Saeid Haghighi Poodeh

NOVEL PATHOMECHANISMS OF INTRAUTERINE GROWTH RESTRICTION IN FETAL ALCOHOL SYNDROME IN A MOUSE MODEL
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Academic Dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 9 of Oulu University Hospital (Kajaanintie 50), on 23 September 2016, at 12 noon

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Abstract

Fetal alcohol syndrome (FAS) is a pattern of anomalies in affected children due to maternal alcohol administration at vulnerable stages of fetal development. Intrauterine growth restriction and facial malformation are the presenting phenotypes of FAS.

In this investigation, novel pathomechanisms of intrauterine growth restriction and facial malformation were the primary aims.

We found by a FAS mouse model that AceCS1 gene expression and polyamines are the immediate targets of fetal alcohol exposure. The AceCS1 product is a precursor for lipid synthesis and protein acetylation and possibly, for polycation acetylation. We cloned the Mus musculus nuclear-cytosolic AceCS1 gene, and showed that its expression is developmentally regulated with a dynamic localization in the cytosolic and nuclear compartment. The enzyme plays an essential role in de novo synthesis of acetyl Coenzyme A.

Fetal alcohol administration targets nutrient supplying networks, which are localized at critical barriers. The main findings were reduced surface of the labyrinthine zone, destruction of gap junctions in the hemotrichorial placenta, reduced syncytiotrophoblastic cell layers and loosening of interaction between cell layers and embryo endothelial cells, reduced Reichert’s membrane thickness with discontinued Reichert’s trophoblast and loss of interaction by Reichert’s-parietal cells, reduction of capillary network and reduced vascularization in the brain area, and perturbed neural crest migration and formation of neural tube defect.

Alteration of angiogenesis-regulating proteins such as VEGF, PIGF, PECAM was detected in FAS, with no significant changes in placental angiogenesis of the labyrinthine zone, but up-regulation of VEGF/PIGF caused permeability changes in the placenta and yolk sac. On the other hand, the PECAM pool in embryos’ brain was reduced, which in turn led to decreased angiogenesis and vascularization.

Keywords: AceCS1, DFMO, PECAM, PIGF, Polyamines, Syncytiotrophoblast cells, VEGF
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Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Oulun yliopisto, Biocenter Oulu; Medical Research Center Oulu; Oulun yliopistollinen sairaala

**Tiivistelmä**


Sikiön altistuminen alkoholille kohdistuu sellaisten ravintoaineiden saatavuuteen, jotka sijaitsevat kriittisissä kudosrajapinnoissa. Päälöydöksinä olivat vähentynyt labyrinttikudoksen pinta-ala, gap-liitosten tuhoutuminen istukan veriessä (hemotrichorial?), ohentunut trofoblastisolut ja Reichertin kalvon paksuus, harventunut verisuonisuonten verkosto sekä verisuonitus aivojen alueella sekä hermopienan solujen siirtymiseseä ja hermostoputken sulkeutumishäiriö.

Verisuonten muodostumista (angiogeneesiä) säätelivien proteiinien (kuten VEGF, PI GF, PECAM) muutoksia todettiin FAS:ssa, mutta merkittäviä muutoksia ei havaittu istukan verisuonistumisessa. VEGF/PIGF-suhteen suureneminen muutti istukan ja ruskuispuussen verisuonisen läpäisevyyttä. Toisaalta sikiöiden aivojen PECAM-määrä pieneni, mikä johti verisuonten ja verisuoniverkoston muodostumisen vähennemiseen.

**Asiasanat:** AceCS1, PECAM, PI GF, polyamiinit, synsytiotrofoblastisolut, VEGF
To all mothers and fathers who sacrifice their desire for drinking by bringing a healthy child to this world, preventing life-lasting pain to an innocent child
Acknowledgments

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Oulu, May 15th 2016
Saeid Haghighi Poodeh
Abbreviations

AceCS1  Acetyl coenzyme A synthetase 1
AceCS2  Acetyl coenzyme A synthetase 2
ACL    ATP-citrate lyase
AdoMetDC S-adenosyl-methionine decarboxylase
ANT    Adenine nucleotide translocator
cAMP   Cyclic adenosine monophosphate
ATP    Adenosine triphosphate
cDNA   Complementary DNA
CNS    Central nervous system
CpG    5′-C-phosphate-G-3′
DAB    Diaminobenzidine
DEM    Dulbecco's Modified Eagle's Medium
DFMO   α-difluoromethylornithine
dpc    Days post coitum
EDTA   Ethylenediaminetetraacetic acid
eIF    Eukaryotic initiation factor
EMT    Epithelial to mesenchymal transition
FAS    Fetal alcohol syndrome
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
G1 phase Gap 1 phase
HAT    Histone acetyltransferase
HDAC   Histone deacetylase
Hif1a   Hypoxia-inducible factor 1-alfa
HMV    High molecular weight
HPLC   High-performance liquid chromatograph
HRP    Horseradish peroxidase
ip     Intraperitoneal
IOM    Institute of Medicine
IUGR   Intrauterine growth restriction
LMW    Low molecular weight
MAT    Methionine adenosyltransferase
mtDNA  Mitochondrial DNA
NAD    Nicotinamide adenine dinucleotide
NTD    Neural tube defect
NC     Neural crest
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NCC</td>
<td>Neural crest cell</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>P450</td>
<td>Cytochrome P450 oxidase</td>
</tr>
<tr>
<td>PAO</td>
<td>Polyamine oxidase</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>Platelet-derived growth factor receptor beta</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>pHi</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>S-phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionione</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate- polyacrylamide gel electrophoresis</td>
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<tr>
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<td>Silent information regulator 2</td>
</tr>
<tr>
<td>SMO</td>
<td>Spermine oxidase</td>
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<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding proteins</td>
</tr>
<tr>
<td>SSAT</td>
<td>Spermidine/spermine N1-acetylspermine</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VYS</td>
<td>Visceral yolk sac</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Wnt1</td>
<td>Wingless-type MMTV integration site family, member 1</td>
</tr>
</tbody>
</table>
List of original articles


1 Introduction

2 Review of literature

3 Aims of the study
4 Materials and methods ................................................................. 41
4.1 Animals and treatments (II, III, IV) ......................................................... 41
4.2 Cell culture (I) ............................................................................. 41
4.3 Polyamine concentrations of in vitro and in vivo models (I, IV) ....... 41
4.4 Northern blot analysis (I, II) .............................................................. 42
4.5 Whole-mount and thin section in situ hybridization (II) .................. 42
4.6 Histology and immunohistochemistry (III) ........................................ 42
4.7 Western blot analysis (III) ............................................................... 43
4.8 Transmission electron microscopy (III) ............................................. 43
4.9 Feto-placental permeability assay (III) .......................................... 43
4.10 Whole-mount immunostaining PECAM (III) ............................... 43

5 Results ................................................................................. 45
5.1 Macroscopic and microscopic phenotype of alcohol-exposed conceputures (IV) .............................................................. 45
5.2 Labyrinth zone or chorioallantoic structure (III) ......................... 45
  5.2.1 Gap junction barrier (III) ............................................................ 47
  5.2.2 Abnormalities of the placenta-yolk sac barrier in alcohol-
   exposed mice (III) ........................................................................... 47
  5.2.3 Impact of permeability-regulating proteins PlGF/VEGF
   on yolk sac-placenta malfunction (III) ........................................... 47
  5.2.4 Defect in heterodimer formation of VEGF and PlGF (III) ......... 47
5.3 The AceSA1 expression is downregulated in response to DFMO (I) .............................................................................. 50
5.4 Whole mount in situ hybridization analysis of AceSA1 (II) ........... 51
5.5 PECAM as an angiogenesis-regulating factor in embryonic and
   extraembryonic tissues (III, IV) ....................................................... 53
  5.5.1 Impact of permeability-regulating proteins PlGF/VEGF
   on yolk sac-placenta malfunction (III) ........................................... 53
  5.5.2 Defect in heterodimer formation of VEGF and PlGF (III) ......... 53
5.6 Polyamine pools are developmentally regulated in embryonic
   and extraembryonic tissues (IV) ...................................................... 55
5.7 DFMO and alcohol have a common target molecular pathway
   but in different manners (I, IV) ....................................................... 56

6 Discussion ............................................................................. 59
6.1 Direct effects of ethanol ................................................................. 61
6.2 Acetaldehyde, the first metabolite of ethanol, as a teratogen ......... 65
6.3 The effects of acetate, the second metabolite of ethanol ............... 68
7 Conclusions  71
References  73
Original publications  85
1 Introduction

Fetal alcohol spectrum disorder (FASD) is a leading cause of maternal disability due to impaired neurodevelopment. Alcohol as a teratogen targets normal fetal programming at vulnerable gestational stages causing epigenetic dysregulation, fetal growth restriction, and postnatal abnormalities (Ungerer et al. 2013). Intrauterine growth restriction (IUGR) is suggested to be a feature of fetal alcohol syndrome (FAS) that might be caused by impaired placentation (Gundogan et al. 2008). In addition to the placenta, the yolk sac may also be affected. Alcohol administration leads to reduced diameter of visceral yolk sac (VYS), decreased protein and DNA content and development of vitelline vessels in the yolk sac in a dose-dependent manner. Moreover, genes related to vasculogenesis and angiogenesis are perturbed (Xu et al. 2005).

At the molecular level, alcohol administration alters several epigenetic modifications (Shyh-Chang et al. 2013). Polyamine biosynthesis and catabolism are some of the molecular pathways that might change after acute and chronic ethanol administration (Sessa & Perin 1997). The polyamines are suggested to promote histone acetyltransferase activity, which leads to chromatin hyperacetylation (Woster 2011) and consequently, to global changes in gene expression.

