Titel: A Compendium of Expression Patterns of Cholesterol Biosynthetic Enzymes in the Mouse Embryo

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Running Title: Expression Patterns of Cholesterol Biosynthetic Enzymes
Abbreviations: CBE, cholesterol biosynthetic enzyme; CBS, cholesterol biosynthesis; Hh, Hedgehog; ISH, in situ hybridization; SEMP, stereotypical energy metabolism pattern; SLOS, Smith-Lemli-Opitz Syndrome; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; TCA, Tricarboxylic acid
Abstract

Cholesterol and its biosynthetic pathway intermediates and derivatives are required for many developmental processes including membrane biogenesis, transmembrane receptor signaling, steroid biogenesis, nuclear receptor activation and posttranslational modification of hedgehog proteins. To perform such multifaceted tasks depends on stringent regulation of expression of cholesterol biosynthetic enzymes (CBEs). We established, for the first time, for a whole organism the 3D expression pattern of all genes required for cholesterol biosynthesis starting from acetyl-CoA and ending with cholesterol. This data was produced by high-throughput in situ hybridization on serial sections through the mouse fetus. The textually annotated image data was seamlessly integrated into the METscout and GenePaint public databases. This novel information helps to understand why CBEs are expressed at particular locations within the fetus. For example, strong CBE expression is detected at sites of cell proliferation and also where cell growth increases membrane surface, such as in neurons sprouting axons and forming synapses. The CBE data also sheds light on the spatial relationship of cells and tissue that express Sonic hedgehog and produce cholesterol, respectively. We discovered that not all cells expressing Sonic hedgehog are capable of cholesterol biosynthesis. This finding suggests novel ways by which cholesterylation of Sonic hedgehog is regulated.

Keywords: ISH, Brain Lipids, Cholesterol/Biosynthesis, Eye, Gene expression, Mouse embryo, Nutrition, Signal transduction, Smith-Lemli-Opitz syndrome.
**Introduction**

Embryogenesis is characterized by significant mass increase which is caused by cell proliferation and cell growth, both of which depend on continuous production of cellular membranes. Because such membranes contain up to 20 weight percent cholesterol, availability of this compound is essential for embryogenesis. Cholesterol maintains membrane integrity and also contributes to and regulates the properties of lipid rafts and lipid microdomains. Rafts and microdomains are the sites where many transmembrane signaling processes occur that play pivotal roles in development (1,2). Cholesterol also has very direct effects. It is covalently bound to the N-terminal fragment of Hedgehog (Hh) proteins which has essential roles in pattern formation and morphogenesis in invertebrates and vertebrates. The C-terminally attached cholesterol is required for the formation of multimeric Hh complexes and is thought to regulate the distribution of extracellular Hh ligand (3). Furthermore, cholesterol is a precursor of steroid hormones, bile acids and oxysterols each of which binds to specific nuclear receptors, which are potent transcriptional activators that control a wide spectrum of developmental and physiological processes (4–6).

The origin of cholesterol is two-fold. First, in the adult cholesterol is taken up from the diet whereas in the embryo cholesterol originates from the maternal circulation (1,2,7). Second, cholesterol is *de novo* synthesized by adult and embryonic tissues along a reaction pathway encompassing >30 different steps catalyzed by >20 cholesterol biosynthetic enzymes (CBEs) located in cytosol, endoplasmic reticulum, mitochondria and peroxisomes (8). The multistep cholesterol synthesis partitions into the presqualene and postsqualene segments (Supplemental Fig. S I). Squalene synthesis initiates with acetyl coenzyme A (CoA) and leads to lanosterol, at which the pathway bifurcates into the Bloch and Kandutsch-Russell pathways, respectively. Both of these pathways eventually lead to cholesterol.

Most compelling evidence for a pivotal role of cholesterol biosynthesis (CBS) for proper mammalian development comes from human and mouse genetic mutations in CBE genes (2,9). The first
human inborn error subsequently associated with a defective cholesterol metabolism is the Smith-Lemli-Opitz Syndrome (SLOS) (10). SLOS is an autosomal recessive disorder caused by mutations in the DHCR7 gene that encodes 7-dehydrocholesterol reductase converting 7-dehydrocholesterol to cholesterol (Supplemental Fig. S I) (11,12). The phenotypic spectrum of SLOS is very broad ranging from death \textit{in utero} to major malformations (skeletal, congenital heart, lung and kidney defects) to mild cases with minor physical defects and impaired learning and behavior. The essentiality of cholesterol for normal mammalian development is further emphasized by the finding that mutations in other human CBE genes often evoke strong phenotypes albeit sometimes different ones for each gene (2,9). Additionally, many mutations in murine CBE homologs evoke strong phenotypes that are, at least in part, reminiscent of those seen in \textit{Homo sapiens}.

