ELSEVIER

Reviews

The function of cholesterol in embryogenesis

George Wolf

Department of Nutritional Sciences, University of California, Berkeley, CA USA

Cholesterol is critical in embryonic development. Inhibition of cholesterol synthesis in experimental animals has caused a birth defect called holoprosencephaly (HPE), which is evidenced by cyclopia (one eye in the middle of the face), monorhinia (protruding single nose above the eye), absence of the pituitary gland, and central nervous system (CNS) abnormalities. In humans, an inherited defect in the cholesterol-synthesizing enzyme 7-dehydrocholesterol reductase depletes cholesterol and results in human HPE, termed Smith-Lemli-Opitz syndrome. In its most severe form, the syndrome leads to cyclopia, monorhinia, and lack of separation of cerebral hemispheres. The cause of the syndrome is a defect in a protein coded by the gene Sonic hedgehog *(*SHH*). The protein SHH is expressed in the notochord of the CNS in the early embryo and is activated by being cleaved autocatalytically, with simultaneous covalent attachment of cholesterol to the N-terminal fragment, which is secreted by cells of the mesoderm layer, signaling the establishment of the neural midline cells. Thus, cholesterol is essential for proper signaling in the development of the normal embryo.* (J. Nutr. Biochem. 10:188–192, 1999)

Keywords: cholesterol; holoprosencephaly; Smith-Lemli-Opitz syndrome; *Sonic hedgehog* gene

Introduction

A high level of serum cholesterol is a high risk factor in cardiovascular disease.[1] Dietary intervention can reduce plasma cholesterol. If this method fails, drugs can lower cholesterol levels, generally by suppressing cholesterol synthesis. The commonest drugs are those that inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (e.g., Mevinolin, Pravachol). Others are inhibitors of 7-dehydrocholesterol reductase (e.g., AY9944, BM15.766). Both enzymes are on the pathway of cholesterol synthesis. Another drug, triparanol, inhibits the conversion of desmosterol to cholesterol.

Cholesterol and teratogenesis

As long ago as 1964, Roux and coworkers[2,3] discovered that the cholesterol synthesis inhibitory drugs AY9944 and triparanol, when given to rats early in pregnancy (second, third, or fourth day of gestation), caused fetal abnormalities, with varying degrees of severity: The most severe cases (25%) demonstrated cyclopia (one eye in the middle of the face) and monorhinia (protruding single nasal structure situated above the eye). Almost 100% of cases lacked the pituitary gland and displayed brain abnormalities that were detected histologically. This syndrome is called holoprosencephaly (HPE). Roux[4] could prevent HPE completely by feeding the animals high doses of cholesterol simultaneously with AY9944. In further support of the hypothesis that hypocholesterolemia was the cause of the teratogenesis of the drug, Roux et al.[5] compared the effect on the action of AY9944 of a hypercholestrolemic diet (33% lard plus 2% cholesterol) with that of a normal diet (4% vegetable oil) fed for 3 weeks before conception and throughout gestation: The hypercholesterolemic diet completely prevented induction of HPE by the drug. In a later study, Roux et al.[6] found that the frequency of malformations in fetuses of rats correlated with maternal plasma levels of cholesterol, which was an expected result because 60 to 70% of embryo cholesterol is derived from the mother.[7] The incidence of malformations was correlated positively to the dose of AY9944. Cholesterol precursors, mainly 7-dehydrocholes-

Address correspondence to Dr. George Wolf, Department of Nutritional Sciences, University of California, 119 Morgan Hall, Berkeley, CA 94720 USA.
Received November 3, 1998; accepted December 8, 1998.

655 Avenue of the Americas, New York, NY 10010
0955-2863/99/$–see front matter
PII S0955-2863(98)00102-8

terol, accumulated.[8] In the laboratory of Roux, Barbu et al.[9] studied the prevention of HPE by feeding cholesterol simultaneously with or after AY9944 administration. When cholesterol was given simultaneously with the drug, prevention was 100%; when given 2 days later, malformations were seen in 7 to 10% of fetuses and were higher (20%) with higher doses of AY9944.

