MOUSE KNOCKOUT OF THE CHOLESTEROGENIC CYTOCHROME P450 LANOSTEROL 14α-DEMETHYLASE (Cyp51) RESEMBLES ANTLEY-BIXLER SYNDROME

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Antley-Bixler syndrome (ABS) represents a group of heterogeneous disorders characterized by skeletal, cardiac and urogenital abnormalities that have frequently been associated with mutations in fibroblast growth factor receptor 2 or cytochrome P450 reductase genes. In some ABS patients, reduced activity of the cholesterogenic cytochrome P450 CYP51A1, an ortholog of the mouse CYP51, and accumulation of lanosterol and 24,25-dihydrolanosterol has been reported, but the role of CYP51A1 in the ABS etiology has remained obscure. To test whether Cyp51 could be involved in generating an ABS-like phenotype, a mouse knockout model was developed that exhibited several prenatal ABS-like features leading to lethality at embryonic day 15. Cyp51−/− mice had no functional Cyp51 mRNA and no immunodetectable CYP51 protein. The two CYP51 enzyme substrates (lanosterol and 24,25-dihydrolanosterol) were markedly accumulated. Cholesterol precursors downstream of the CYP51 enzymatic step were not detected, indicating that the targeting in this study blocked de novo cholesterol synthesis. This was reflected in the up-regulation of 10 cholesterol synthesis genes, with the exception of 7-dehydrocholesterol reductase. Lethality was ascribed to heart failure due to hypoplasia, ventricle septum, epicardial and vasculogenesis defects, suggesting that Cyp51 deficiency was involved in heart development and coronary vessel formation. As the most likely downstream molecular mechanisms, alterations were identified in the sonic hedgehog and retinoic acid signaling pathways. Cyp51 knockout mice provide evidence that Cyp51 is essential for embryogenesis and present a potential animal model for studying ABS syndrome in humans.

Cholesterol is a structural component of membranes, as well as a precursor for bile acid and steroid hormone synthesis (1). It plays an essential role in development as it is required to bind and activate the morphogenic sonic hedgehog (SHH) (2,3) and retinoic acid (RA) (4) signaling pathways during early gestation. A relatively high number of human birth defects have been shown to be disorders of metabolism caused by mutations of enzymes mediating cholesterol biosynthesis (reviewed in (5,6)). ABS (7) represents a heterogeneous pool of disorders in steroid and steroid metabolism. Clinical features include brachycephaly, facial hypoplasia, bowed ulna or femur, synostosis of the radius, camptodactyly, and cardiac and urogenital abnormalities. ABS is a genetically heterogeneous disorder for which mutations in two causal genes have been identified thus far. Recessive mutations in the cytochrome P450 reductase (POR) gene (8-10) are frequently found in an ABS form that includes disordered steriodogenesis (ABS1; MIM 201750). A predominantly skeletal phenotype without genital anomalies or disordered steriodogenesis can be caused by a dominant mutation in a fibroblast growth factor receptor 2 gene, FGFR2 (ABS2; MIM 207410). However, ABS-like phenotypes have also been reported in response to fluconazole, an inhibitor of fungal CYP51 used to treat fungal infections. High-dose fluconazole exposure during early pregnancy resulted in inhibition of the CYP51A1 gene product and ABS-like defects in four reported cases (11-13). Two ABS case studies reported impaired CYP51 activity and markedly increased levels of lanosterol and 24,25-dihydrolanosterol, substrates of CYP51 (14). However, a direct causative CYP51–ABS relationship has not yet been proven in any of the aforementioned cases.
Lanosterol 14α-demethylase (in humans CYP51A1; in mouse, CYP51) is the most evolutionarily conserved member of the cytochrome P450 superfamily (15) and is present in all phyla. It has been studied in many species, including human (16), mouse (17), and fungi where it represents a target for azoles (18). The enzyme catalyzes demethylation of lanosterol or 24,25-dihydrolanosterol in the cholesterol synthesis pathway using POR as an obligatory redox partner (16). To test whether Cyp51 deficiency can cause an ABS-like phenotype, and to assess the phenotypic effects of CYP51 ablation, we generated a Cyp51<sup>−/−</sup> mouse knockout model. Cyp51 knockout mice show ABS-like features with skeletal and cardiac malformations leading to embryonic lethality in the late-midgestation period. As the most likely downstream molecular mechanisms, we identified alterations in the SHH and RA signaling pathways.