Although cholesterol deficiency is a hallmark of all these disorders, it has been proposed that accumulating cholesterol synthesis intermediates and their metabolites also contribute to the disease phenotypes. Knowing when in development and in which organ a particular CBE is expressed thus helps in understanding the physiology and pathophysiology of CBE disorders. More broadly, such data sheds light on (i) the spatiotemporal dynamics of cholesterol metabolism in the context of other metabolic and signaling pathways, (ii) puts CBE expression into the context of its upstream regulators such as sterol regulatory element-binding proteins (SREBPs), and (iii) can lead to the identification of sites of accumulation of potentially toxic intermediates and metabolites of CBS. As a contribution towards advancing knowledge in these areas we have determined the expression patterns of the 25 genes encoding CBEs using high-throughput \textit{in situ} hybridization (ISH). CBE gene expression was analyzed to conform with and match to the ISH data of the transcriptome-wide www.genepaint.org and www.eurexpress.org digital atlases (13,14) that house thousands of gene expression patterns including those for the hedgehog signaling pathway and for the steroid, oxysterol and bile acid-controlled gene transcription networks. Additionally, CBE gene expression data were integrated into the METscout database.
(www.metscout.mpg.de) a powerful database in which cholesterol metabolism is integrated into the global metabolic network of the mouse (15).
Materials and Methods

**DNA template production and RNA probe synthesis**

DNA template production and probe synthesis was performed using previously described procedures (16,17). Template sequences are deposited on the genepaint.org database and can be retrieved on the set viewer page for each of the genes examined.

**Tissue collection, sectioning and in situ hybridization**

The general method for the dissection of E13.5 or E14.5 embryos, tissue collection, tissue sectioning, fixation and *in situ* hybridization was performed as previously reported (16,17). The E14.5 embryo expression pattern for each gene is documented in 22 - 24 sagittal sections.

**Imaging and data management**

The detailed procedures were previously described (17). Images were uploaded on to the GenePaint and METscout databases (13,15,16).

**Databases**

All primary ISH data on which this publication is based are accessible on the METscout database ([www.metscout.mpg.de](http://www.metscout.mpg.de)) by typing the gene symbol (provided in the second column of Fig. 1) into the gene entry field found on the front page. For a description of METscout see (15). Additionally, data were uploaded onto [www.genepaint.org](http://www.genepaint.org) and are accessed therein using the GenePaint Set ID found in the rightmost columns of Fig. 1 and Supplemental Table S I.
Results

CBE mRNAs reveal a stereotypical energy metabolism pattern

The spatial expression profiles of 25 genes encoding CBEs (see Supplemental Table S I for a listing of components and Supplemental Fig. S I for the pathway) were determined at stage E14.5 of mouse embryonic development by ISH. At this stage of development, organs begin to differentiate and some of them have started to carry out their particular metabolic and/or endocrine functions. Additionally, embryos undergo exponential growth and hence depend extensively on cellular membrane biosynthesis. E14.5 is also a time in development where many of the developmental signals such as Hedgehog are still highly active.

ISH results are summarized in the color chart in Fig. 1 in which the level of expression of mRNAs encoding the CBEs is shown for a series of signature tissues. Strongest expression is observed in the central nervous system, in liver and in adrenal glands. It is evident that the levels of expression of the pathway components differ quantitatively in any given tissue. Moreover, a particular CBE transcript shows considerable variation across the tissue spectrum, reminiscent of the situation in adult tissues in which some cells produce cholesterol in excess while others take up cholesterol. Although not every tissue expresses the full complement of CBE, this would not imply that cholesterol synthesis is absent in those tissues because precursors are found in and exchanged via the bloodstream. At E14.5 blood circulation is functional such that tissues have access to blood-borne metabolites. Also note that ISH data represents mRNAs of CBEs which are predictive of expression of the cognate proteins. However, it is widely appreciated that levels of mRNA and of protein activity are not always proportional. The data shown in the color chart can readily be confirmed by a visual inspection of the comprehensive ISH primary data on www.metscout.mpg.de or www.genepaint.org.