In an extensive study of HPE in the fetuses of rats, Salen et al.[10] fed the dams the 7-dehydrocholesterol reductase inhibitor BM15.76611. Plasma cholesterol levels dropped 67% and 7-dehydrocholesterol concentration rose from almost zero to 17 mg/dL. When these dams were dosed with cholesterol, their plasma cholesterol levels rose 3.7-fold and 7-dehydrocholesterol declined by 88%. In similar experiments, Dehart et al.[11] described the midline facial abnormalities that appeared in embryos on gestational day 12 and the neural defects of the cell populations of the forebrain, lower midbrain, and hind brain in HPE as a result of stomach-tube administration of BM15.7661 to the pregnant rats.

The occurrence of craniofacial malformations in lambs newborn to ewes feeding on the range plant *Veratrum californicum* (corn lily) in the western states of the United States, led to the identification in these plants of two steroidal alkaloids that had structures resembling cholesterol.[12] These compounds—jervine and cyclopamine—produce malformations in lambs that closely resemble HPE, with craniofacial abnormalities of varying degrees of severity. The most severely affected lamb displayed a single median eye (cyclopia), a nose consisting of a tubular proboscis above the eye, and a brain severely reduced in size with a collapsed cortex. Beachy et al.[13] found that, when treated with jervine, COS-7 cells in culture metabolically labeled by ^{3}H-mevalonic acid synthesized much less labeled cholesterol and instead increased levels of labeled zymosterol. Other compounds structurally resembling cholesterol, which were isolated from potato sprouts, were found to be teratogenic, inducing craniofacial abnormalities in fetuses of Syrian hamsters.[14] These compounds were the steroidal alkaloids α-solanine and α-chaconine. It is possible that these plant toxins inhibit cholesterol synthesis because of their structural similarity to cholesterol: They may compete with cholesterol for covalent binding to protein (see below).

Not only can inhibition of cholesterol synthesis cause HPE, but inhibition of the transport of cholesterol into embryonic cells also can cause HPE. A family of receptors for low-density lipoprotein (LDL), called megalin, is expressed in embryonic neuroectoderm, among other tissues. These receptors take up LDL-bound cholesterol into cells. Mouse embryos deficient in megalin suffer from HPE, presumably because of a defect in the supply of cholesterol.[15]

Other inhibitors of cholesterol synthesis (e.g., drugs such as Pravacol or Mevinolin) that inhibit the synthesis of mevalonate, an early compound on the pathway from acetate to cholesterol, also produced fetal abnormalities in rats.[16] These can be prevented by the administration of mevalonate to the mother. However, these malformations were of an entirely different nature from HPE and could not be prevented by cholesterol supplementation. Newer cholesterol-lowering drugs such as Atorvastatin, another HMG-CoA reductase inhibitor, produced no fetal defects in either rats or rabbits.[17]