The polyamine metabolism also involves the decarboxylation of S-adenosylmethionione (SAM). The SAM itself is engaged in methyl-donating process, where it transfers methyl groups to the protein, DNA, RNA and intermediary metabolism (Jones & Takai 2001, Meyer & Niggemann 2012). Alcohol intoxication perturbs the metabolite pools, methyl donating acetic acid, and consequently alters the epigenetic codes of DNA and histones in cells (Resendiz et al. 2014).

This thesis was carried out to search for novel pathomechanisms of FAS in a mouse model. The normal transition of anaerobic state into aerobic state in pregnant mice at critical gestational stages, 8.5-14.5 dpc, is perturbed by alcohol intoxication. The applied methods enabled us to follow how alcohol exposure deregulates interaction of the fetus, yolk sac and placenta. Novel methods were applied to visualize the malformation and consequently, altered permeability of the yolk sac and placenta. We visualized vascularization and angiogenesis in the capillary of brain and placenta tissue by platelet endothelial cell adhesion molecule (PECAM) immunostaining. The fetomaternal barriers that separate two blood circulations are the major targets in the FAS model, and the normal structure and function of the
barriers were perturbed. In addition, impaired angiogenesis and vascularization in FAS brain may impose irreversible, life-lasting disability.
2 Review of literature

2.1 Diagnosis criteria of Fetal alcohol syndrome (FAS)

Alcohol exposure during pregnancy causes a wide range of irreversible abnormalities and birth defects, including physical and mental retardation, which are features of FAS. The FAS syndrome was published for the first time in 1968 (Lemoine et al., 1968), although the diagnostic scheme for FAS came much later as the partial form of the disorder varies due to the methodology of the study.

2.2 Alcohol has many targets

Alcohol may have many targets in a fetus. Lipid solubility is one of the physical properties of ethyl alcohol that makes it capable to pass through maternal-embryonic barriers into all embryonic and extraembryonic tissues. Due to this ability, blood ethanol concentration reaches its peak level in mouse rapidly, 15 min after intraperitoneal (ip) injection (Simm & Murdoch 1990). Ethanol acts as teratogen directly or through its metabolites, acetaldehyde (Bosco & Diaz 2012; Garro et al. 1991) and acetate (Long et al. 2013). In the embryo at differentiation and organogenesis stages, alcohol leads to irreversible damages of developing embryonic and extraembryonic tissues.

Alcohol causes various damages to the fetal central nervous system (CNS), such as growth deficiencies, mental retardation, neurodevelopmental disorders and craniofacial anomalies (O'Leary et al. 2010). Low birth weight with craniofacial features are classic diagnostic signs of FAS. The severity of damages caused by alcohol depends on gestational stage, genetic background and exposing doses. In a mouse model, two intraperitoneal (ip) injections of 3 g/kg alcohol at four-hours intervals, at critical gestational stages of 7-9 dpc, cause development of FAS (Sulik et al. 1981).

Teratogens such as alcohol acutely disrupt embryogenesis by specific mechanisms that target the conceptus. The embryo-yolk sac-placenta (Sulik & Johnston 1982, Xu et al. 2005, Gundogan et al. 2008) is a closed system, in close interaction with each other and the dam blood circulation. Although maternal and fetal blood alcohol levels are approximately equal in the beginning (Waltman & Iniquez 1972), a high dose of alcohol is totally cleared within 6 hours from the mouse dam blood circulation (Simm & Murdoch 1990). The ability of the embryo
to metabolize alcohol is limited due to lack of functional hepatic alcohol dehydrogenase. Therefore the penetrated alcohol in the embryo has to be eliminated by passive back diffusion across the placenta to the mother, where it is eliminated (Waltman & Iniquez 1972). On the other hand, amniotic fluid alcohol clearing is approximately half compared with maternal blood circulation, causing high alcohol concentrations in amniotic fluid. Thus, amniotic fluid may act as a reservoir of alcohol for a longer than expected period if compared with maternal alcohol in circulation (Brien et al. 1983). In addition, each organ has a critical teratogenic period depending on the timing of differentiation during development. Under normal development, the intrauterine environment is under stable condition; in the event of teratogen exposure, the risk is greatest during the phase of organogenesis.

2.3 The diagnostic criteria for FAS recommended by Burd (Burd et al. 2003)

1. Maternal alcohol exposure has to be confirmed.
2. A typical FAS characteristic pattern of facial anomalies, including short palpable fissures, and abnormalities of the premaxillary zone (e.g. flat upper lip, flat -tended philtrum, flat midface) has to be detectable.
3. Growth restriction, such as low birth weight, lack of weight gain over time, disproportional low weight to height
4. Neurodevelopmental abnormalities of the CNS of the FAS child, such as small head size at birth; structural brain abnormalities.

The most urgent concern is that many FAS children with minor phenotypes might not be correctly diagnosed, and are therefore the most vulnerable FAS individuals.

2.4 Embryogenesis and embryonic nutrient supplies during early stages of mouse development

The vast majority of studies on embryogenesis in mammals have been conducted in mice due to the short period of pregnancy. The embryonic and fetal development in mice takes about 20 days whereas in humans the process normally takes 38 weeks. Many aspects of embryogenesis in mice resemble that of humans, although they differ in several other important respects. After implantation, in humans an embryonic disk develops but in mice an egg cylinder. In this process the embryo is coordinated for cell division, specialization, migration and programmed cell death.
(Hogan 1999, Pelton et al. 1998). In the second stage the fertilized egg produces a blastocyst, composed of an outer layer of cells and of a hollow sphere inside. The outer layer of cells will form the placenta and other supporting tissues essential for fetal development while the inner cell mass cell is capable of forming all of the tissues of the body. The morula is the first stage in which any cell specialization occurs. In the fourth stage of embryonic development, called gastrula, three embryonic germ layers - endoderm, mesoderm, and ectoderm - appear that will ultimately give rise to all the cell types of the body.

2.4.1 Nutrient delivery during first trimester of embryo development

During embryogenesis, prior to mid-gestation, the embryo is reliant on a diffusion-dependent nutrient delivery system. In all species, nutrition of the conceptus is initially histiotrophic, which means uptake of oviductal and uterine secretions by the trophoblast (Enders & Carter 2006). In later stages of development in mammalians, the histiotrophic switches to hemotrophic uptake. Histiotrophic nutrition – exchange between fetal and maternal circulation – takes place within the placenta. In humans, the uptake of oviductal and uterine secretions by trophoblasts functions only during the very early stage of development (Enders & Carter 2006).

As development advances, vitelline circulation provides to the embryo a more efficient system of vascular conduits for supplying nutrients into its tissues (Jollie 1990). In humans, at six weeks of gestation endometrial glands are highly active and discharge uterine milk into the coelomic cavity, from where nutrient uptake into the embryonic tissues takes place.

In rodents, during early stage of development (4-7 dpc) the yolk sac acts as a primary placenta, the sole or principal materno-fetal transporting system (Mossman 1991) that is in charge of nutrient supply. Both the placenta and the yolk sac appear for intermediate cholesterol passage in mouse (Yoshida & Wada 2005). Also, an investigation in rat embryo at 9.5 dpc indicated yolk sac lysosomal degradation of medium serum protein as a major source of amino acids supplying roughly about 86% of that required during this phase of embryonic development (Rowe & Kalaizis 1985). The role of the visceral yolk sac is important in fetal supply of amino acids derived from degradation of maternal plasma proteins (Richardson et al. 2000). In rat and mouse, 90% of amino acid transportation during organogenesis is by protein uptake and subsequently, its breakdown (Burton et al. 2001).
2.4.2 Placenta and yolk sac

The placenta is the first organ to develop during mammalian embryogenesis. It is a unique, autonomous and transient organ that is vital for mammalian embryo development. It is localized between fetal and maternal environments with several functions, such as exchange of gases, nutrients and waste products between the fetus and mother. It is also an important source of pregnancy-associated hormones and hormonal functions that are required for maintenance of pregnancy and fetal well-being. Human and mouse placenta differ in gestational stages of morphogenesis and endocrine functions and in the time and triggering of uterine decidualization. (Rossant & Cross 2001).

During the early stages of rodent development, which have a hemotrichorial placenta prior to establishment of full blood circulation in rodents, the yolk sac acts as a primary placenta. In rodents, the nutrient supply of the embryo through the vitelline blood vessel is thus totally dependent on the yolk sac during early stages (Gabriel et al. 1998) and essential for the survival and growth of the embryo during early development (Fig. 2E).

In humans, chorionic villus, the definitive functional and structural unit in the placenta, is formed by day 21 after ovulation. At the corresponding developmental stage in mice (Malassiné et al. 2003, Xu et al. 2005) before the formation of chorioallantoic placenta, the yolk sac has several essential roles in embryo development, prior to via yolk sac, as an expansion or satellite of the fetal gut. In mouse during stages 8.5-9.5 dpc, the vasculature is established, and the circulatory and cardiac system are partially functional. At 8.5 dpc, the chorion is fused to allantois and at 10.5 dpc, chorioallantoic placenta is formed. There are several studies where in vivo (Shi et al. 1985) and in vitro models (Gitlin & Perricelli 1970, Gitlin 1975) have shown that although fetal liver is capable of producing sufficient amount of proteins, including plasma proteins (albumin, pre-albumin and transferrin, α-fetoprotein, α1-antitrypsin and transferrin), the yolk sac endodermal layer is still the main source of proteins. It is also suggested that the yolk sac, not the embryo proper, is the primary organ in the mouse for blood cell production (Palacios & Imhof 1993). In the yolk sac, some blood islands appear, fuse and form the yolk sac vasculature, which contains a large number of primitive nucleated red blood cells.