The distribution of expression strength of CBE mRNAs across signature tissues resembles that of “stereotypical energy metabolism pattern” (SEMP) that characterizes glycolytic and TCA cycle
components (18). For example, expression of *Hmgcr* encoding the rate-limiting 3-hydroxy-3-methylglutaryl-CoA reductase, varies considerably across the signature tissues (Fig. 1; Fig. 2a). There is one marked difference with SEMP: *CBEs* are mostly not detectable in the heart (Fig. 1 and 2a), except for *Fdps* and *Hmgcs2*, whose transcripts are found in the valves of the developing heart and the developing blood vessels (*Hmgcs2* only) (Fig. 2b-d). Elevated but variable expression of *CBEs* is seen in the mesencephalon, the pons and the medulla, the dorsal root ganglia, the cranial ganglia, in the liver, in the adrenal and in the kidney (Fig. 1 and 2a).

Most CBEs are strongly expressed in the developing cerebral cortex (Fig. 3). The innermost cortical layer, at this developmental stage referred to as the ventricular zone, contains the cell bodies of mitotic cells that express the mitotic marker *Mki67* (Fig. 3, top left) whereas the intermediate and marginal zones are mostly populated by postmitotic neuronal progenitors. The intermediate zone expresses very low levels of *CBEs* while the two other zones express *CBEs*. Additionally, most *CBEs* are strongly expressed in a semi crescent-shaped domain extending along the outer boundary of the olfactory bulb (Fig. 3). Apparently proliferating neuronal progenitors in the ventricular zone require cholesterol for making more cell membrane in the process of spinning off daughter cells. The need for cholesterol in postmitotic neurons of the marginal zones in the cortex and the olfactory bulb could reflect cell growth and in particular the formation neuronal networks that begin to emerge in these regions. Establishment of these networks depends on cholesterol to as there is a substantial increase of the cell surface and synaptic connections are formed (19).

The embryonic retina contains both, proliferating neuronal precursors (outer neuroblastic layer, expressing the proliferation marker *Mki67*; Fig. 4, top left) and postmitotic, growing neurons (inner neuroblastic layer). Interestingly, most mRNAs encoding CBEs are detected in the inner neuroblastic layer, i.e. in postmitotic cells that undergo differentiation, which is, as noted for the marginal zone of the cortex, accompanied by a marked increase in the membrane surface due to the formation of axons of the optic tract plus the formation of synaptic connections.
Apart from its contribution to the formation of cellular membranes, cholesterol might be required by mitotic cells of the developing cortex in lipid raft microdomains that contain transmembrane signaling proteins such as growth factor receptors. For example, FGFR1 and -2 receptors are expressed in the ventricular zone (Genpaint set ID EH130 and FG35) and FGFR2 is present in rafts (20).

**CBEs in the adrenal glands**

The components of the CBS pathway are expressed in the adrenal gland, even at embryonic stage E14.5 (Figs. 1, 5). In this tissue cholesterol serves as a precursor of for adrenal steroid biosynthesis (Supplemental Fig. S II). The six catalytic activities required for the synthesis of glucocorticoids, mineralocorticoids and the sex hormone precursor androstenedione are all expressed in the developing adrenal. Of the five Hsd3b isozymes (1, 2, 4, 5 and 6) Hsd3b1,-5 and -6 are expressed. Expression patterns of Cyps and Hsb3s are nearly uniform except for Cyp17a1 that is predominantly found along the margin of the adrenal and is required for androstenedione and cortisol synthesis. Therefore, already during development a medulla/cortex regionalization in steroid synthesis can be seen.

**Cholesterol biosynthesis along midline structures**

Cholesterol is conjugated to Hedgehog proteins (including Sonic hedgehog) which raises the question about the relative relationship of the expression domains of Shh and the CEB mRNAs. In contrast to CEBs the Shh gene is expressed in a highly restricted manner often in signaling regions that control pattern formation and morphogenesis in the surrounding tissues. The expression pattern of Shh in the developing brain is highly dynamic (21). A sagittal section through the midline of an E14.5 embryo reveals a prominent expression domain of Shh in the shape of a narrow band found on the floor of the midbrain and extending into the hypothalamic region of the forebrain (Genpaint Set ID MH459, midline sagittal section). As judged from midline sagittal sections through E14.5 embryos subjected to ISH with CBE probes, the majority of CBEs replicate this pattern. For the majority of CBEs elevated expression is detectable along the mesencephalic and telencephalic midline with decreasing strength in the more lateral neuronal tissue (see e.g. the SLOS-causative gene Dhcr7, GenePaint Set ID MH2386). To fully capture...
the overlap between the expression domains for of CBEs and Shh, cross sections through the floor of the hypothalamic and mesencephalic regions of an E13.5 embryo were prepared. Extending previous work (21), hybridization with a Shh probe revealed a V-shaped expression domain of Shh that coincides with the ventricular zone (Fig. 6a, right). Hmgcr, the rate-limiting regulatory enzyme of the pathway is broadly expressed in the wall of the hypothalamus but the strength of expression is markedly upregulated in the ventricular zone (Fig. 6a, left). Taken together, there is, within the ventricular zone, a substantial but not complete overlap of expression of Shh and Hmgcr. The transverse sections through the floor region of the mesencephalon (Fig. 6b) shows restricted expression of Shh in a ventralmost region and widespread expression of Hmgcr throughout the nervous tissue peaking in cells adjacent to the expression domain of Shh. Therefore, the data shown in Fig. 6b emphasize that there is but a limited overlap between the two expression domains. Fig. 6c illustrates the expression domains of Shh and Hmgcr in the primordium of the fascial vibrissae. The sickle-shaped expression domain of Shh is substantially overlapping with that of Hmgcr. It is obvious that for forming a covalent adduct, SHH and cholesterol have to colocalize and at least as judged on the localization of Shh and Hmgcr transcripts, this is not always the case. In the case shown in Fig. 6a coexpression is limited to a specific region which could potentially serve as a very restricted and hence precisely defined source of cholesteryl-Shh. In the situations exemplified in Fig. 6b there is virtually no overlap between the expression domains but exogenous cholesterol present in cerebrospinal fluid filling the ventricles, could act as a cholesterol source. This exogenous cholesterol could also be of maternal origin.

