subjects with homozygous hypobetalipoproteinemia or abetalipoproteinemia who

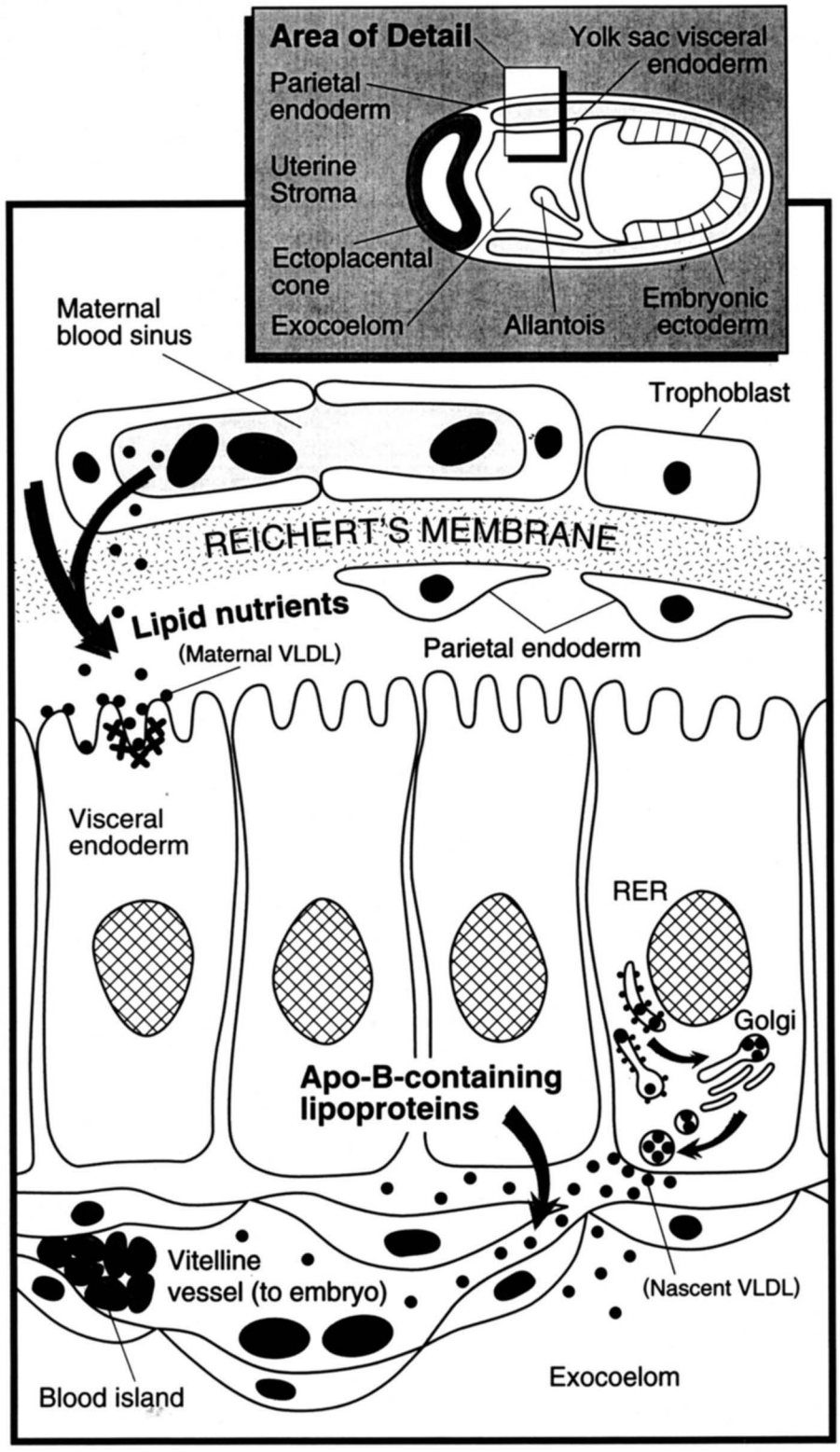


Fig. 7. Hypothetical model for the role of apoB and lipoproteins in facilitating lipid transport to the developing mouse embryo via the yolk sac endoderm. Lipid nutrients from maternal decidual tissues or maternal capillaries are transported across Reichert's membrane and the noncontiguous parietal endodermal cell layer to the apical surface of the visceral endodermal cells. Here lipids either undergo hydrolysis or are internalized via receptors. The visceral endodermal cells subsequently repackage maternally derived lipids or endogenously synthesized lipids into apoB-containing lipoproteins for secretion at the basal aspect of these cells for transport via the vitelline circulation to the developing embryo.

have few, if any, apoB-containing lipoproteins in their plasma and who apparently had no developmental problems. However, it is still possible that the lack of apoB-containing lipoproteins could lead to unrecognized fetal demise in some kindreds. In any case, our findings suggest that the transport of certain lipids across the maternal–fetal barrier is essential for embryonic development. It is possible that certain species, such as humans, which rely primarily on chorioallantoic placentation rather than yolk sac placentation for nutrition, may have evolved an alternative means for transporting essential lipid nutrients.

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