**Experimental Procedures**

**Generation of Cyp51<sup>−/−</sup> knockout mice-** The Cyp51 homology arm fragments for the targeting construct were isolated from the bacterial artificial chromosome (BAC) clone 519E21 that was identified in the mouse BAC library prepared from 129/Sv genomic DNA. The targeting construct also contained a thymidine kinase (TK) negative selection cassette, a phosphoglycerate kinase-neomycin (PGK-neo) positive selection cassette flanked by FRT sites (FRT-neo-FRT) inserted in intron 3, and loxP sites together with a new HindIII restriction site in introns 2 and 4 (Fig. 1A). The linearized construct was electroporated into ES cells isolated from mouse strain 129 (19). After double selection with G418 and ganciclovir, targeted clones carrying the Cyp51<sup>lox-FRT-neo</sup> allele were identified using PCR in the 3loxP, 5loxP and neo region as shown in Fig. 1A (primers used for genotyping: 3loxF, 5'-AACATAGCCCCATTTAAGCA-3'; 3loxR, 5'-TTCCGACCTACTGATTTT-3'; 5loxF, 5'-CAGACCTTGATGGAAGAGAT-3'; 5loxR, 5'-TTAGCTTTATGCTCGGGTCTC-3'; 3NeoF, 5'-AGGATTGGGAAAGAATAGC-3'; 3NeoR, 5'-CTAAAGGCGATGCCTCAG-3'; 5NeoF, 5'-CTGATCTTCTGTTGTGGA-3'; 5NeoR, 5'-CTAAAGGCGATGCCTCAG-3'). Homologous recombination was confirmed by Southern blot analysis (Fig. 1B) on HindIII and BamHI - digested ES clone genomic DNA using probes located outside the 5' and 3' homology arms. Probes were labeled with <sup>32</sup>P-dCTP in a random priming reaction using the Rediprime II DNA labeling system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The PGK-neo cassette was excised from the Cyp51<sup>lox-FRT-neo</sup> ES clones by electroporating a eukaryotic expression vector encoding the FLP recombinase to generate a conditional Cyp51<sup>lox</sup> allele. Correct excision of the selection cassette in ES cells was confirmed by PCR (using the 5Neo and 3Neo primer pairs) and sequencing across all important elements. Mouse chimeras were generated by injecting two independently-targeted Cyp51<sup>lox</sup> ES clones into C57BL/6JOlaHsd (C57BL/6; Harlan, Italy) mouse blastocysts. Heterozygous Cyp51<sup>lox/lox</sup> offspring were backcrossed to C57BL/6 for at least four generations to generate the transgenic line B6;129Sv<sup>Cyp51tm1Bfro</sup>. The Cyp51<sup>lox</sup> allele was detected in segregating progeny by PCR from ear clips using the 3loxF and 3loxR primers (Fig. 1C). The knockout mouse line B6;129Sv<sup>Cyp51tm1Bfro</sup> carrying the Cyp51<sup>−/−</sup> allele lacking exon 3 and 4 was generated by crossing homozygous Cyp51<sup>lox/lox</sup> females with transgenic males carrying EIIa-Cre (strain B6.FVB-Tg[EIIa-Cre]C5379Lmgd/J, Jackson Laboratories, Maine, USA). To obtain knockout embryos, F<sub>1</sub> Cyp51<sup>−/−</sup> mice were set up for mating at the end of the light cycle period. The day a vaginal plug was detected was designated embryonic day 0.5 (E0.5). Pregnant females at E9.5, E10.5, E11.5, E12.5, E13.5, and E14.5 were sacrificed with cervical dislocation, and embryos were isolated in PBS. After photographing, leg tissue of the embryos was dissected away for PCR genotyping (Fig. 1D & E), and the rest of the embryos were processed for paraffin embedding using standard protocols. Subsequent sections (5 µm) were used for hematoxylin and eosin staining and histology analysis. Alcian staining of E14.5–E15.0 embryos was performed as previously described (20). The Chi-square test was used to compare the observed frequencies in genotypic classes with the expected 1:2:1 Mendelian ratio. Some animal experiments were performed in accordance with institutional guidelines and were approved by the Veterinary Administration of the Republic of Slovenia. Immunohistochemistry and in situ hybridization-Immunohistological studies using paraffin sections of staged mouse embryos were performed according to our previously published protocols (21) using a goat polyclonal anti-
platelet/endothelial cell adhesion molecule-1 (PECAM-1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse monoclonal anti-Connexin-43 (Transduction Laboratories, Lexington, KY, USA). BrdU incorporation as a measure of cell proliferation was detected as described (22). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) analysis was performed as described (23). For in situ hybridization experiments, the GenPoint™ Amplified Detection System (Dako, Carpinteria, CA, USA) was employed using a previously described protocol (24). All plasmids for SHH pathway in situ hybridization probes were kindly provided by G. Fishell.

**Measurements of cholesterol synthesis intermediates**- Quantitative analysis of nine cholesterol precursors and sterols was performed on F2 embryos of all three genotypes at E12.5 and E14.5 using a gas chromatographic/mass spectrometric method exactly as described (25).

**Western blot analysis**- Frozen E14.5 embryos were pulverized and homogenized with a Polytron homogenizer in lysis buffer (5 mM Tris pH 7.4, 2 mM EDTA, protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany)). The homogenate was centrifuged 15 min at 500 g and 4 °C to remove cell debris. The supernatant was collected and centrifuged 15 min at 45,000 g and 4 °C to remove cell debris. The pellet was resuspended in resuspension buffer (75 mM Tris, 4 °C. The pelleted membrane fraction was separated by 14% SDS-PAGE for 60 min at 60 V, and transferred onto a PVDF membrane (Millipore, Eschborn, Germany) for 2 h at 200 mA (Bio-Rad Laboratories, Hercules, CA, USA). After blocking overnight with 5% (w/v) nonfat dry milk in PBST containing 0.05% (v/v) Tween-20, the membrane was incubated for 2 h with rabbit polyclonal anti-CYP51 antibodies (1:500) and anti β-actin antibodies (1:200, Sigma, Steinheim, Germany). The anti-CYP51 antibody against the CYP51 peptide QRLKDWSAERLDFNPDRY recognizes all mammalian CYP51 proteins (D. Rozman et al., unpublished). After three washes for 10 min each in PBST, the membrane was incubated for 2 h with goat anti-rabbit IgG peroxidase, diluted 1:2000 with 5% nonfat dry milk in PBST. The membrane was visualized using the enhanced chemiluminescence detection system (Thermo scientific, Rockfor, IL, USA) and detected with a camera (Fujifilm LAS-400, Valhalla, NY, USA).