Discussion

Although open access ISH data have previously been used to characterize cholesterol homeostasis in the mouse hippocampus (22), the present study is the first one to provide a comprehensive view of the spatial organization of cholesterol biosynthesis for a whole organism. From the wealth of data present in the CBE expression atlas three aspects were selected that shed light on and are relevant for cholesterol’s
physiological roles: (i) in the biogenesis and stability of membranes, (ii) as precursor for adrenal steroids and (iii) as essential covalent modifier of Hedgehog proteins.

The expression patterns of CBEs strongly resemble that of enzymes mediating cellular energy metabolism that constitute Krebs cycle and glycolysis. These enzymes, like the CBEs, provide building blocks for cellular structures. In the case of the rapidly growing embryo such building blocks are constantly and ubiquitously required. Nonetheless, neither TCA, nor glycolytic and CBEs are uniformly expressed but there are substantial differences in expression levels between and within tissues. Emblematically, CBE expression levels in developing neocortex and retina were examined. Both tissues are characterized by the presence of proliferating and postmitotic, differentiating neurons. We found that highest levels of CBE expression in the retina is found in the inner neuroblastic layer, devoid of any substantial cell proliferation but characterized by neuronal differentiation tied to growing neurites that eventually have to reach distant targets in the brain. In addition, the developing retina is characterized by the formation of synaptic connections. Both axon genesis and synapse formation require the formation of new membrane bilayers. In the neocortex, highest CBE levels are seen in the ventricular zone in which cells divide at high rates and therefore membrane synthesis is critical in the ventricular zone. Additionally, neuronal progenitors of the ventricular zone receive growth factor signals from the cerebrospinal fluid-filled ventricle. These factors bind to their cognate transmembrane receptors some of which are organized in cholesterol-containing lipid rafts.

It is interesting to compare the CBE expression data with measurements of total sterols in the mouse embryo (23). Based on a comparative analyses of Dhc7 knock out (a SLOS model) and wild type mice, it was concluded that in the brain, cholesterol is chiefly of maternal origin until about E10-11. In liver, and lung, the dam provides cholesterol until about E12-14. These data are in strong agreement with our ISH data that definitely show the presence of expression of CBEs by E14.5.
The present study also clarifies the spatial relationship of the localities of cholesterol biosynthesis and of Shh expression. As a proxy for the former, the expression of Hmgcr was used, since this gene encodes a key regulatory enzyme of cholesterol biosynthesis. The most important finding in this part of the study is that Shh and Hmgcr are overlapping but not always and not completely; we show cases in which expression domains of the two are adjacent. In this situation one would hypothesize that cholesterol would have to be taken up by Shh-expressing cells either from neighboring tissues that synthesize this compound or from cerebral spinal fluid that fills the space of the brain ventricles.

Because the expression patterns of CBEs seamlessly fit into the data deposited on Genpaint and Eurexpress databases, the CBE data can readily be integrated into that body of transcriptome-scale data. We illustrate this point by combining expression data for CBEs with those of the enzymes that convert cholesterol to adrenal steroids. This way the complete pathway starting from acetyl-CoA could be reconstructed showing that even embryonic adrenals are equipped with all the components required for the synthesis of all adrenal steroids.