Smith-Lemli-Opitz syndrome

A birth defect first described by Smith, Lemli, and Opitz in 1964,[18] termed Smith-Lemli-Opitz syndrome (SLOS), is an inherited autosomal recessive disorder, with normal heterozygotes.[19] The clinical phenotype, HPE, appears in approximately 1 in 20,000 births. An estimated 1 to 2% of the North American caucasian population carries the gene.[20] The disorder presents with craniofacial abnormalities: microcephaly, anteverted nares, cleft palate, and micrognathia. In some cases, incomplete development and septation of midline structures of the central nervous system (CNS), limb and urogenital abnormalities, and heart defects may be present. HPE in SLOS can occur with widely differing clinical severity. Kelley et al.[21] described the most severe form (usually stillborn), as alobar HPE, with cyclopia, a primitive nasal structure above the eye (proboscis) and lack of separation of the cerebral hemispheres; intermediate HPE is described as semilobar, with partial separation of the hemispheres; the least severe form presents as microforms, with microcephaly, midfacial clefting, and median cleft lip. Thus, the clinical phenotype corresponds closely to HPE described in animals dosed with cholesterol synthesis inhibitors.[6] Indeed, some (but not all) SLOS homozygotes show an inherited deficiency of the enzyme 7-dehydrocholesterol reductase,[10] which results in low levels of plasma cholesterol and accumulation of the cholesterol precursor 7-dehydrocholesterol. Tint et al.[22] reported that in 33 SLOS patients, plasma cholesterol concentrations were very low (below the fifth percentile for age- and gender-matched control children). 7-Dehydrocholesterol levels in plasma had risen several thousand-fold. There was an inverse correlation between plasma concentration of cholesterol and the number of malformations.[23] In a subsequent report, Tint et al.[24] observed that, although total tissue sterol concentration in a homozygous fetus was comparable to that of normal matched fetuses, tissue cholesterol concentrations were approximately ⅒ to ½₀ of normal. Cerebral cortex cholesterol concentrations were 2.2 mg/g in normal fetuses (20 weeks' gestation) versus 0.09 mg/g in the homozygote. 7-Dehydrocholesterol, which was undetectable in tissues of normal fetuses, rose so much in the homozygote that total tissue sterol concentrations were nearly normal. These authors[24] concluded that the malformations were a result of not only low maternal plasma cholesterol but, because of the low tissue cholesterol, defective local tissue cholesterol synthesis in the fetuses also must have been responsible for the SLOS.

Shefer et al.[25] developed a method for rapid identification of SLOS caused by an inherited defect in the enzyme 7-dehydrocholesterol reductase, both for heterozygotes and homozygotes. Fibroblasts from SLOS heterozygotes, homozygotes, and normal subjects were grown in cholesterol-depleted medium. 7-Dehydrocholesterol reductase activity was lower in both heterozygotes and homozygotes compared with that from normal control subjects. Homozygotes showed a 30-fold increase in fibroblast 7-dehydrocholes-

terol, whereas heterozygotes displayed no such accumulation. By this comparison, SLOS homozygotes and heterozygotes can both be diagnosed.

The *Sonic hedgehog* gene

Because cholesterol is a precursor of steroids and because steroids are involved in embryogenesis, investigators proposed that HPE caused by cholesterol synthesis inhibitors or by a mutated cholesterol synthesis gene that produced low levels of cholesterol gave rise to "sterol perturbations"[9] and thus to defective embryogenesis. However, a quite unexpected finding shifted the focus to a gene that controlled embryonic development at a deeper level. The gene in question, termed *hedgehog* (*hh*), was first described by Nusslein-Volhard and Wieschaus[26] as a gene that was required to establish local polarity with respect to the anterior-posterior axis in each segment in developing Drosophila larvae. The gene was characterized and sequenced by Lee et al.[27] The formation of the segments' anterior boundary depends on expression of the *wingless* (*wg*) gene, and of the posterior boundary, on expression of the *engrailed* (*en*) gene. In order for the *wg* and *en* genes to be continually expressed during embryogenesis, *hh* also must be expressed. If the gene product, the Hh protein, does not function, defective patterning results. The character of Hh was that of a secreted protein involved in localized extracellular signaling.

In a later paper, Lee et al.[28] reported that the Hh protein was cleaved internally by an autocatalytic proteolysis. The amino acid sequence of the Hh protein harbored a seven-residue sequence with similarities to serine proteases, with a histidine residue that acted as a base catalyst. Induced mutations in the N-terminal domain had no effect on the cleavage reaction, whereas mutations in the C-terminal domain of Hh prevented cleavage, suggesting that the C domain was the catalyst in the cleaving proteolysis. Functionally, to maintain the expression of the *wg* gene, the C domain mutations rendered Hh inactive. Therefore, autoproteolysis was deemed essential for function, suggesting that the N fragment, which was cell associated, was the active signal. By studying the patterning of larval structures of Drosophila, studies[28,29] found that Hh directs morphogenetic patterning by induction of expression of other downstream genes.