**Quantitative RT-PCR**- Total RNA was extracted from embryos or embryo hearts using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (2.5 or 1 (in hearts) µg) was treated with amplification grade DNase I, and reverse transcribed with the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was performed using a LightCycler 480 detection system using SYBR Green I Master, Probes Master for SYBR green, or TaqMan chemistry according to the manufacturer's instructions (Roche). Primers were designed using the Primer Express 1.5 software and validated for PCR efficiency. PCR was performed with the following PCR parameters: 95 °C for 10 min, then 95 °C for 10 s, 60 °C for 30 s, 72 °C for 5 s for 45 cycles plus a dissociation step (60–95 °C). The relative expression ratios were calculated as described (26). Actin beta (Actb), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), hypoxanthine guanine phosphoribosyl transferase (Hprt1) or Actb, ribosomal protein, large, P0 (Rplp0), UTP6, small subunit processome component, homolog (yeast) (Utp6c) were selected as described (27) and used as internal reference genes in embryo hearts and whole embryos, respectively. RNAs for three different mouse Cyp51 exons were also quantified using TaqMan® Assay-on-Demand gene expression assays and a β-actin TaqMan® Pre-Developed Assay (Applied Biosystems, Foster Cit, CA, USA) were used as internal controls. Relative expression was calculated with the standard ΔΔCt method and analyzed with GraphPad software. The list of primers and pre-developed assays used in quantitative RT-PCR is provided in Supplemental Tables 2 and 3.

**RESULTS**

**Generation of Cyp51 knockout mice.** We generated a conditional knockout Cyp51<sup>tm1Bfro</sup> allele of the mouse Cyp51 gene, referred to here as the Cyp51<sup>flox</sup> allele (MGI: 4357830), by placing two loxP sites in intron 2 and 4 of Cyp51 (Fig. 1). A deletion of exon 3 and 4 should shift the reading frame in the Cyp51 transcript generating a premature stop codon and truncated CYP51 protein. There is no evidence for...
alternative splicing of Cyp51 or multiple CYP51 proteins in mice or other mammals, though an additional transcript in the testis arising from alternative polyA signal selection does exist in several mammalian species. Our knockout strategy was designed to create null alleles for both the somatic and the shorter testis-specific transcript, since both Cyp51 polyA signals are located downstream from the chosen recombination event in exons 3 and 4. Germline founders carrying the Cyp51\textsuperscript{flx} allele were backcrossed to C57BL/6JOLA Hsd mice for at least four generations and then to EIIa-Cre deleter line mice to generate knockout Cyp51\textsuperscript{m1.1Bfro} allele mice, referred to here as the Cyp51\textsuperscript{-/-} allele (MGI:4836362). Heterozygous Cyp51\textsuperscript{+/-} mice were intercrossed to obtain homozygous Cyp51\textsuperscript{-/-} knockout embryos.

To test the pattern of RNA transcription from the Cyp51 knockout allele in various parts of the Cyp51 locus, we performed a quantitative RT-PCR (qRTPCR) analysis with probes covering exons 1–2, 3–4, and 8–9 in embryos at E12.5 and E14.5 (Fig. 2A). Cyp51\textsuperscript{-/-} embryos did not express exons 3 and 4, as expected, given that this segment should have been deleted. From the knockout allele, there was approximately 30\% transcription of non-coding exons 1 and 2, and 20\% of exons 8 and 9 compared to the 100\% wild-type allele transcription level. However, as deletion of exons 3 and 4 generates a premature stop codon, any remnant read-through transcription would generate a truncated transcript, as well as a protein devoid of the cysteine-binding site in exon 10 that is crucial for CYP51 activity. Our Western blot analysis (Fig. 2B) supports the above Cyp51 mRNA analysis and demonstrates that there is no immuno-detectable CYP51 protein, even in the truncated form, in Cyp51\textsuperscript{-/-} embryos. Therefore, our assays at the DNA, RNA, and protein levels demonstrate that gene targeting was successful in generating a complete loss of function Cyp51 allele.

Quantification of mRNA and cholesterol precursors from the cholesterol biosynthesis pathway of F\textsubscript{2} embryos. We quantified mRNA expression of 11 genes from the cholesterol biosynthesis pathway and nine intermediates from lanosterol to cholesterol. A deletion of one functional Cyp51 allele in embryos at E14.5 resulted in statistically significant decrease of Cyp51 mRNA while other cholesterogenic genes did not differ significantly between heterozygotes and wild-type (Fig. 3A). The sterol measurements in embryos at the same stage corroborate the above mRNA analyses in heterozygous embryos (Fig. 3B). Interestingly, in Cyp51\textsuperscript{-/-} embryos, where both Cyp51 alleles were nonfunctional, expression of all cholesterogenic genes except 7-dehydrocholesterol reductase (Dhcr7) was significantly increased. The major accumulating sterol in Cyp51\textsuperscript{-/-} embryos was confirmed to be 24,25-dihydrolanosterol. Cholesterol synthesis intermediates downstream of the CYP51 reaction step were not detected in Cyp51\textsuperscript{-/-} embryos (Fig. 3B) demonstrating that a complete block in the cholesterol synthesis pathway occurred in Cyp51\textsuperscript{-/-} knockouts. These embryos nevertheless contained measurable amounts of the final product cholesterol, though significantly less than wild type and heterozygotes. RNA expression analyses and sterol measurements showed similar trends at stage E12.5 (data not shown).