The yolk sac contains trophoblast, Reichert’s membrane, and visceral endoderm (Fig. 2E). Reichert’s membrane is a specialized basement membrane that surrounds the embryo and passively filters nutrients (Gardner & Seeger 1983). The
structural analysis of Reichert’s membrane by light microscopic immunostaining suggests the presence of laminins α1, α2, α3, β1, β2, γ1, γ2, nid-1, nid-2, α1 collagen type IV and perlecan (Gersdorff et al. 2005).

Reichert’s membrane might play a crucial role in the normal embryo development. An investigation indicated that collagen IV deficiency might be engaged in immunological embryo toxicity (Leung et al. 1977).

A second type of placentation, the placenta proper in rodents, forms a separate maternal-fetal transportation system, which functions as a barrier to the passage of macromolecules such as albumin, and also has a crucial role during the early development of the rodent embryo. That barrier consists of Reichert’s membrane within a trophoblast cell layer on the maternal side and the parietal endodermal cells on the embryonic side (Ogura et al. 1998).

Two vascular systems, maternal and fetal, are separated in the mouse by a barrier structure consisting of three layers of trophoblast, two syncytiotrophoblast layers (II, III), and one layer of cytotrophoblasts. In humans, the fetal blood circulation is separated by one layer of endothelial cells and trophoblasts (Fig. 2A).

In rodent placentation, the cells become organized to form two vascular systems, the maternal and fetal, in order to form a sieve barrier. The barrier dynamically separates the maternal blood circulation from its fetal counterpart. The fetal blood circulation is separated with one layer of endothelial cells. Although in mouse, four cell layers ensure that maternal and fetal blood circulation are separated, functionally the syncytiotrophoblastic layers II and III serve as a major barrier (Matsubara et al. 1993). The two barriers exchange substances from the maternal circulation to the fetus and vice versa. The barriers can be viewed as an ultrafilter on a semipermeable membrane.

In hemotrichorial placenta, passage of large molecules into the fetal blood circulation is limited by gap junctions that connect layer I and II syncytiotrophoblast. The gap junction membrane structures (which enable adjacent cells, through direct cytoplasmic exchange of ions and low molecular weight metabolites, glucose, hormones, polyamines, to regulate animal physiology including propagation of electrical signals and coordinating cell signaling) control proliferation, differentiation and homeostasis (Kumar & Gilula 1992, Risek & Gilula 1996, Nielsen et al. 2012, Takata & Hirano 1997).

Increased activity of ornithine decarboxylase and polyamine pools regulates gap junction communication in connexin 43-expressing cells (Shore et al. 2001). The histone deacetylase (HDAC) inhibitors inhibit deacetylation of cytosolic
proteins, and thereby affect gap junction function independent of transcription (Colussi et al. 2011).

2.4.3 Angiogenesis and permeability in embryonic and extraembryonic tissues

Formation or sprouting of new blood vessels from preexisting ones is called angiogenesis. In embryonic tissues, sprouting endothelial cells form new blood vessel attract platelets by producing Platelet Derived Growth Factor Receptor β (PDGF-β) expressing pericytes. The pericytes align to the newly formed vessel (Bergers & Song 2005). During embryonic development, pericytes in most vessels originate from mesenchymal stem cells (Yamashita et al. 2000) with the exception of central nervous system (CNS) cardiac vessels that arise from the neural crest cells (Bergwerff et al. 1998, Creazzo et al. 1998, Etchevers et al. 2001).

Pericytes and endothelial cells interact in the regulation of vascular formation, remodeling and function. Endothelial cells and pericytes form adherens junction, where pericytes and endothelial cells connect their cytoskeleton through cytoplasmic membranes with catenins, cadherins and gap junctions. Adjacent cell membranes either fuse or remain separated by a 2- to 3-nm space (Sims 1986). The cell-cell contacts between pericytes and endothelial cells play a crucial role in the regulation of vascular formation, its stabilization, remodeling and function. In mammalian embryos circulation is established in a stepwise pattern. Specialized vascular hematopoietic environment exist only after circulation is fully developed after 10 dpc (McGrath et al. 2003). Prior to that stage, at 8.5 dpc, PECAM expression indicated the vascular development to be more widespread as compared with the distribution of red blood cells (McGrath et al. 2002).

Angiogenesis can also be induced by polyamines. Treatment of chick yolk sac by spermine and spermidine results in the induction of angiogenesis in the membrane. However, the angiogenic activity of spermine is stronger than that of spermidine, with no induction by putrescine (Takigawa et al. 1990). Kucharzewska (Kucharzewska et al. 2010) also suggested that ornithine decarboxylase and extracellular polyamines regulate microvascular sprouting. Hypoxia is developmentally regulated (Shepard et al. 1997) and hypoxia-inducible factor 1-alfa (Hif1α), vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and platelet endothelial cell adhesion molecule (PECAM-1) can be used as markers for its progress. Previous reports have indicated that the VEGF and PIGF pools regulate the permeability of the placenta (Debieve et al. 2001). Transcripts
encoding mouse PlGF and VEGF are abundant in trophoblast giant cells and yolk sac parietal cells (Achen et al. 1997). Upregulation of PlGF/VEGF plays a role in the regulation of permeability with no significant changes taking place in placental angiogenesis (Dvorak 2006). In fact, VEGF became first known as a permeability regulator and later as a regulator of angiogenesis. Although hypoxia during embryogenesis is normal and has an essential role in development, it also affects vascularization, angiogenesis, hematopoiesis and chondrogenesis (Giaccia et al. 2004). Excess of hypoxia leads to abnormal development and even postnatal deficit (Anderson et al. 2000). Complications caused by fetal hypoxia/anoxia are among the top ten causes of fetal deaths. Several factors including living at high altitude, hypertension, anemia, pulmonary disease, preeclampsia, drug abuse and smoking are suggested to contribute to fetal hypoxia (Anderson et al. 2000).

The three distinct types of hypoxia as suggested by Kingdom and Kaufmann (1997) are:

(A) pre-placental hypoxia (the mother, the placenta, the fetus) caused by living in high altitude
(B) uteroplacental hypoxia (the placenta and fetus hypoxic) caused by preeclampsia
(C) post-placental hypoxia (normoxic mother, hypoxic embryo), VEGF is decreased, PlGF is increased

2.4.4 Teratogens

The word teratogen (“teras” meaning malformation or monstrosity) refers to any environmental factor, agent or an influence that causes physical defects in the developing embryo, impairs prenatal development and leads to birth defects or even death. Drugs, chemicals, stressors, viruses and malnutrition might possess the capability to cause damage during the prenatal period. The damage depends on a complex interplay of many factors (Gilbert-Barness 2010).

In mammalians such as rodents and humans, the embryo is protected from environmental teratogens by structural barriers between the mother and embryo. During embryogenesis and differentiation of organs the embryo is vulnerable to teratogenic agents causing alterations of growth and morphology of the fetus. A search in the NCBI yields more than 1,460 papers for “teratogen and embryo development in mammalians”, but only 88 for yolk sac and 128 for placenta. In humans, craniofacial malformations are the most common birth defect (Young et al. 2000). Although the list of teratogens is rising all the time their pathogenic
mechanism still remains obscure. There are several basic factors affecting teratogenesis including timing, critical period in gestation, exposing dosage or threshold effect of the drug or chemical, physical properties of the drug or chemical, passing protective barriers and altering the genotype of the embryo and genetic variability.

2.4.5 Neural tube formation during critical stages of 7-9 dpc

Neurulation is a landmark event during the gestational stages of 7-9 dpc in mouse. It proceeds by initial generation of the nerve system that begins by formation of a neuron plate (at 8 dpc embryo with 1-7 somites), folding of the plate and further closure of the neuron tube (Golden & Chernoff 1993).

Later on, at the stage 8.5 dpc, rotation of the embryo already begins and initial elevation of neural folds proceeds at 9 dpc. Consequently, fusion of neural fold is completed to form the hollow neural tube. During 8-10 dpc in mice, the neural plate goes through a series of complex morphogenetic events that results in formation of the neural tube. The pattern of closure moves like a wave from initiation sites to closing points, the neuropores (Macdonald et al. 1989, Copp et al. 1990, Golden & Chernoff 1993).

In mice head, on day 9 the last regions of the neural tube elevation and fusion come to close position (Theiler & Varum 1985, Theiler 1988, Kaufman 2004, Kaufman 1979). Any perturbation in neural tube closing might cause the development of neural tube defect (NTD). In mice, more than 190 neural tube defect closure mutants have been reported to date, including 150 known single-gene mutations and 33 unidentified genes, and eight multifactorial strains have been reported so far (Harris & Juriloff 2007, Harris & Juriloff 2010).