In a later work,[29] the Beachy group obtained the purified Hh protein by means of expressing the *hh* gene in bacteria. Cleavage of Hh within Drosophila cells resulted in a 25-kDa C-terminal fragment and a 19-kDa N-terminal fragment,[30] the split being between glycine 257 and cysteine 258. The group tested the phenotypic expression by transgenic Drosophila embryos expressing the N domain alone, the C domain alone, or the complete Hh protein with cysteine replaced by methionine. These experiments proved that the C domain was the determinant of autocatalytic cleavage and that the N domain was the active signaling portion of Hh, but only if derived from *native* Hh by cleavage; a truncated artificially constructed N domain (Hh-N) exhibited abnormal distribution. This result suggested that some alteration to the N domain had occurred during cleavage.

In further experiments, the Beachy group[30] explored the

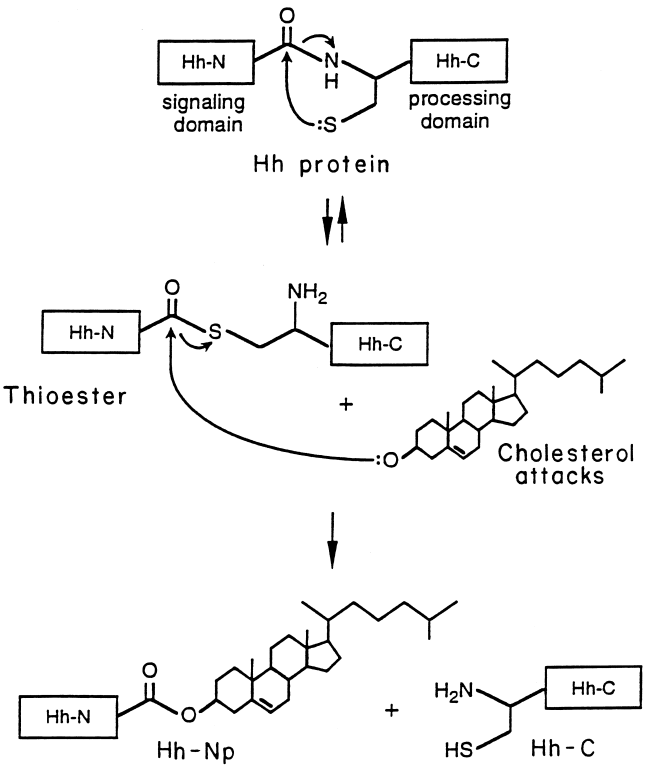

Figure 1 Diagrammatic representation of autoprocessing and protein self-splicing of the Hedgehog protein. (Adapted from Beachy et al.,[13] with permission.)

autoproteolysis of Hh and the consequent spatial patterning in Drosophila. They used the GAL4 transcriptional activator from yeast, which was controlled by the *en* gene promoter expressing the full-length *hh* gene or the upstream activating sequence with the truncated *hh*-N gene. Transgenic flies that expressed these constructs were generated. Homozygotes were mated and the resulting embryos were analyzed for their phenotypes: Full-length *hh* constructs showed localized expression of Hh protein and normal phenotype. The flies with the *hh*-N construct exhibited multiple defects, including *hh*-N defective cuticle and embryo death.