Cyp51 knockout causes an ABS-like phenotype and results in midgestation lethality. No viable Cyp51\textsuperscript{-/-} mice were identified among 185 weaned progeny derived from heterozygous matings. The ratio of Cyp51\textsuperscript{+/-} and Cyp51\textsuperscript{-/-} adult mice did not deviate significantly from the expected 1:2 Mendelian ratio indicating that lethality did not occur among heterozygotes. To determine the developmental stage of lethality, embryos were genotyped at seven different embryonic stages from E9.5 to E15.5 (Table I). The results presented in Table I were pooled for two lines derived from two independently-targeted embryonic stem cell clones, as both lines gave identical outcomes (Supplemental Table S1). Genotype ratios from E9.5 to E14.5 were in accordance with the expected F\textsubscript{2} Mendelian ratios. At stage E15.5, however, all Cyp51\textsuperscript{-/-} embryos were in an advanced stage of resorption. Examples of embryos in earlier stages of resorption from different developmental points are presented in Supplemental Fig. S1. As the majority of Cyp51\textsuperscript{-/-} embryos at E13.5 and E14.5 (37 out of 41) still had a beating heart, while none could be noted at E15.5, we conclude that lethality of Cyp51\textsuperscript{-/-} embryos occurred at E15. Loss of Cyp51 function resulted in several developmental malformations. Knockout embryos were first distinguished at E10.5 by kinked, shortened tails (arrow in Fig. 4A) and embryos displayed an increased nuchal translucency most clearly distinguished at E14.5. Severe cases of nuchal translucency were accompanied by hematomas in the dorsolateral and pericardial regions (Fig. 4B). Transverse
Histological sections of nuchal regions demonstrated a highly expanded jugular lymph sac, up to 20-times larger compared to wild-type structures (Fig. 5A). Close examination of endothelial cells lining the lymph sacs showed no obvious changes in endothelial cell morphology, but an increase in the number of surrounding smooth muscle cells (Fig. 5B). Skeletal abnormalities assessed at E14.5 exhibited shortened fore and hind limbs in all examined specimens (micromelia). The radius and tibia were bowed and synostosis of the femur and tibia was observed (Fig. 4D). Tarsal and carpal elements were underdeveloped and distal phalanges were absent. Compared to the forelimbs, the abnormalities in hind limbs were more pronounced and variable in severity between Cyp51\(-/-\) embryos (Fig. 4C). Forelimbs displayed digital joint contractures (campodactyly) resulting in soft tissue syndactyly of second and third finger. A differential phenotype between the left and right hind limbs appeared in approximately half of embryos. The third distal tarsal element was absent from both hind limbs resulting in syndactyly of the second and third metatarsal bones on the left foot and the second, third and fourth metatarsal bones on the right foot. Bone syndactyly of the second and third right metatarsus was complete, while the fourth metatarsus was joined only proximally. Proximal hind limb phalanges bifurcated and formed additional fingers (Fig. 4E). Histological examination of embryos from different stages revealed an absence of the palatal shelf cartilage resulting in cleft palate (Fig. 4F). Knockout embryos also exhibited some craniofacial abnormalities, including brachycephaly, micrognathia (shortened mandible), and shortened or absent tongue (Fig. 4F & G).

*Cyp51* knockout lethality occurs most likely due to heart failure. Macroscopically visible pericardial bleeding was the first sign of heart failure in E14.5 knockout embryos (Fig. 5C). In addition, *Cyp51\(-/-\)* embryos exhibited increased nuchal translucency that has been frequently associated with congenital heart defects or underdevelopment of the lymphatic system resulting in over-distension of the jugular lymphatic sacs in humans (reviewed by (28)). In agreement with the human situation, we observed a dramatic jugular lymph sac extension in the knockout embryos (Fig. 5A). Whole mount analysis of the hearts at E14.5 revealed smaller hypoplastic hearts with dilation of the atria, a very prominent interventricular groove, which might result from abnormal development of the interventricular septum, but normal structure and positioning of the great arteries (Fig. 5C). Histological analysis of embryos at E12.5, E13.5 and E14.5 confirmed hypoplastic development of ventricles and showed enlarged endocardial cushion tissues in the knockout embryos compared to wild-type littermate embryos (Fig. 5D). In serial transversal sections, we noted ventricle septum defects in the knockout embryos at E14.5, which were further confirmed under high-power magnification by the presence of blood cells in the communicating opening between ventricles (arrow in Fig. 5E). Examination under high-power magnification supported not only the hypoplastic myocardium at E14.5 (arrows in Figure 5F), but also revealed epicardial defects between E12.5 and E14.5 with a partial lack of cells in the epicardial layer.

To investigate the potential underlying causes for the hypoplastic heart development, we analyzed proliferation and apoptosis. Proliferation was reduced in knockout hearts at E12.5 (Fig. 6A), which became even more pronounced at E14.5 (Fig. 6B), whereas apoptosis was not different between wild-type and knockout hearts at the investigated time points (data not shown). As histological analysis revealed abnormalities in the myocardium and epicardium, qRT-PCR analyses of several genes involved in epicardial-myocardial signaling (29) were performed. We found significantly decreased expression of retinoic X receptors (*Rxra*) and retinoic acid receptors (*Rara*) in knockout hearts at E12.5 and E14.5 and of NK2 transcription factor related, locus 5 (Drosophila) (*Nkx2-5*) at E14.5. No significant change in expression levels was noted for forkhead box C1 (*Foxc1*), Wilms tumor 1 homolog (*Wt1*), vascular endothelial growth factor A (*Vegfa*) or nuclear receptor subfamily 1, group H, member 2 (*Nr1h2*) (Fig. 6E). In addition, lower expression of connexin 43 (*Gja1*) in the remaining epicardial cells of the knockout hearts at E14.5 was demonstrated by qRT-PCR and confirmed with immunostaining (Fig. 6C). We additionally investigated expression of the SHH pathway members by high sensitivity in situ hybridization. *Shh* and *slit* homolog 1 (*Drosophila Slit1*) were down-regulated in the knockout hearts, whereas GLI-Kruppel family member GLI1 (*Gli1*) was up-regulated. Distal-less homeobox 2 (*Dlx2*) and patched homolog 1 (*Ptc1*) showed no obvious differences.
(Supplemental Fig S2). As Shh, RA, and connexin 43 (Gja1) are all critically involved in epicardial-myocardial signaling directing coronary vasculogenesis (for review see (29)), we hypothesized that in addition to the cardiac abnormalities mentioned above, the Cyp51 deficiency might be involved in coronary vessel formation. In support of that hypothesis, immunostaining for PECAM-1 at E12.5 revealed a significantly lower number of proliferating cardiac vessels, a difference which was even more pronounced at E14.5 (Fig. 6D).