2.4.6 Neural crest and facial formation

The neural crest (NC) cells are a transient embryonic cell population. Their formation is orchestrated by a gene regulatory network (Sauka-Spengler & Bronner-Fraser 2008). The NC cells are multipotent, with stem-cell-like properties giving rise to many types of cells (Li et al. 2015). They contribute to formation of the majority of bone, cartilage, connective tissue and peripheral nervous tissue in the head (Santagati & Rijli 2003). During embryogenesis, frontonasal mass and branchial arches together form the facial organs that originate from neural crest cells migrating from the lateral edge of the prosencephalon (Osumi-Yamashita et
Alteration in neural crest migration and possible death of the neural crest cells during this stage of development might be one of the pathomechanisms causing facial malformations (Yamada et al. 2005). The cranial neural crest, which arises from the dorsal margins of the neural fold, participates in the formation of head skeleton. Wnt1 (Wingless-type MMTV integration site family, member 1) might be an essential and sufficient molecule for induction and generation of neural crest cells (García-Castro et al. 2002). Wnt1 expression depends on sonic hedgehog gene expression. The sonic hedgehog protein requires cholesterol molecule for signaling. In the processing of hedgehog proteins they are truncated at the normal site of internal cleavage. The produced activated proteins signal at the extreme carboxyl-terminal residue, which is targeted by a nucleophilic attack of cholesterol. In this way the amino-terminal product of the cleavage receives a covalent cholesterol adduct (Porter et al. 1996). The reaction is essential for the release of the active signal from the precursors and shift in the properties of this signal within cells and tissues. Thus, the mature protein signal, via insertion into lipid bilayer of the cell membrane, leads to cellular responses in developing tissues and functions.

2.4.7 Molecular pathways regulating neural crest migration

Several findings indicate that neural crest cell migration is a highly dynamic process, in which migrating NC cells adapt their behavior by permanently probing their local environment and interacting with other NC cells or neighboring cell types (Carmona-Fontaine et al. 2008, Cerny et al. 2004, Hall 2008, Kulesa & Fraser 2000).

One of the critical events during neurulation is delamination, in which mouse cephalic NC cell migration proceeds at open neural plate stage, after which the cells undergo an epithelial mesenchymal transition. At this stage the neural crest cells start to migrate within a few hours (Nichols 1987). By formation and closure of anterior neuropore the delamination is completed.

There is diversity in the cellular behavior that takes place during neural crest cell migration and epithelial-to-mesenchymal transition (EMT). Several different mechanisms in parallel might regulate the detachment and delamination of NCCs from the neuroepithelium. Polyamines modulate epithelial-to-mesenchymal transition (Compagnone et al. 2012).
2.5 Master metabolites in embryonal and fetal development

2.5.1 Polyamines

The polyamines (putrescine, spermidine and spermine) are low molecular weight polycation amines that are protonated in physiological environment. The level and composition of polyamine pools are extremely sensitive and vulnerable to any acute changes in the chemical and physical conditions in the cellular environment (Yatin *et al.* 2001). Their positive charges favor them to interact with negatively charged nucleic acids and proteins. They can be detected in all mammalian cells, fungi, protozoa, and bacteria. In mammalians polyamines are synthesized *de novo* in the cytoplasm or by taken up from diet. The polyamines contain two amino acids: L-methionine and L-ornithine. In fact, mammalian cells take up L-ornithine from plasma. It is a side product of the urea cycle and L-methionine can be synthesized *de novo* from arginine by the action of arginase. L-ornithine is further converted to putrescine by the action of ornithine decarboxylase, which is rate-limiting for polyamine synthesis that can be irreversibly inhibited by α-difluoromethylornithine (DFMO) (Fig. 4). For the synthesis of the second member of polycations spermidine requires two enzymes: *S*-adenosyl-methionine decarboxylase (AdoMetDC) for the synthesis of the aminopropyl donor and a transferase enzyme (spermidine synthetase or spermine synthetase). The aminopropyl group is transferred to the primary group of putrescine or spermidine.

The intracellular pool of polyamines is regulated by interaction of metabolism and catabolism. During metabolism, putrescine is converted into spermidine and spermine, spermidine synthase. In catabolism spermine is converted vice versa to spermidine by direct action of spermine oxidase (SMO) or alternatively, spermine can be converted back to spermidine by spermidine/spermine N1-acetylspermine (SSAT) and *polyamine oxidase* (PAO) (*Wang et al.* 2001). Acetylation is the rate-limiting step in this interconversion of spermine and spermidine using acetyl-CoA. The acetylated molecules then become substrates for peroxisomal *polyamine oxidase* (PAO). It has been suggested that the PAO prefers acetylated polyamines as a substrate (*Wang et al.* 2001). The outcome of a balanced diet appears homeostatically partitioned between SSAT acetylation of polyamines and fatty acid metabolism; altered SSAT expression can affect fat metabolism (*Jell et al.* 2007) (Fig. 4).

The uptake of polyamines is dependent on pH of cellular compartments and seems to involve a vesicular compartment (*Soulet et al.* 2004). Polyamines play a
major regulation role in ionic and osmotic stress in most living cells (Tabor & Tabor 1976). Ionic insults, such as acid stress, K+ and Mg2+ deficiency, high concentration of SO2 or low extracellular pH increase the biosynthesis of putrescine. Vice versa, polyamine depletion by DFMO causes stable intracellular acidification (Poulin & Pegg 1995).

The polyamines have a role in several other biological pathways’ interaction with negatively charged DNA, RNA and phospholipids (bind electrostatically to negatively charged molecules), regulation of gap junctions, regulation of microvascular sprouting and arrangement of actin cytoskeleton dynamic in endothelial cells, as well as modulation of fetal EMT transition.

When the DFMO treatment is applied to inhibit endogenous polyamine synthesis in an in vivo mouse model, the reduction of the putrescine pool can be found, but surprisingly, the spermine pool is increased as a compensation due to uptake from exogenous sources. Furthermore, the results of the in vivo experiment showed that DFMO might irreversibly cause malformation of mouse embryo although the treatment does not cause death (López-García et al. 2008).

2.5.2 One-carbon donation and methylation during embryogenesis

This one-carbon donating group causes dynamic structural modifications of chromatin. The chromatin is modified by DNA methylation via two different pathways. Firstly, by gene imprinting the same parental allele is always epigenetically silenced (Jirtle 1999). In this way the methylated DNA of a gene is determined to be silent in zygote cells. Secondly, DNA methylation occurs at CpG islands, which is a dynamic phenomenon. Alcohol exposure is suggested to induce aberrant change in DNA methylation which is associated with gene expression (Liu et al. 2009) (Fig 4).

One-carbon metabolism, such as insufficient folate or methionine uptake or impaired methionine synthase activity, is suggested to be behind skeletal malformation in FAS (Padmanabhan et al. 1984). Also S-adenosylmethionine (SAM) serves as the methyl donor for enzyme of DNA methyltransferase. The first metabolite of alcohol, acetaldehyde, is suggested to inhibit DNA methyltransferase activity (Garro et al. 1991). Depletion of SAM has a minimal effect on the polyamine pool due to increases in ODC (Kramer et al. 1987, Kramer et al. 1988) (Fig. 4). Ornithine decarboxylase (ODC) enzyme activity, a key regulatory member in polyamine biosynthetic pathways, is developmentally regulated in mouse conceptus (López-García et al. 2008). ODC activity is at the lowest level during
gestation 6-7 dpc, abruptly increased at 8 dpc, with a dramatic decrease at gestation 9-10 dpc. During the second half of mouse pregnancy, ODC activity and protein levels are higher in placenta and especially in labyrinthine zone, compared with fetus and yolk sac (López-García et al. 2008).

2.5.3 Two-carbon donor sources

The two-carbon donating molecules are thought to be metabolic master regulators that are engaged in various biological functions (Schroeder et al. 2014). These include Krebs cycle for ATP production, lipid and steroids synthesis, protein acetylation, and induction of RNA polymerase II transcription and promoter binding by transcription factor IID in the absence of histones (Galasinski et al. 2000).

Acetyl-CoA has a role as a gauge of cellular metabolism in an organism. Two metabolites, CoA and ATP, are essential for activating acetate in the liver (Fig 4). Acetate and acetaldehyde are products of oxidative metabolism of alcohol in the liver of a mother exposed to ethanol. Acetyl-CoA is a CoA thioester, which in a biological pathway is placed at a junction of multiple catabolic and anabolic pathways (Tsuchiya et al. 2014). Cellular localization of acetyl-CoA is divided into cytosolic, mitochondrial and nuclear origin. In mitochondria, the acetyl-CoA is from catabolic sources. It is catabolized from glucose and beta-oxidation of fatty acids which can be further oxidized in Krebs cycle and produce energy for the cell. The acetyl-CoA from cytosolic source incorporates in lipid and cholesterol synthesis. In addition, both mitochondrial and nucleocytoplasmic acetyl-CoA are suggested to supply acetyl-CoA-substrate for acetylation of proteins.

Metabolism may connect to various cellular functions by modulating intracellular metabolites including acetyl-CoA, polyamines and Nicotinamide adenine dinucleotide (NAD) which act as co-factors for posttranslational modification affecting enzyme function or the epigenetic status of chromatin (Kaelin & McKnight 2013).

AceCS1 catalyzes the ligation of acetate with CoA to produce acetyl-CoA, an essential molecule utilized in various metabolic pathways including fatty acid and cholesterol synthesis and the tricarboxylic acid cycle. AceCS1 transcription is induced by sterol regulatory element-binding proteins (SREBPs). The gene encodes acetyl-CoA synthetase (AceCS1), the cytosolic enzyme that activates acetate so that it can be used for lipid synthesis or for energy generation.
In ruminant animals, *acetyl-CoA synthetase* plays a key role in the catabolism of acetate produced by microorganisms in the rumen. In non-ruminant mammals, acetate production occurs in the liver from ethanol by alcohol dehydrogenase and acetaldehyde dehydrogenase and by acetyl-CoA hydrolase (Crabtree *et al.* 1989). Furthermore, *AceCS1* is postulated to play a key role in the recycling of acetate released by acetylcholine esterase for the formation and release of acetylcholine in cholinergic nerve terminals (Sterri & Fonnum 1980, Carroll 1997). *AceCS1* from various microorganisms has revealed that the enzyme belongs to the firefly luciferase super family (Toh 1991) which includes mammalian long chain acyl-CoA synthetases (Fujino & Yamamoto 1992, Fujino *et al.* 1996, Oikawa *et al.* 1998). There are cardiac and hepatic type enzymes. Subcellular fractionation revealed that the hepatic type enzyme *AceCS1* is a cytosolic enzyme (Luong *et al.* 2000, Fujino *et al.* 2001) and the cardiac enzyme (termed *AceCS2*) is located in the mitochondrial matrix. According to Fujino *et al.*, *AceCS2* (2001) provides acetyl-CoA that is utilized mainly for oxidation under ketogenic conditions, meaning oxidation through the tricarboxylic acid cycle to produce adenosine triphosphate (ATP) and CO2 in the mitochondrial matrix.