With cultured Drosophila cells (S2),[30] immunofluorescence in cells expressing full-length *hh* showed cell-surface localization of Hh-N protein, whereas expression of *hh*-N resulted in diffuse distribution of the protein. The conclusion was that the Hh-N domain formed from expression of full-length *hh* construct was distinct from that formed from the *hh*-N construct. The investigators[30] termed the protein formed by autoprocessing from full-length Hh Hh-N_p; the truncated protein expressed by the *hh*-N gene construct was termed Hh-N. Hh-N_p was more hydrophobic upon electrophoresis and during solvent extraction than was Hh-N. Mass spectrometry revealed an extra mass of approximately 400 daltons linked covalently to the C terminal of Hh-N_p.

The Hh protein could be cleaved in vitro nonenzymatically by dithiothreitol (DTT). The split was between Gly 257 and Cys 258; thus: Hh→Hh-C + Hh-N. The investigators[30] proposed that Cys 258 attacks Gly 257, forming a thioester intermediate (*Figure 1*). This then could be cleaved by a nucleophile such as DTT. However, the

nucleophilic attack also could be by a hydrophobic molecule, forming a hydrophobic adduct with formation of Hh-N_p. Indeed, when the in vitro reaction was carried out in the presence of microsomes, the hydrophobic Hh-N_p was obtained instead of Hh-N.

The Beachy laboratory went on to establish that the nucleophilic adduct to Hh-N was cholesterol.[31] When they added bulk lipid from Drosophila S2 cultured cells to Hh in the presence of low concentrations of DTT, Hh-N_p was obtained. Fractionation of the bulk lipid and test of the fractions resulted in identification of cholesterol as the adduct. Autoprocessing of Hh with pure cholesterol also formed Hh-N_p, and ^{3}H-cholesterol formed ^{3}H-Hh-N_p. The covalent linking of the cholesterol was demonstrated by base hydrolysis of Hh-N_p, which yielded Hh-N.

Thus, the Beachy laboratory has established the mechanism whereby a cell-to-cell signaling protein is formed in early embryogenesis. To recapitulate, a two-step process occurs: (1) the SH of Cys 258 within the Hh protein attacks the carbonyl of the neighboring peptide bond of Gly 257 and produces a thioester intermediate (*Figure 1*); (2) the same carbonyl is then attacked by cholesterol, which results in cleavage, with formation of the cholesteryl ester Hh-N_p and the Hh-C peptide (*Figure 1*). The process was shown to be intramolecular, because its rate was independent of the concentration of the Hh protein. The Hh-N_p is secreted from the cell and tethered to cell surfaces by the hydrophobic cholesterol moiety, thus limiting its spatial distribution and increasing its local concentration.

An important discovery by Echelard et al.[32] demonstrated the occurrence of a gene in vertebrates, called *Sonic hedgehog* (*Shh*) that is closely related to the insect *hh* gene. The authors showed that it was expressed in the notochord, the floorplate, and the zone of polarizing activity of the CNS of the early mouse embryo. It was a controlling factor in the regulation of CNS polarity. The Shh protein signal is secreted by cells of the mesoderm layer of CNS to establish midline identity of the overlying neural plate. The neural midline cells then give rise to ventral brain, floor plate, and motor neurons.[33] Mutations in *Shh* result in defective midline structures.

The *Shh* gene has since been isolated from tissues of many species, including frog, fish, chicken, mouse, rat, and human.[34] Chiang et al.[34] experimented with mice carrying a transfected *Shh* construct to study the direct role of *Shh* in vertebrate embryonic patterning. They constructed a vector with deletions that caused the loss of 97 of the 198 residues of the N-terminal domain of Shh protein and, by inserting it into their embryonic stem cells, created mice homozygous for the disrupted gene. Heterozygous mice were normal; homozygous mice bore embryos that died at or just before birth. The mutant embryos showed defects in the future forebrain, smaller brains, absence of lateral eyes, extreme abnormalities in craniofacial structures, and defects in heart, lung, kidney, foregut, and skeleton. The authors[34] showed that, though a normal notochord was formed in the mutant embryos, floorplate cells and motor neurons of the embryonic CNS were not induced by the notochord. They suggest that the autocrine secretion of Shh protein represents a maintenance function for the notochord. Chiang et al.[34] concluded that the expression of *Shh* in prechordal plate mesoderm is the "signal to establish the ventral midline of the brain and eye field." Thus, the phenotype of the $Shh^{-/-}$ mice closely resembles SLOS and mouse HPE.