DISCUSSION

We successfully developed a global knockout mouse model of the Cyp51 gene to gain insight into the phenotypes connected to human malformations of the ABS-like phenotype, and to further understand the importance of de novo cholesterol synthesis during embryogenesis. In Cyp51 knockout embryos, Cyp51 mRNA was absent or significantly reduced; however, mRNA of 10 genes catalyzing various steps in the cholesterol pathway was significantly increased (Fig. 3A). This observation is in line with predictions that deficiencies in cholesterol or intermediates up-regulates the entire pathway in knockout embryos to compensate for the lack of de novo cholesterol synthesis, which has been observed previously in other knockouts of late cholesterol synthesis such as NAD(P)-dependent steroid dehydrogenase-like knockout mice (Nsdhl-/-) (30). Explanation of possible mechanisms could be drawn from early literature studying effects of CYP51 inhibitors administration. Chronic treatment with the inhibitor ketoconazole, an inhibitor of the fungal CYP51, which also inhibits the human CYP51A1 (18), mimics our Cyp51 knockout state. Under such conditions, enhanced hepatic 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) activity was reported (31). Similarly, chronic administration of ketoconazole to HepG2 cells gave rise to increased HMGR activity (32). Enhanced HMGR activity may have been due to biological action of endogenously-produced oxysterols operating via the sterol regulatory element binding transcription factor 2 (SREBF2) or the nuclear receptor subfamily 1, group H, member 3 (NR1H3) (33). The balance of natural oxysterol regulators is chronically disrupted in Cyp51 knockout embryos, which could cause the increase in expression of Hmgcr and other cholesterogenic genes, as observed in our case. An exception was Dhcr7, whose expression in knockout embryos was not significantly different from the wild-type or heterozygous embryos. This was not expected since in the adult tissues and cells, transcriptional regulation of Dhcr7 is in line with the cholesterol feedback loop and transcription factors of the SREBP family. Dhcr7 mRNA is inducible under sterol starvation or when treated with cholesterol synthesis inhibitors in the liver (34) and the brain (35). Proteomic studies confirmed the increased expression of cholesterol synthesis enzymes in brains of the Dhcr7 null mice (36). However, data from pig embryos identified Dhcr7 as one of the imprinted, maternally-expressed genes (37). It remains to be elucidated whether Dhcr7 is also imprinted in mouse and whether this is the reason for the unchanged expression of Dhcr7 in Cyp51-/- embryos. In heterozygous embryos, the Cyp51 mRNA level was decreased by half when compared to wild-type littermates, whereas for other cholesterogenic genes no significant differences between heterozygotes and wild-types could be detected (Fig. 3A). Sterol analyses of the Cyp51 knockout embryos show a block of cholesterol synthesis at the CYP51 enzymatic step. The two substrates of CYP51, lanosterol and 24,25-dihydrolanosterol, that are present in minute amounts in wild-type embryos, represent the only sterol intermediates that are detectable in the Cyp51 knockout embryos (Fig. 3B). Downstream from lanosterol and 24,25-dihydrolanosterol, no cholesterol intermediates were detected, suggesting that the measured cholesterol in knockout embryos could be of maternal origin unless some as yet unknown alternative cholesterol biosynthesis pathway exists.

A role for cholesterol synthesis in development was already indicated in 1959 by data showing that ingestion of steroidal alkaloids is teratogenic (38). Such a role is also suggested by the phenotypes of the six existing mouse models with engineered mutations in cholesterol pathway genes. Loss of function of pre-squalene Hmgcr, mevalonate kinase (Mvk) and farnesyl-diphosphate farnesyltransferase 1 (Fdf1l) genes results in early embryonic lethality, suggesting a developmental requirement for endogenous sterol(s). The Hmgcr knockout (39) causes preimplantation lethality and early embryonic death was also reported for Mvk knockout (40). Mice lacking Fdf1l (41) die between E9.5 and E10.5. Our Cyp51 knockout is the next known model in the order of post-squalene enzymatic
steps causing lethality later in development (E15.0) than the three aforementioned pre-squalene knockouts. There are four engineered knockouts of genes later in the pathway. Ablation of hydroxysteroid (17-beta) dehydrogenase 7 (Hsd17b7) results in embryonic lethality at E10.5 (42,43). Early lethality of Hsd17b7 knockout could be explained by its additional role in estradiol synthesis. A knockout mutation in the sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (Sc5d) results in stillborn pups (44). Targeted disruption of Dhcrr7 in two independent studies (45,46) led to death of pups within 24 hours of birth. Two separate studies on 24-dehydrocholesterol reductase knockout mice (Dhcr24+) (47-49) demonstrate either perinatal lethality or viability to adulthood in some animals clearly indicating a genetic background effect. Our results fit into a general picture in which defects in genes active early in the pathway are early embryonic lethal while those later in the pathway tend also to survive later in development, with exception of Hsd17b7.