Purification of murine *AceCS1* and *AceCS2* revealed that these two enzymes preferentially utilize acetate with similar affinity among C2-C5 short and medium chain fatty acids. Although there are numerous studies showing acetate utilization in the hepatic mitochondria, apparently no *AceCS2* transcripts were detected in the liver. Hepatic carnitine acetyltransferase, which catalyzes the reversible transfer of short chain acyl groups between CoA and carnitine, may play a role in the transportation of cytosolic acetyl-CoA into the mitochondrial matrix (Fujino *et al.* 2001). A NAD⁺-dependent deacetylase enzyme, Sir2 (Silent information regulator 2), modulates gene silencing, aging and energy metabolism. Mammalian acetyl-CoA synthetases (AceCSs) are regulated by reversible acetylation. Sir2 activates AceCSs by deacetylation of mouse AceCS1 on lys-661 (Hallows *et al.* 2006).

### 2.5.4 Energy sensors and embryonic cell differentiation

There are several homeostatic systems that act as energetic sensors for the cell that sense the abundance of metabolic products such as ATP, NAD⁺, polyamines and acetyl-CoA. Ethanol and hypoxia both disrupt the cellular energy metabolism by hyper-reduction of the NAD⁺-dependent part of the respiratory chain and consequently decrease the ranges of ATP synthesis during oxidative phosphorylation (Korneev & Komissarova 1993).
Calories are converted into ATP, acetyl-CoA, S-adenosyl-methionine (SAM) and reduced NAD$^+$ that are precursors for several molecular pathways regulating global gene expression. Under normal conditions when calories are plentiful, it might turn on an orchestrated biological network for the production of ATP and acetyl-CoA for the phosphorylation and acetylation of chromatin (Fig. 4). This leads to the DNA-chromatin relaxation and unwinding allowing transcription and (or) replication. Vice versa, while supply of calories for the organism is insufficient, the chromatin phosphorylation and acetylation disappear and consequently cause reclosing of DNA strands and the suppression of gene expression, where thousands of bioenergetic genes disperse across the chromosomes and mitochondrial DNA (mtDNA) (Feinberg 2007, Feinberg 2008, Gerhart-Hines et al. 2007, Asher et al. 2008).

### 2.5.5 Protein acetylation and the enzymes utilizing acetate

At the moment, there are over 6,800 acetylation sites known in mammalian proteins indicating the importance of this modification (Lakshminarasimhan et al. 2013, Rauh et al. 2013, Kim et al. 2006). Several of these acetylation sites are relatively uncharacterized and sirtuins might serve as a deacetylase for several of them. Acetate is generated in the colon by bacterial function, and its production is increased by a high fiber diet. It is also generated within the nucleus of all cells by the function of histone deacetylases and transported via the portal vein to the liver, where it is activated by AceCS1 and AceCS2. Also, a relatively high amount of acetate is generated from acetyl-CoA within cells by \textit{acetyl-CoA hydrolase}, a ubiquitous cytosolic enzyme. Ingested ethanol is oxidized in adult liver, first to acetaldehyde and then to acetate, which must be converted to acetyl-CoA. A small amount of ethanol may be oxidized by the \textit{P450 oxidase complex} but mainly it is metabolized in the liver by the enzyme alcohol dehydrogenase.

Ethanol is metabolized in the liver to acetate:

\begin{align}
\text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ &\rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ & (1) \\
\text{CH}_3\text{CHO} + \text{NAD}^+ + \text{H}_2\text{O} &\rightarrow \text{CH}_3\text{CO}_2^- + \text{NADH} + \text{H}^+ & (2)
\end{align}

Most of the acetate produced is converted by extrahepatic tissues to acetyl-CoA and enters the tricarboxylic acid (TCA) cycle. According to Simm & Murdoch (Simm & Murdoch 1990), acetate indicates alcohol-induced responses. After acute administration of ethanol (3.5 g/kg, i.p.) to pregnant mice (9 dpc) a significant
increase in the levels of glucose, glucose-6-phosphate and fructose-6-phosphate of the uterus is detected; alcohol thus induces alterations in uterine glucose metabolism. In the absence of ethanol sodium acetate is rapidly cleared from the circulation in a concentration-dependent manner (Simm & Murdoch 1990). A large amount of generated NADH causes metabolic disturbances. The mitochondrial shuttle systems become saturated (malate aspartate shuttle, glycerol phosphate shuttle) making the cytosolic NADH very high and NAD⁺ low. This slows down further oxidation of ethanol as well as other NAD⁺-requiring pathways (e.g. glycolysis) (Murdoch & Simm 1992).

2.6 Embryonic cell cycle and pH

2.6.1 Embryonic cell cycle and intracellular pH (pHi)

The pHi adjustment is dynamic and cell cycle-dependent. Several studies have indicated alkalization of cell interior as a prerequisite for cell division (Epel 1978, Moolenaar 1986, Soltoff & Cantley 1988). When cells are stimulated to enter mitogenesis, pHi rises due to activation of a membrane protein, a regulating mechanism for exchange of extracellular sodium for intracellular hydrogen. Histone acetylation also regulates intracellular pHi. Histone acetylation increases as pHi rises, such as when resting cells are induced to proliferate. In the rat, during organogenesis cells of many organ systems begin to change from a proliferative mode into a differentiated state. Intracellular pH is developmentally regulated as the pHi of day 11.5 dpc rat embryo decreases readily compared to day 14 dpc. Because there is a concomitant fall in the proliferative rate over this span of development, this correlation might be additional evidence of an association between proliferation and alkalization of the cell interior (McBrian et al. 2013).

Neural tube development of chick embryo is also affected by pH (Kotwani et al. 1995, Gillespie & McHanwell 1987).showed that during embryonal stage of 4-22 somites in the extracellular space of the segmental region, pH range was 7.8–8.4, while caudally at the regressing primitive streak in the segmental plate the pH was consistently lower. The buffering capacity of the extracellular space in the segmental region was lower compared with the segmental plate. In other words, ectodermal and endodermal epithelium form an effective barrier between the inside of the embryo and the exterior.
2.6.2 Alcohol and DFMO affect embryonic cell cycle

During organogenesis, DNA, RNA and protein synthesis are developmentally regulated. During early stage of development prior to proliferation, the cell cycle is longer than normally. The total amount of DNA, mRNA and protein during organogenesis is developmentally regulated (Abe et al. 2013). During proliferation the DNA, mRNA and protein pools change and are regulated by the cell cycle. Polyamines and lipogenesis play an essential role during these developmental stages.

Teratogens, such as ethanol and DFMO, halt both the cell cycle at the G1 stage. Alcohol administration causes collective misregulation of protein expression which is pivotal to the regulation of the G1 phase of the cell cycle, triggering premature induction of DNA synthesis (S-phase) (Anthony et al. 2008).

In humans, maternal alcohol abuse possibly causes also transient hypercapnia and acidosis in the fetus and mother (Cudd et al. 2001), and acidosis further leads to cell losses both in neurons and glial cells (Goldman et al. 1989, Nedergaard et al. 1991, Staub et al. 1990). Furthermore, alcohol-induced maternal hypercapnia directly results in fetal hypercapnia producing both intracellular and extracellular brain acidosis (Ramadoss et al. 2007). Alcohol exposure possibly alters maternal arterial pH and consequently results in damage to the developing fetal brain.

2.7 Chromatin post modifications and the role of acetyl-CoA

Chromatin has a dynamic structure which is actively altered during the cell cycle, and the cell cycle during embryogenesis is developmentally regulated (Abe et al. 2013).

The histones are post-modified by acetylation, deacetylation, methylation (Fig 4), phosphorylation, sumoylation, ubiquitinylation, and ADP-ribosylation. These modifications are dynamic and carried out at specific residues (Kouzarides 2007). Depending on specific histone proteins and the site of modification the histone methylation, acetylation or ubiquitination can either activate or repress gene expression, but in general, histone phosphorylation promotes gene transcription, DNA repair and cell apoptosis (Wang et al. 2012) (Fig. 4).

The histone N-terminal tail is acetylated by histone acetyltransferase (HATs) or deacetylated by histone deacetylase (HDACs). The histone acetylation and deacetylation are the most studied forms of chromatin post-modification. Histone acetylation can lead to changes in chromatin structure by reducing the histone-DNA
interaction which makes the DNA more accessible for gene transcription. Thus, in this way acetylation generally leads to transcriptionally active chromatin while deacetylation leads to transcriptional downregulation (Pang & Zhuang 2010).