The report of Chiang et al.[34] was quickly followed by the discovery of a mutation in the human homologue of the *Shh* gene by Roessler et al.[35] The locus known as HPE3, located on chromosome 7q36 in humans, had been known to be linked to the human genetic defect of HPE.[36] The human gene (called *SHH*) is one of three genes homologous to Drosophila *hh*. Roessler et al.[35] pinpointed the mutation in 30 autosomal dominant HPE families, with defined defects in the SHH protein. This protein normally is cleaved in the early embryo, with cholesterol required for the cleavage reaction, which is bound covalently to the mature signaling peptide. Any events that prevent or retard this process could give rise to HPE malformations: a defective enzyme in cholesterol synthesis (7-dehydrocholesterol reductase), administration of drugs that limit the supply of cholesterol (e.g., AY9944), or a mutation in the gene for the signaling protein itself (*SHH*).

A question that arises is the following: What is the downstream recipient of the SHH signal? At least in Drosophila, the protein expressed by a gene named *Patched* (*ptc*) is the receptor for the Hh protein. Ptc is a negative regulator, the activity of which is inhibited by Hh binding. Only after Hh binding can Ptc signal downstream. The large number of genes on the pathway of Ptc signaling was discussed by Tabin and McMahon.[37] Recent work by the Beachy group found a new role for cholesterol in Shh signaling, possibly involving the Ptc protein.[38] Using chick embryo explants known to respond to added recombinant Shh, these authors[38] showed that some of the teratogens that block cholesterol synthesis (e.g., jervine, AY9944) inhibited the normal signaling response of the embryonic tissue even in presence of purified Shh protein. Therefore, the teratogens must function by some mechanism that inhibits not only cholesterol synthesis, but also the response of the embryonic target tissues to Shh signaling. Cooper et al.[38] speculate that these cholesterol-synthesis inhibiting teratogens function by acting through the sterol-sensing domain of the Ptc protein, thereby influencing the response to the Shh signal. This process would represent a second role for cholesterol in embryogenesis, possibly more important than the activation of Shh by cholesterol attachment.

Summary

In summary, cholesterol is involved in three different, but related, ways whereby birth defects are produced. The first is by drugs that inhibit cholesterol synthesis; the second is by a genetic, heritable defect in the enzyme that synthesizes cholesterol. In both cases the level of cholesterol that is available to the cells of the early embryo is lowered at a critical period of development of the CNS. Both processes converge on a signaling protein SHH, which is secreted by the cells of the prechordal mesoderm and requires covalently-bound cholesterol for its formation. Therefore, the third way is in a mutation of the SHH protein itself.

References

1 Stamler, J., Wentworth, D., and Neaton, J.D. (1986). Is the relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded? *J. Am. Med. Assoc.* **256,** 2823–2828

2 Roux, C. (1964). Action tératogène du triparanol chez l'animal. *Arch. Franc. Pediatr.* **21,** 451–464

3 Roux, C. and Aubry, M.M. (1966). Action tératogène chez rat d'un inhibiteur de la synthèse du cholestérol, A 9944. *Compt. Rend. Soc. Biol.* **160,** 1353–1357

4 Roux, C. (1968). Essai de prévention de l'action tératogène d'un hypocholéstèrolemiant. *Compt. Rend. Soc. Biol.* **162,** 1671–1675

5 Roux, C., Horvath, C., and Dupuis, R. (1979). Teratogenic action and embryo lethality of AY 9944: Prevention by a hypercholesterolemia-provoking diet. *Teratology* **19,** 35–38