Characterization of our Cyp51 mouse knockout model revealed that several developmental defects resemble the ABS-like phenotype of humans such as skeletal and heart abnormalities. Skeletal abnormalities in ABS include craniosynostosis, brachycephaly, facial hypoplasia, bowed ulna or femur, synostosis of the radius and camptodactyly. In Cyp51 knockout animals, we observed also facial hypoplasia, brachycephaly, bowed and jointed bones of the extremities with camptodactyly. The reason why craniosynostosis could not be observed in our mouse model lies in the fact that they die before the cranial development is complete. Syndactyly was also noticed in patients with ABS due to POR mutations whereas polydactyly and hypoplastic left heart syndrome have been reported as potential teratogenic effects in response to intrauterine azole exposure. Thus, hypoplastic heart development in Cyp51 knockout embryos most likely corresponds to the inhibition of CYP51, similar to azole treatments in the human situation. In addition, we observed ventricular septal defects in the knockout embryos, which are also found in some patients of ABS-like syndrome (6) together with epicardial defects and impaired cardiac vessel formation. As potential underlying mechanisms for this complex cardiac phenotype, we suggest down-regulation of Shh, Rxra, Rara, Nkx2-5, and connexin 43 (Gja1). Ablation of SHH signaling in the heart has been shown to result in loss of cardiac blood vessels leading to lethality due to heart failure (50). RA signaling (Rxra, Rara) in heart development has also been extensively studied using different knockout mouse models. From these reports, it became clear that RA signaling is involved in differentiation of embryonic ventricular cardiomyocytes (51) and is necessary to avoid cardiac hypoplasia (52), and epicardial and coronary vessel defects (53). Growth of Nkx2-5−/− mutant hearts arrests during the looping heart stage when a complete lack of ventricle septum formation, severe cardiac hypoplasia, and underdevelopment of the outflow tract (54,55) are observed. Connexin 43 deficiency results in partial detachment of the epicardium and disturbed coronary vessel formation (56,57). Down-regulation of all the above mentioned genes in the hearts of Cyp51 knockout embryos could be expected to contribute to the observed cardiac phenotype. However, the exact molecular mechanism leading to this down-regulation in the Cyp51 deficient hearts remains to be determined.

ABS has been most frequently associated with recessive mutations in POR (ABS1 form), but also in some cases with dominant mutations in FGFR2 (ABS2 form). A mouse knockout of the FGFR2 ortholog does not model ABS2 in humans as the heterozygote mice are normal (no dominant effect) while homozygous nulls die at the blastocyst stage (58). Two mouse knockouts exist for the Por gene and exhibit a wide range of defects including neural tube, eye, heart, limb, and retarded growth, and embryos do not survive beyond E10.5 (59) or beyond E13.5 (60). Although some developmental abnormalities were similar to the ones reported in our Cyp51 knockout study, it is clear that Por null embryos have a broader range of developmental abnormalities possibly because Por is involved as electron donor in multiple biochemical pathways (61). Additionally, the Por knockout may not be the most suitable animal model for ABS1 because there is only one copy of Por in the mouse genome (62) whereas, in humans, the POR gene maps to a duplication region of chromosome 7 (63) likely providing some redundancy. By generating a knockout model of the Cyp51 gene, we therefore generated a genetic inactivation model that can help to characterize the importance of Cyp51 in the observed ABS-like phenotype in humans and indicate the possibility of a new form of ABS syndrome.
where mutations in the human orthologous gene CYP51A1 may be involved.

A lesson from the human cholesterol biosynthesis syndromes and mouse knockouts is that a complete inhibition of cholesterol synthesis results in embryonic or early postnatal lethality. However, the time when lethality occurs depends on the step of the pathway that a particular gene catalyzes. The earlier the gene acts in the pathway, the earlier embryonic lethality occurs. It still remains unclear whether the phenotypes reported are a consequence of cholesterol deficiency, accumulation of intermediates upstream of the knockout gene, lack of intermediates downstream of the knockout, perturbations of pathways branching from the “main” cholesterol pathway, or an interaction of these aforementioned factors. Perturbations in levels of sterol intermediates and their novel oxysterol metabolites have already been demonstrated to have a role in the pathophysiology of embryo development. It has been shown recently that phenotypes like micrognathia, cleft palate and limb malformations are a direct consequence of oxysterol accumulation due to the activity of sterol 27-hydroxylase CYP27A1), rather than altered cholesterol levels (reviewed in (64)). Although future studies may find some common mechanisms to explain developmental phenotypes of cholesterol biosynthesis gene knockout or deficiency models, it is also likely that some models have their own underlying molecular basis and that one unifying mechanism may not exist. Our comparative study with cholesterol human syndromes reveals though that Cyp51 knockout phenotype resembles many features of ABS and can hence serve as an animal model for this syndrome. As we developed a conditional Cyp51floxed allele, this enables development of conditional knockouts to study tissue- or time-specific effects of CYP51 deficiency or generating partial loss of Cyp51 function to provide hypomorphic ABS models suitable for studies in adult animals.

REFERENCES


FOOTNOTES

*Authorship note: Rok Keber and Helena Motaln contributed equally to this work.