Depending on the cell strain and physiological conditions, the acetyl-CoA is supplied from different sources. The main source of acetyl-CoA for the histone acetylation is from the action of ATP-Citrate Lyase (ACL), but under abolished physiological level of ACL, the histone acetylation is rescued by AceSA1 (Wellen et al. 2009) (Fig.4). During embryogenesis, acetaldehyde, the metabolite of alcohol dehydrogenase, impairs pyruvate dehydrogenase activity and consequently reduces the acetyl-CoA pool which is going to enter Krebs’ cycle (Hard et al. 2001).

Histone acetylation is increased during differentiation of murine 3T3-L1 and is regulated by ACL and nutrition availability (Yoo et al. 2006). Silencing of ACL reduces histone acetylation (Hard et al. 2001). On the contrary, histone deacetylation releases acetate anions that have to be transported out of the cell to prevent the change in pHi. ACL activity has less impact on the net acetylation of the core histone even in the absence of substantial histone deacetylation (Hard et al. 2001).

Metabolism may connect to various cellular functions by modulating intracellular metabolites including acetyl-CoA, polyamines and NAD⁺, which act as cofactors for posttranslational modification affecting enzyme function or the epigenetic status of chromatin (Kaelin & McKnight 2013, Lu & Thompson 2012).

The acetyl-CoA pool per se changes the global gene expression without any histone acetylation (Galasinski et al. 2000). Several sources supply acetyl-CoA molecules on the basis of cell biological status demand, including β-oxidation, ALC, AceSA1, AceSA2 and pyruvate dehydrogenase complex.

Pairing of folic acid, methionine and polyamines molecular pathways, are suggested to provide the methyl group for DNA methylation (Mato & Lu 2007).
3 Aims of the study

This study was carried out to investigate the effects of binge alcohol administration on the development of FAS during mouse development. The study focused on pathways that are engaged in the transition phase of the embryo from anaerobic into aerobic conditions during days 8-9.5 of the embryonic development. The aim of this study was to answer the following questions:

1. Why are the gestational stages 7-9 dpc so vulnerable to development of FAS?
2. Why does acute alcohol exposure cause a long-lasting intrauterine growth restriction?
3. What is the pathomechanism behind the development of neural tube defect and facial malformations?
4 Materials and methods

A more detailed description of the materials and methods can be found in the original papers (I-IV).

4.1 Animals and treatments (II, III, IV)

CD-1 mice were used to study the acute effects of alcohol on embryogenesis and placentation. The experiments were carried out in the laboratory animal center of the University of Oulu with the permission of the National Animal Experiment Board.

The pregnant mice were treated with two intraperitoneal (ip) injections of ethanol at 4-hr intervals. The injections contained 30% ethanol (3 g/kg) or Phosphate Buffered Saline (PBS) solution and were administered at 8.75 days post coitum (dpc). Presence of a copulatory plug on the noontime of the plug detection day was considered as 0.5 dpc. The mice were sacrificed by decapitation on pregnancy days 9.5, 10.5, 12.5 and 14.5 dpc. For whole-mount (II) experiments embryos’ head (IV), trunk (IV), or extraembryonic tissues, placenta and yolk sac (III), (IV) were isolated from CD1 mouse line for the planned experiments.

4.2 Cell culture (I)

The effect of DFMO on non-transgenic Rat-2 fibroblasts (ATCC, Rockville, MD, USA) was analyzed by differential display analysis (I). The Rat-2 cells were plated on 10 cm tissue culture dishes and grown in DEM supplemented with 2 mM glutamine, 100 µg/mL streptomycin and 100 units/mL penicillin and 5% (v/v) fetal bovine serum in 5% CO₂, 95% air at 37°C. For cell growth analysis, the cells were seeded on 10 cm dishes and maintained and treated with 5 mM DFMO for 90 min or 48 h prior to differential display and for 48 hours before exposure to 4 µM actinomycin D for the mRNA half-life assays.

4.3 Polyamine concentrations of in vitro and in vivo models (I, IV)

In vitro model polyamine concentrations in Rat 2 cells treated by DFMO were determined as described earlier (Gilbert et al. 1991) (I). Polyamine concentrations of the in vivo samples from the embryos of 9.5 dpc – 12.5 dpc: briefly, the embryonic and extraembryonic samples were homogenized in 300 µl of ice-cold
buffer (25 mM Tris pH 7.4, 0.1 mM EDTA, 1mM dithiothreitol). Aliquots (100 µL) of homogenates were precipitated with 100 µL of 10% sulfosalicylic acid containing 20 mM dianminoheptane. After centrifugation, the supernatant fractions were used for high-performance liquid chromatograph (HPLC) analysis (Hyvönen et al. 1995).

4.4 Northern blot analysis (I, II)

Total RNA (Chomczynski & Sacchi 1987) of Rat-2 cells (I) and adult mice tissues – kidney, ovaries, testes, lungs, brain and muscles (II) were fractionated (15-25 g/lane) on a 1% agarose gel containing formaldehyde, blotted onto nitrocellulose and hybridized with complementary DNA (cDNA) fragments labeled with [α-32P]dCTP using the random-priming method (Boehringer Mannheim). Membranes were hybridized with the full-length AceSA1 cDNA and stained with methylene blue as a loading control.

4.5 Whole-mount and thin section in situ hybridization (II)

Expression and localization of AceSA1 in embryos and tissues was studied by whole-mount and thin section in situ hybridization method according to standard procedures (Wilkinson 1992). The digoxigenin-labeled AceSA1 probe was prepared from the clone AF216873 in the pBluescript SK-Vector. We isolated and cloned AceSA1 from mouse kidney 16.5 dpc cDNA library.

4.6 Histology and immunohistochemistry (III)

The mouse placenta tissue samples for histology and immunohistochemistry were fixed in 4% para-formaldehyde (PFA), dehydrated through increasing ethanol series and then embedded in paraffin. The thin sections of control and alcohol treated embryonic tissues were immunostained by using the primary antibody of the VEGF, mouse anti-human VEGF (clone 26503 Sigma-Aldrich, MO, USA) dilution (1:200), and labeled secondary antibody, rat anti-mouse Alexa Fluor 488 (Jackson ImmunoResearch Laboratory), was used as a stain.
4.7 Western blot analysis (III)
Proper amounts (100 µg) of protein from alcohol-exposed and control placentas (9.5 dpc –14.5 dpc) were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto Immobilon-P membranes (Millipore) and probed with the following primary antibodies: anti-VEGF (BFD31, NeoMarkers), anti-PlGF (AF465, R&D Systems), and anti-α-tubulin (B-6199, Sigma-Aldrich). Antibodies were detected with HRP-conjugated secondary antibodies (Dako) and detection reagents (Thermo Scientific).

4.8 Transmission electron microscopy (III)
Placenta specimens were harvested at 10.5 dpc and 11.5 dpc, fixed and embedded in Epon LX 112 (Ladd Research Industries, Williston, Vermont, USA). Thin sections were cut with Leica Ultracut UCT ultramicrotome, stained in uranyl acetate and lead citrate and examined in a Philips CM100 transmission electron microscope. Images were captured with a Morada CCD camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

4.9 Feto-placental permeability assay (III)
The permeability of the placentas at 9.5 dpc–14.5 dpc was demonstrated as described previously (Pöschl et al. 2004) by injecting Evans blue (Sigma-Aldrich) into the tail vein of alcohol-exposed and control mice. Penetration of Evans blue color from the dam’s vein into the placenta and yolk sac was visualized and the dye concentration was measured at 640 nm. The obtained value was reported for each unit.

4.10 Whole-mount immunostaining PECAM (III)
Embryo heads were removed at 9.5 dpc and analyzed for immunocytology. The intrinsic endothelial cells were detected with an antibody against CD-31/PECAM, an endothelial adhesion molecule (BD Bioscience). For immunostaining, the samples were first fixed; then the secondary antibodies were horseradish peroxidase (HRP) anti-rabbit antibodies from Molecular Probes (USA). The primary antibody was incubated o/n at +4°C, washed and the secondary antibodies were added. Diaminobenzidine (DAB) (ZYMED, USA) was used as a substrate for
the color reaction. After developing the stain, the samples were post-fixed washed with 50% glycerol and stored in 80% glycerol for photographing. When a fluorescent secondary antibody was used no post-fixation with PFA was performed. The samples were photographed with a digital camera (Hamamatsu ORCA-ER) using an Olympus Cell M video microscope and the images were processed with Adobe Photoshop CS and Corel 12 software.
5 Results

This study aimed at reproducing FAS in the mouse model at the critical stage of 8.75 dpc. This enabled us to investigate further novel pathomechanism(s) causing intrauterine growth restriction and facial malformations in FAS. Alcohol administration caused malformation of both embryonic and extraembryonic tissues during mouse development, including the development of the yolk sac and placenta.

5.1 Macroscopic and microscopic phenotype of alcohol-exposed conceptuses (IV)

Alcohol-exposed embryos appeared smaller in size compared with controls (Fig. 1F). This is the most common phenotype in human FAS. In addition, defects in embryo turning and neural tube defects comprised alcohol-caused embryonic malformations at 9.5 dpc. The organs were visualized by light microscopy (III). Light microscopic analysis of the embryos showed that the morphology of ethanol-exposed embryos was altered in various organs, including open hemisphere, abnormal dilation of the cerebral vessels and dilated vascularization (Fig. 1F), which was visualized in the head area by whole-mount PECAM/CD31 immunostaining.