6 Roux, C., Dupuis, R., Horvath, C., and Talbot, J.-N. (1980). Teratogenic effect of an inhibitor of cholesterol synthesis (AY 9944) in rats: Correlation with maternal cholesterolemia. *J. Nutr.* **110,** 2310–2312

7 Chevallier, P. (1964). Transferts et synthèses du cholestérol chez le rat au cours de sa croissance. *Biochim. Biophys. Acta* **84,** 316–339

8 Dvornik, D. and Hill, P. (1968). Effect of long-term administration of AY9944, an inhibitor of 7-dehydrocholesterol Δ^7-reductase, on serum and tissue lipids in the rat. *J. Lipid Res.* **99,** 587–595

9 Barbu, V., Roux, C., Lambert, D., Dupuis, R., Gardette, J., Maziere, J.-C., Maziere, C., Elefant, E., and Polonovski, J. (1988). Cholesterol prevents the teratogenic action of AY 9944: Importance of the timing of cholesterol supplementation to rats. *J. Nutr.* **118,** 774–779

10 Salen, G., Shefer, S., Batta, A.K., Tint, G.S., Xu, G., Honda, A., Irons, M., and Elias, E.R. (1996). Abnormal cholesterol biosynthesis in the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **37,** 1169–1180

11 Dehart, D.B., Lanoue, L., Tint, G.S., and Sulik, K.K. (1997). Pathogenesis of malformations in a rodent model for Smith-Lemli-Opitz syndrome. *Am. J. Med. Genet.* **68,** 328–337

12 Evans, H.E., Ingalls, T.H., and Binns, W. (1966). Teratogenesis of craniofacial malformations in animals. *Arch. Environ. Hlth.* **13,** 707–714

13 Beachy, P.A., Cooper, M.K., Young, K.E., von Kessler, D.P., Park, W.-J., Hall, T.M.T., Leahy, D.J., and Porter, J.A. (1997). Multiple roles of cholesterol in hedgehog protein biogenesis and signaling. *Cold Spring Harbor Symp. Quanti. Biol.* **62,** 191–204

14 Gaffield, W. and Keeler, R.F. (1996). Induction of terata in hamsters by solanidane alkaloids derived from *Solanum tuberosum. Chem. Res. Toxicol.* **9,** 426–433

15 Willnow, T.E., Hilpert, J., Armstrong, S.S., Hammer, R.E., Burns, D.K., and Herz, J. (1996). Defective forebrain development in mice lacking gp330/megalin. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 8460–8469

16 Minsker, D.H., MacDonald, J.S., Robertson, R.T., and Bokelman, D.L. (1983). Mevalonate supplementation in pregnant rats suppresses the teratogenicity of mevinolinic acid, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Teratology* **28,** 449–456

17 Dostal, L.A., Schardein, J.L., and Anderson, J.A. (1994). Developmental toxicity of the HMG-CoA reductase inhibitor Atorvastatin in rats and rabbits. *Teratology* **50,** 387–394

18 Smith, D.W., Lemli, L., and Opitz, J.M. (1964). A newly recognized syndrome of multiple congenital anomalies. *J. Pediatr.* **64,** 210–217

19 Pober, B. (1990). Smith-Lemli-Opitz syndrome. In *Birth Defects Encyclopedia* (M.L. Buyse, ed.), pp. 1570–1572, *Blackwell Scientific*, Cambridge, MA, USA

20 Opitz, J.M. and de la Cruz, F. (1994). Cholesterol metabolism in the RSH/Smith-Lemli-Opitz syndrome: Summary of an NICHD conference. *Am. J. Med. Genet.* **50,** 326–338

21 Kelley, R.I., Roessler, E., Hennekam, R.C.M., Feldman, G.L., Kosaki, K., Jones, M.C., Palumbos, J.C., and Muenke, M. (1996). Holoprosencephaly in RSH/Smith-Lemli-Opitz syndrome: Does abnormal cholesterol metabolism affect the function of *Sonic Hedgehog*? *Am. J. Med. Genet.* **66,** 478–484