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The abbreviations used are: SHH, sonic hedgehog; RA, retinoic acid; ABS, Antley-Bixler syndrome; BAC, bacterial artificial chromosome; BrdU, 5-bromodeoxyuridine; EDTA, ethylenediaminetetraacetate; ES cell, embryonic stem cell; GC-MS, gas chromatography–mass spectrometry; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PGK-neo, phosphoglycerate kinase-neomycin; P450, Cytochrome P450, qRT-PCR, quantitative reverse transcription PCR; RA, retinoic acid; SHH, sonic hedgehog; TK, thymidine kinase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

FIGURE LEGENDS

Fig. 1. Generation of Cyp51\(^{-/-}\) mice. A. Targeting strategy. The neomycin resistance cassette (gray box) flanked with two FRT sites (encircled black triangle) was inserted into intron 3. Exons 3 and 4 (black boxes) were flanked by two loxP sites (squared white triangle) inserted into introns 2 and 4 (together with new HindIII restriction sites). The thymidine kinase (TK) gene (gray arrows) was used for negative selection of non-targeted insertion events. Diagnostic HindIII (H) and BamHI (B) restriction sites as well as probes for Southern analysis (black bars) located 5’ (5’-out) and 3’ (3’-out) to both homology arms are indicated. Gray arrows show PCR primer positions used for genotyping. Crossed bars indicate homologous recombination. The neomycin selection cassette was removed in ES cell clones with Flp recombinase generating a conditional Cyp51\(^{\text{flox}}\) allele. EIIa-Cre recombinase was used to obtain the knockout Cyp51\(^{-}\) allele lacking exon 3 and 4. B. Southern blot of HindIII or BamHI digested DNA from ES cell clones carrying one Cyp51\(^{\text{flox-FRT-neo}}\) allele hybridized with 5’Eout and 3’Eout probes, respectively. Lane 2 shows a targeted ES cell line. C. Ear clip DNA genotyping of F\(_2\) offspring carrying conditional Cyp51\(^{\text{flox}}\) allele using 3loxF/3loxR primers. D. PCR genotyping of F\(_2\) embryos carrying knockout Cyp51\(^{-}\) allele using 5loxF/3loxR primers. A 187-bp fragment indicates Cyp51\(^{-}\) allele. E. The full knockout Cyp51\(^{-/-}\) genotype was further confirmed by the absence of a 159-bp fragment in 3loxF/3loxR PCR screen. Wild-type, floxed and knockout Cyp51 alleles are designated +, flox and -\(^{-}\), respectively.

Fig. 2. Cyp51 knockout embryos did not express functional Cyp51 mRNA and produced no immunodetectable CYP51 protein. A. Quantification of mRNA of various Cyp51 exons in F\(_2\) embryos. To test the expression pattern of the Cyp51 knockout allele in various parts of the Cyp51 locus, a qRT-PCR analysis was performed on probes covering exons 1 to 2 (Ex 1–2), exons 3 to 4 (Ex 3–4) and exons 8 to 9 (Ex 8–9) in embryos at E12.5 (upper panel) and E14.5 (lower panel). Cyp51 knockout embryos did not express exons 3–4 (this segment was deleted) but retained ~30% transcription of exons 1–2 and 20% of exons 8–9. Heterozygotes expressed all tested Cyp51 exons at levels between 60–80% of the wild type. Bars show mean + SEM (N=4), * P< 0.05. B. Cyp51 knockout embryos did not express the CYP51 protein. Membrane protein isolates (40 µg) from E14.5 embryos of the three F\(_2\) genotypes were probed with anti-CYP51 and anti β-actin antibodies. Western blot demonstrated that there is no immunodetectable CYP51 protein (53 kDa band) in the Cyp51 knockout embryos whereas it is observed in the wild type and heterozygotes. Isolated recombinant bovine CYP51 was used as a
positive control (lane C+) for the CYP51 detection and β-actin as a loading control. A 42 kDa CYP51 band of wild-type, heterozygous, and knockout embryos are designated +/+, +/-, and -/-, respectively.

Fig. 3. Quantification of cholesterogenic gene expression and sterol intermediates in F₂ embryos at E14.5. A. Gene expression in embryos at E14.5. Cyp51 knockout embryos exhibited significant increases in mRNA of all cholesterogenic genes, with the exception of Dhc7. In knockout embryos, there was approximately 20% read-through transcription of the truncated Cyp51 transcript compared to the 100% wild-type allele (see Fig. 2). Heterozygous embryos displayed a significant decrease in Cyp51 expression, while the expression of other cholesterogenic genes was not affected. (B) Quantification of sterol intermediates in embryos at E14.5. Upstream intermediates (lanosterol and 24,25-dihydroxolanosterol) were highly accumulated in knockout embryos, significantly increased in heterozygotes, but barely detectable in wild-type embryos. Cholesterol intermediates downstream of the CYP51 catalytic step were below the limit of detection in the Cyp51 knockout embryos (no values) compared to heterozygous and wild-type embryos. High amounts of 24,25-dihydroxolanosterol and lanosterol masked the measurements of low amounts of other sterols in the GC-MS method. A significant decrease in cholesterol levels was found in knockout embryos. These results demonstrate a block of de novo cholesterol biosynthesis in Cyp51 knockout embryos. Quantity of FF-MAS is shown on the right axis. Bars show mean + SEM (N= 4), P < 0.05. Cyp51 wild-type, heterozygous and knockout embryos are designated +/+, +/-, and -/-, respectively.