Haematoxylin-staining of the whole-mount β-galactosidase-stained embryos showed that neural crest cell migration was altered at 10.5 dpc. Histology of double-stained embryos at 10.5 dpc indicated neural tube defect in some of the alcohol-exposed embryos. Additionally, microscopic analysis of alcohol-exposed tissues showed malformations of the feto-maternal barriers (III) (Fig 2B, D, F). All feto-maternal structural barriers located between maternal and fetal sides were affected by alcohol administration. The affected tissues were: cell layers barriers, cell-cell connections (Fig. 2B, F), cell–Reichert’s membrane (Fig. 2F) connection, and gap junction (Fig. 2D) within the barrier cells.

5.2 Labyrinth zone or chorioallantoic structure (III)

Histological sections of placenta from control and alcohol-exposed pregnant mice indicated that labyrinth layer thickness was reduced in alcohol-exposed placenta (Fig. 1B).
Fig. 1. Microscopic (A, B) and macroscopic (C, D) morphology of FAS-placenta (11.5 dpc) confirmed that the labyrinth zone is reduced (B), as visualized by hematoxylin-staining of sagittal sections of control (A) and alcohol-exposed mice (B). Light microscopy showed that the alcohol-exposed embryos at 9.5dpc showed reduced vasculature in the brain, smaller head and facial morphology changes (F) compared with controls (E). Malfunction of the placenta-yolk sac, premature permeability, was visualized by passage of Evans blue dye through placenta-Reichert’s membrane barriers (G) and whole yolk sac in (E) control and (F) alcohol-exposed mice. Evans blue passage was increased in the alcohol-exposed yolk sac.
In rats and mice, the labyrinth zone is composed of syncytiotrophoblasts, chorionic trophoblasts, blood vessels and stroma. Of the three basic cell layers, two layers of trophoblasts (layer II and III) and one layer of cytotrophoblasts separate the maternal blood from the fetal blood. In alcohol-exposed placentas the normal barrier was affected. Transmission electron microscopy revealed the barrier in more detail. At 11.5 dpc, the trilaminar trophoblast layer appeared much thinner in alcohol-exposed placentas when compared with controls. Additionally, the membrane of the vessel endothelium was also thinner with irregularity in the shape (Fig. 2B, F).

5.2.1 Gap junction barrier (III)

The cytoarchitecture of alcohol-exposed labyrinth zone at 11.5 dpc was studied by transmission electron microscopy. Alcohol exposure had destructed the gap junctions that are located between layers II and III trophoblast cells (Fig. 2D). The structure of gap junction in mammalians is composed of two hemi-channels named connexins. They connect neighboring cells from each side of the channel (Fig. 2C). In control tissue, the transmembrane channels were tightly associated in the intracellular structure (Fig. 2C), but after alcohol exposure hemi-channels existed partially and were partially destroyed. Therefore, their association in the intracellular space might be destroyed.

5.2.2 Abnormalities of the placenta-yolk sac barrier in alcohol-exposed mice (III)

In CD-1 mice, placenta-yolk sac permeability is developmentally regulated during pregnancy. Reduced thickness of Reichert’s membrane and loss of tight connections with adjacent trophoblasts implied the disruption of the structure of the feto-maternal barrier and discontinued and lost connection between the Reichert’s membrane and parietal cells (Fig. 2F). In mice, disappearance of the decidua capsularis and Reichert’s membrane, permeability is developmentally regulated, but quantification of permeability in the placenta and yolk sac in the interval 10.5–14.5 dpc indicated a drastic increase in passive permeability in alcohol-exposed yolk sacs compared with controls (Table 4). The premature permeability was visualized by applying Evans Blue dye at different stages of development (9.5–14.5 dpc).
Fig. 2. (A, B) The cytoarchitecture of the labyrinth zone at 11.5 dpc showed that it consists of trilaminar trophoblast layers located between the maternal (M) and fetal (F) part. Of the layers, a bilayer of syncytiotrophoblast (II, III) cells surrounds the fetal blood vessel endothelium (E) which has a significantly reduced cellular volume in alcohol-exposed (E) mice. The other malformed barrier is the gap junction which is located between labyrinth layers II and III (C, D). The gap junction selectively allows small essential molecules such as glucose to pass into the fetal part. (E, F) Feto-maternal barriers in the placenta and yolk sac, which support the embryo as a primary placenta, were examined by transmission electron microscopy. Sections of Reichert's membrane (RM) at 11.5 dpc showed reduced thickness in alcohol-exposed (F) compared with control (E) mice. The membrane has two functions, a protective effect and as a main nutrient pathway at the early stage of embryo development. The barrier consist of Reichert's membrane, a layer of parietal endodermal cells (white arrow) which is superimposed on it, and the layer of parietal endodermal cells located within the yolk sac cavity. Of the corresponding parts of the barrier the giant trophoblast cells are located in the labyrinth (black arrow) which seems to have a loose connection and reduced contacts with Reichert's membrane (F).
5.2.3 Impact of permeability-regulating proteins PIGF/VEGF on yolk sac-placenta malfunction (III)

In addition to Reichert’s membrane malformation, an association between increased premature yolk sac-placenta permeability with PIGF/VEGF ratio was also detected. Under normal conditions VEGF decreases from 8.75 dpc to 14.5 dpc, while PIGF stays at the same level during normal embryonic development. However, under alcohol administration during 9.5–11.5 dpc both PIGF and VEGF were upregulated (Table 3).

5.2.4 Defect in heterodimer formation of VEGF and PIGF (III)

Western blotting of the placental proteins was performed to examine candidate genes that might potentially possess a regulatory role in the development of the premature permeability of yolk sac-placenta barriers. Under normal conditions, both PIGF and VEGF were developmentally regulated, but both were regulated by a specific pattern. The PIGF pool was significantly upregulated in FAS placenta, followed by falling down below the level of the control at 14.5 dpc. In control placentas the angiogenesis-regulating VEGF protein pool was analyzed by Western blotting of placental tissues at 9.5–14.5 dpc. VEGF was expressed as two isoforms, the low molecular weight (LMW-VEGF) and high molecular weight (HMW-VEGF) isoform, with different expression patterns. In placentas, due to alcohol administration, expression of the LMW-VEGF isoform was increased at 9.5 dpc and fell below the level of the control placentas. Concerning HMW-VEGF, the level of isomer was higher in the alcohol-exposed mice at 9.5 and 11.5 dpc and decreased at 14.5 dpc to a level even lower than the controls. Under the alcohol treatment, the ratio of PIGF to both isoforms of VEGF was higher at 11.5 and 14.5 dpc. In agreement with previous results, native Western blotting of placental tissues showed that intracellular PIGF/VEGF heterodimer increased. In parallel, VEGF/DAPI immunostaining indicated that the pool of VEGF was mostly accumulated inside the cells of the syncytium. It has previously been shown that VEGF may stimulate angiogenesis if the protein is localized in the extracellular space and on the other hand, is co-expressed in the same cell with PIGF (Table 3).
5.3 The AceSA1 expression is downregulated in response to DFMO
(I)

Differential display was repeated twice in Study I. In response to DFMO treatment after 48 hours, polyamine pools were depleted (Table 1).

<table>
<thead>
<tr>
<th>Gene/metabolite</th>
<th>Methods</th>
<th>Treatment</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>AceSA1</td>
<td>Northern blot</td>
<td>DFMO</td>
<td>AceSA1 ↓</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td>GAPDH ↑</td>
</tr>
<tr>
<td>S-AdoMetDC</td>
<td></td>
<td></td>
<td>S-AdoMetDC ↑</td>
</tr>
<tr>
<td>Acetoacetyl-CoA thiolase</td>
<td></td>
<td></td>
<td>Acetoacetyl-CoA ↓</td>
</tr>
<tr>
<td>L3</td>
<td></td>
<td></td>
<td>thiolase ↑</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td>L3 ↑</td>
</tr>
<tr>
<td>ADP/ATP translocase</td>
<td></td>
<td></td>
<td>β-actin ↑</td>
</tr>
<tr>
<td>3Sa</td>
<td></td>
<td></td>
<td>ADP/ATP translocase ↑</td>
</tr>
<tr>
<td>Polyamines</td>
<td>HPLC</td>
<td>DFMO (48h)</td>
<td>Putrescine ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spermidine ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Spermin</td>
</tr>
<tr>
<td>S3a</td>
<td>Northern blot</td>
<td>DFMO</td>
<td>S3a ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Putrescine</td>
</tr>
</tbody>
</table>

The Rat-2 cell samples were analyzed and we identified 35 mRNAs in response to 48 hours of DFMO treatment. We detected 35 cDNA bands and 16 of them were downregulated. The results of differential display were further confirmed by Northern blot analysis of 11 bands. Out of the 11 clones two are engaged in the production of acetyl-CoA (Table 1).

The AceSA1 was one of those clones that were downregulated; it was characterized as a cytosolic acetyl-CoA synthetase (EC 6.2.1.1) and the other was Acetoacetyl-CoA thiolase (EC 2.3.1.9), which was upregulated. The AceSA1 needs one molecule of ATP to activate acetate and ligate it with CoA. On the other hand, acetoacetyl-CoA thiolase is implicated in the beta-oxidation of fatty acids. In agreement with our present data, an investigation by Dierks-Ventling & Cone (Dierks-Ventling & Cone 1971) indicated that after 24 hours’ starvation thiolase activity reaches a high level in the brain and after 48 hours in the liver. This was found to be the case in our preliminary experiments as well (Table 1).
AceSA1 gene expression in adult mouse tissues (II) by Northern blot hybridization of adults’ tissues (ovaries, testes, kidney, liver, lung, heart, brain, muscle) showed the highest expression of AceSA1 in the kidneys, ovaries, and testes (Table 2).

In this study, the full sequence of mouse cDNA clone (AF216873) AceSA1 gene was isolated and sequenced for the first time. It was isolated from the cDNA library of a 16-dpc embryonic kidney.