22 Tint, G.S., Irons, M., Elias, E.R., Batta, A.K., Friedin, R., Chen, T.S., and Salen, G. (1994). Defective cholesterol biosynthesis associated with Smith-Lemli-Opitz syndrome. *N. Engl. J. Med.* **330,** 107–113

23 Tint, G.S., Salen, G., Batta, A.K., Shefer, S., Irons, M., Elias, E., Abuelo, D.N., Johnson, V.P., Lambert, M., Lutz, R., Schanen, C., Morris, C.A., Hoganson, G., and Hughes-Benzie, R. (1995). Severity and outcome correlate with plasma sterol levels in type I and type II variants of the Smith-Lemli-Opitz syndrome. *J. Pediatr.* **127,** 82–87

24 Tint, G.S., Seller, M., Hughes-Benzie, R., Batta, A.K., Shefer, S., Genest, D., Irons, M., Elias, E., and Salen, G. (1995). Markedly increased tissue concentrations of 7-dehydrocholesterol combined with low levels of cholesterol are characteristic of the Smith-Lemli-Opitz syndrome. *J. Lipid Res.* **36,** 89–95

25 Shefer, S., Salen, G., Honda, A., Batta, A., Hauser, S., Tint, G.S., Honda, M., Chen, T., Holick, M.F., and Nguyen, L.B. (1997). Rapid identification of Smith-Lemli-Opitz homozygotes and heterozygotes (carriers) by measurement of deficient 7-dehydrocholesterol reductase activity in fibroblasts. *Metabolism* **46,** 844–850

26 Nusslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature* **287,** 795–801

27 Lee, J.J., von Kessler, D.P., Parks, S., and Beachy, P.A. (1992). Secretion and localized transcription suggest a role in positional signaling for products of the segmentation gene *hedgehog. Cell* **71,** 33–50

28 Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., and Beachy, P.A. (1994). Autoproteolysis in *hedgehog* protein biogenesis. *Science* **266,** 1528–1537

29 Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee J.J., Moses, K., and Beachy, P.A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374,** 363–366

30 Porter, J.A., Ekker, S.C., Park, W.-J., von Kessler, D.P., Young, K.E., Chen, C.-H., Ma, Y., Woods, A.S., Cotter, R.J., Koonin, E.V., and Beachy, P.A. (1996). Hedgehog patterning activity: Role of a lipophilic modification mediated by the carboxy-terminal autoprocessing domain. *Cell* **86,** 21–34

31 Porter, J.A., Young, K.E., and Beachy, P.A. (1996). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274,** 255–259

32 Echelard, Y., Epstein, D.J., St.-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., and McMahon, A.P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules is implicated in the regulation of CNS polarity. *Cell* **75,** 1417–1430

33 Blader, P. and Strahl, U. (1998). Casting an eye on cyclopia. *Nature* **395,** 112–113

34 Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., and Beachy, P.A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383,** 407–413

35 Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S.W., Tsui, L.-C., and Muenke, M. (1996). Mutations in the human *Sonic Hedgehog* gene cause holoprosencephaly. *Nature Genetics* **14,** 357–360

36 Muenke, M., Gurrieri, F., Bay, C., Yi, D.H., Collins, A.L., Johnson, V.P., Hennekam, R.C., Schafer, G.B., Weik, L., and Lubinsky, M.S. (1994). Linkage of a human malformation, familial holoprosencephaly, to chromosome 7 and evidence for genetic heterogeneity. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 8102–8106

37 Tabin, C.J. and McMahon, A.P. (1997). Recent advances in Hedgehog signalling. *Trends in Cell Biology* **7,** 442–446

38 Cooper, M.K., Porter, J.A., Young, K.E., and Beachy, P.A. (1998). Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science* **280,** 1603–1607