Many of the CBE encoding genes are characterized by sterol regulatory elements (SREs) which are binding sites for Sterol Regulatory Element-Binding Proteins 1 and 2 (8). The transcripts (Srebp1 and Srebp2) for both proteins show expression patterns reminiscent of those of the CBE genes. Strong expression of Srebp1 is seen in the ventricular zone of the forebrain, in liver and in the Shh-positive midline of the midbrain (Genpaint set ID MH946). Srebp2 transcripts are also found in the ventricular zone and in midbrain, but to a lesser extent in liver. Furthermore, Srebp 2 but not Srebp1 is strongly expressed in the peripheral nervous system. It appears that CBE regulators Srebp1 and Srebp2 are characterized by both, coexpression in some tissues and complementary expression in others.

Human congenital disorders tied to cholesterol metabolism and the corresponding mouse models have strong embryonic phenotypes (for a review see 2). At least to some extent, knowing all sites of expression of CBEs can rationalize developmental abnormalities and hence guide further research. Thus
the digital compendium of \textit{CBE} expression patterns in conjunction with expression data for genes up- and downstream of cholesterol biosynthesis provides a most useful resource for the interpretation of biological, genetic data and disease data.
Acknowledgements

We thank Ana Martinez Hernandez, Maren Brockmeyer, Sarah Dettmer, Sarah Dunker, Frauke Grabbe, Barbara Negelen, Dirk Reuter, Markus Uhr and Christine Kauck for technical assistance. This work was funded by grants from the Scientific and Technological Research Council of Turkey (Project no: TBAG-2223, 106T175), T. R. Prime Ministry State Planning Organization (Project no: 2006/34) and the Max Planck Society.
References


Figure legends

**Figure 1. Summary diagram of CBE expression patterns in 25 signature tissues.** For each tissue the expression level (strong, medium, weak, and not detected) of each transcript of the 25 cholesterol biosynthetic enzymes (CBEs) is shown in shades of caramel. The GenePaint set ID in the rightmost column identifies the ISH reference data set which can be fully viewed at [www.genepaint.org](http://www.genepaint.org) or [www.metscout.mpg.de](http://www.metscout.mpg.de).

**Figure 2. CBEs exhibit a stereotypical energy metabolism pattern (SEMP) in the E14.5 mouse embryo.** (a) Hmgcr expression is emblematic for an SEMP, except for the absence of expression in the heart. (b, c) Fdps and Hmgcs2 are the only CBEs detected in the developing heart. (d) In contrast to other CBEs, transcripts of Hmgcs2 localize predominantly to blood vessels passing through the brain parenchyma, meninges, and choroid plexus. **Abbreviations:** ag, adrenal gland; ac, axial cartilage; bv, blood vessels; cb, cerebellum; cp, choroid plexus; cx, neocortex; drg, dorsal root ganglion; h, heart; inbl, inner neuroblastic layer; iz, intermediate zone; ki, kidney; li, liver; lu, lung; m, medulla oblongata; mes, mesencephalon; mz, marginal zone; ob, olfactory bulb; oe, olfactory epithelium; onbl, outer neuroblastic layer; pc, pancreas; pn, pons; sc, spinal cord; si, small intestine; st, stomach; vz, ventricular zone; vf, vibrissae follicles.

**Figure 3. Expression of CBEs in the developing forebrain.** In all cases CBE transcripts are found in the ventricular zone that houses proliferating neuronal progenitor cells (Mki67 ISH image top left) but frequently also in regions with postmitotic cells such as the marginal zone and a crescent-shaped domain in the olfactory bulb anlage. For abbreviations see legend of Fig. 2.

**Figure 4. Expression of CBEs in the developing eye.** Transcripts of all CBE genes are detected in the inner neuroblastic layer which contains postmitotic neuronal progenitors that do not express the proliferation marker Mki67 (see ISH picture top left). For abbreviations see legend of Fig. 2.
Figure 5. Expression of CBEs in the developing adrenal gland. All components of the CBS pathway are represented by at least one expressed isoenzyme in the developing adrenal gland. Expression of CBEs not necessarily overlaps with proliferating cells (Mki67 ISH image top left) but is in strong agreement with the expression patterns observed for steroid hormone synthesis (bottom rows separated by a dashed line). For abbreviations see legend of Fig. 2.

Figure 6. Expression domains of Hmgcr and Shh in the E13.5 mouse embryo. (a) The ventricular zone of the hypothalamic and (b) the mesencephalic area and (c) the vibrissae follicles of the snout illustrate in contralateral (a, b) and ipsilateral (c) adjacent coronal sections that the expression domains of Hmgcr and Shh show substantial but not complete overlap. For abbreviations see legend of Fig. 2.
Figures

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