Fig. 4. Morphological developmental abnormalities in Cyp51 knockout embryos. A. Cyp51 knockout embryos were first distinguished at E11.5–E12.5 by kinked and short tails (arrow). Nuchal translucency extending dorsolaterally from the nuchal region was observed in knockout embryos from stage E12.5 to lethality which occurred at E15.0. At stage E15.5, all knockout embryos were in an advanced stage of resorption (right most knockout embryo). B. Example of nuchal translucency with hemorrhages (arrow) at E14.5. C. Variable phenotypes, ranging from mild to severe (left-right), of soft tissue syndactyly in the right hindlimb at E14.5. D. Representative limb skeletal abnormalities demonstrated by Alcian blue dye staining. Micromelia (shortened limbs) was observed in all examined specimens. Radius and tibia were shortened and bowed (arrows). High magnification demonstrated signs of femuro-tibial synostosis (arrow). E. Tarsal and carpal elements were underdeveloped and distal phalanges were absent. Joining of fingers or toes (syndactyly) was a prominent feature as well as post-axial polydactyly. Camptodactyly was most obvious in left forelimb. FL-forelimb, HL-hindlimb (Left/Right). F. Gross histological examination of embryos at E14.5. Extended subcutaneous, underdevelopment of tongue and absence of palatal shelf bone (arrow) was visible in knockout embryos. G. The most prominent head abnormality, micrognathia, is shown in Alcian blue stained specimens. Arrow points to the tongue in a wild-type embryo. The tongue did not develop normally in knockout embryos. Scale bars indicate 1 mm. Cyp51 wild-type, heterozygous and knockout embryos are designated +/+, +/-, and -/-, respectively.

Fig. 5. Developmental heart defects in Cyp51 knockout embryos. A. Sagittal and transverse section of wild-type (+/+) and knockout (-/-) embryos at E14.5 shows enlarged jugular lymph sac. B. High power magnification of jugular lymph sac endothelial lining. Endothelial cells of jugular lymph sacs appeared normal while the number of surrounding smooth muscle cells was greatly increased. C. Whole mount hearts at E14.5 show normal positioning of aorta and pulmonary trunk, dilation of the atri, and a very prominent interventricular groove in knockout embryos. D. Histological sections of wild-type and knockout embryo hearts at E12.5–E14.5. Note the hypoplastic development of the myocardium and the prominent endocardial cushions in knockout embryos. E. Transverse sections of E14.5 hearts displaying the ventricular septum defect (arrow). F. High-power magnification of the heart of E12.5 and E14.5 wild-type and knockout embryos. Note the reduction in the compacted layer of the myocardium at E14.5 (arrows) and the partially detached or missing epicardial cell lining in the knockout animals. Scale bars indicate 1 mm in A and 100 µm in B, C, D, E and F.

Fig. 6. Analyses of proliferation, apoptosis and gene expression in hearts of Cyp51 knockout embryos. A. BrdU immunostaining of E12.5 hearts and subsequent counting of BrdU-positive cells revealed lower proliferative activity in knockout hearts (N=3, P<0.05). B. BrdU immunostaining of
E14.5 hearts and subsequent counting of BrdU-positive cells showed an even more pronounced inhibition in cardiac cell proliferation in knockout embryos at later stages of development (N=3, P<0.05). C. Quantitative RT-PCR and immunostaining for connexin 43 (Gja1) as an important mediator of epicardial integrity and coronary vessel formation revealed reduced expression of connexin 43 in knockout hearts at E14.5. D. Immunostaining of hearts for platelet endothelial cell adhesion molecule-1 (PECAM-1, CD31) at E14.5. Note the significantly reduced number of proliferating vascular cells in Cyp51 knockout hearts. Scale bars indicate 100 µm. E. Quantitative RT-PCR analysis of genes involved in epicardial-myocardial signaling in embryo hearts at E12.5 (left) and E14.5 (right). Rxra and Rara were down-regulated in knockout hearts at E12.5 and E14.5, while Nkx2-5 was down-regulated in E14.5. Bars indicate mean ± SEM (N=6), * P<0.05.
**TABLES**

Table 1. Genotype frequency analysis of F$_2$ offspring from Cyp51$^+/-$ heterozygote crosses. No homozygous Cyp51$^{E/E}$ pups were present among 185 screened. Embryo analysis showed lethality between stage E14.5 and E15.5 where live Cyp51 knockouts were no longer present. P-values of stages where significant difference from the expected Mendelian ratio was detected are underlined. Examples of embryos in early stage of resorption from different developmental points are presented in Supplemental Fig. S1.

<table>
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<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
<th>Total</th>
<th>P(CHI-square)</th>
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<td>185</td>
<td><strong>3.95 \times 10^{-14}</strong></td>
<td>NA$^b$</td>
</tr>
</tbody>
</table>

$^a$Genotyping of most embryos in advanced stages of resorption was ambiguous because of small amount and degraded DNA and hence no genotype could be ascribed.

$^b$ NA, not applicable.
Figure 1

A. Wild type allele

Targeting construct

Targeted ES allele

Floxed allele

Knockout allele

B

C

D

E

Flp-mediated deletion of PGKneo in ES cells

Cre-mediated deletion of exon 3 and 4 in germline

1707 bp
187 bp
159 bp

159 bp
Figure 2

A

E12.5

Relative mRNA levels

Ex 1-2  Ex 3-4  Ex 8-9

E14.6

Relative mRNA levels

Ex 1-2  Ex 3-4  Ex 8-9

B

C+  +/-  -/-  +/-  

53 kDa

42 kDa
Figure 3
Figure 5
Figure 6

(A)

(B) Figure 6

(C) Relative mRNA levels

(D) PECAM1-positive vessels

(E) E12.5

(E14.5)
Mouse knockout of the cholesterogenic cytochrome P450 lanosterol 14α-Demethylase (CYP51) resembles Antley-Bixler syndrome
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Jure Acimovic, Damjana Rozman and Simon Horvat

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