<table>
<thead>
<tr>
<th>Table 2. Expression of AceSA1 in embryo, fetus and adult tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment</strong></td>
</tr>
<tr>
<td>In vivo</td>
</tr>
<tr>
<td>Embryo</td>
</tr>
<tr>
<td>Fetus</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td>In vivo</td>
</tr>
<tr>
<td>adult tissues</td>
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</tbody>
</table>

AceSA1 gene expression in adult mouse tissues (II) (ovaries, testes, kidney, liver, lung, heart, brain, muscle) by Northern blot hybridization showed the highest expression of AceSA1 in the kidneys, ovaries, and testes (Table 2).

In this study, the full sequence of mouse cDNA clone (AF216873) AceSA1 gene was isolated and sequenced for the first time. It was isolated from the cDNA library of a 16-dpc embryonic kidney.

5.4 Whole mount in situ hybridization analysis of AceSA1 (II)

In this study, the expression of AceSA1 was localized in CD-1 mice during development. We found that the expression of AceSA1 is developmentally regulated. At embryonic stage 9.5 dpc the AceSA1 expression was localized in the cephalic region and in later stages, at 10.5 dpc, the transcripts also appeared in the...
spinal cord and dorsal root ganglions. Whole mount in situ analysis of alcohol-exposed mouse embryo CD-1 indicated that alcohol inhibits AceSA1 gene expression in the embryos when compared with controls (unpublished data). During organogenesis at 11.5 dpc stage of development AceSA1 expression was detected in the liver, and at stage 12.5 dpc, in the interstitial Leydig cells. During progressing embryo development, expression of AceSA1 was visualized at 13.5–15.5 dpc in the ovarian interstitial component. At 16.5 dpc, transcripts of AceSA1 appeared in the renal tubules (Table 2).
5.5 PECAM as an angiogenesis-regulating factor in embryonic and extraembryonic tissues (III, IV)

Whole-mount immunostaining of the mouse embryo at 9.5 dpc with CD-31/PECAM (an endothelial adhesion molecule) was performed to investigate the formation of blood vessel network in the early stages of embryonic development (Table 3) and placental tissue development. In the embryo head exposed to ethanol, we detected abnormal dilution of the cerebral vessels as a dominant defect with reduction of vessel branching and altered normal vascular development. In placenta no significant change was detected in angiogenesis. In fact, the labyrinth zone was altered in ethanol-exposed placentas with anatomical abnormalities, with a reduced surface area in the labyrinth (Table 4).

5.5.1 Impact of permeability-regulating proteins PIgf/VEGF on yolk sac-placenta malfunction (III)

In addition to Reichert’s membrane malformation, an association between increased premature yolk sac-placenta permeability with PIgf/VEGF ratio was also detected. Under normal conditions VEGF decreases from 8.75 dpc to 14.5 dpc, while PIgf stays at the same level during normal embryonic development. However, under alcohol administration during 9.5–11.5 dpc both PIgf and VEGF were upregulated (Table 3).

5.5.2 Defect in heterodimer formation of VEGF and PIgf (III)

Western blotting of the placental proteins was performed to examine candidate genes that might potentially possess a regulatory role in the development of the premature permeability of yolk sac-placenta barriers. Under normal conditions, both PIgf and VEGF were developmentally regulated, but both were regulated by a specific pattern. The PIgf pool was significantly upregulated in FAS placenta, followed by falling down below the level of the control at 14.5 dpc. In control placentas the angiogenesis-regulating VEGF protein pool was analyzed by Western blotting of placental tissues at 9.5–14.5 dpc. VEGF was expressed as two isoforms, the low molecular weight (LMW-VEGF) and high molecular weight (HMW-VEGF) isoform, with different expression patterns. In placentas, due to alcohol administration, expression of the LMW-VEGF isoform was increased at 9.5 dpc and fell below the level of the control placentas. Concerning HMW-VEGF, the level
of isomer was higher in the alcohol-exposed mice at 9.5 and 11.5 dpc and decreased at 14.5 dpc to a level even lower than the controls. Under the alcohol treatment, the ratio of PIGF to both isoforms of VEGF was higher at 11.5 and 14.5 dpc. In agreement with previous results, native Western blotting of placental tissues showed that intracellular PIGF/VEGF heterodimer increased. In parallel, VEGF/DAPI immunostaining indicated that the pool of VEGF was mostly accumulated inside the cells of the syncytiu m. It has previously been shown that VEGF may stimulate angiogenesis if the protein is localized in the extracellular space and on the other hand, is co-expressed in the same cell with PIGF (Table 3).

Table 3. Placenta yolk sac VEGF/PIGF and PECAM localization and pools are developmentally regulated.

<table>
<thead>
<tr>
<th>Experiment/gestation</th>
<th>DNA/Gene/tissues</th>
<th>Methods</th>
<th>Treatment</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5 dpc DNA staining</td>
<td>H&amp;E sections (paraffin sections)</td>
<td>EtOH/PBS</td>
<td>Vocules ↑ labyrinth zone reduced ↓</td>
<td></td>
</tr>
<tr>
<td>PECAM/CD31 VEGF DAPI</td>
<td>Whole mount immunostaining (paraffin sections)</td>
<td>EtOH/PBS</td>
<td>PECAM ± no change in angiogenesis VEGF ↑</td>
<td></td>
</tr>
<tr>
<td>9.5-11.5 dpc VEGF</td>
<td>Western blotting</td>
<td>EtOH/PBS</td>
<td>LMW-VEGF ↑ HMV-VEGF ↑ induced permeability</td>
<td></td>
</tr>
<tr>
<td>PIGF</td>
<td>Western blotting</td>
<td>EtOH/PBS</td>
<td>PIGF ↑ Heterodimer</td>
<td></td>
</tr>
<tr>
<td>PIGF/VEGF Barriers/placenta Dam, conceptus -Yolk sac</td>
<td>Native Western blotting Spectrophotometric (640 nm)</td>
<td>EtOH/PBS/ EvansBlue</td>
<td>PIGF/VEGF ↑ Permeability ↑</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Response of the CD-1 mouse extraembryonic tissues to alcohol

<table>
<thead>
<tr>
<th>dpc stage</th>
<th>Barrier cells</th>
<th>Localization</th>
<th>Methods</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5</td>
<td>Trilaminar trophoblast</td>
<td>Placenta</td>
<td>Transmission electron microscopy</td>
<td>Much thinner</td>
</tr>
<tr>
<td>11.5</td>
<td>Vessel endothelium</td>
<td>Placenta</td>
<td>Transmission electron microscopy</td>
<td>Thinner and irregular</td>
</tr>
<tr>
<td>11.5</td>
<td>Syncytiotrophoblasts trophoblast layers II and III</td>
<td>Placenta</td>
<td>Transmission electron microscopy</td>
<td>Gap junctions destroyed</td>
</tr>
<tr>
<td>11.5</td>
<td>Parietal – Reichert's membrane</td>
<td>Yolk Sac</td>
<td>Transmission electron microscopy</td>
<td>Discontinue and lost contact between parietal cells and Reichert's membrane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yolk Sac-placenta</td>
<td>Transmission electron microscopy</td>
<td>Trophoblast not well aligned, and had lost tight connections with adjacent trophoblasts</td>
</tr>
<tr>
<td>11.5</td>
<td>Reichert's membrane</td>
<td>Yolk sac</td>
<td>Transmission electron microscopy</td>
<td>Reduced in thickness</td>
</tr>
<tr>
<td>9.5-11.5</td>
<td>Placenta-Yolk Sac permeability and PIGF/VEGF ratio</td>
<td>Placenta-Yolk Sac</td>
<td>Spectrophotometry</td>
<td>Albumin-Evans normally does not cross the placenta. Higher leakage of the tracer. permeability ↑</td>
</tr>
</tbody>
</table>

5.6 Polyamine pools are developmentally regulated in embryonic and extraembryonic tissues (IV)

In this investigation we studied polyamine pools during gestational stages of 9.5 dpc–12.5 dpc in CD-1 embryos. The polyamine pools are developmentally regulated, and under alcohol administration the polyamine levels were altered with different patterns. The perturbation depended on gestational stage, tissue type and the type of polyamine. It was found that in the brain, alcohol treatment harshly targeted polyamine pools at 9.5 dpc. Brain showed an abolishment of polyamines
at 9.5 dpc, which was compensated at gestational stage 12.5 dpc (Table 5). Furthermore, embryonic tissue, head and trunk, and extraembryonic tissues, yolk sac and placenta, did not respond to polyamine changes with the same pattern.

**Table 5. Alcohol administration alter polyamine pools, PECAM, angiogenesis and vascularization, and development of neural tube defect in CD-1 mouse embryonic and extraembryonic tissue at 9.5dpc**

<table>
<thead>
<tr>
<th>Gene/metabolite/embryo</th>
<th>Tissue</th>
<th>Methods</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo brain</td>
<td>light microscopy</td>
<td>Defect in bending and neural tube defect</td>
<td></td>
</tr>
<tr>
<td>Hemisphere Brain</td>
<td>Paraffin- transverse sections toluidin blue</td>
<td>Altered hemisphere closure</td>
<td></td>
</tr>
<tr>
<td>Hemisphere Brain</td>
<td>Eosin+Hematoxyline X-gal staining sagittal sections</td>
<td>Altered hemisphere closure</td>
<td></td>
</tr>
<tr>
<td>PECAM/CD31 Embryo head</td>
<td>Whole-mount immunostaining</td>
<td>Abnormal dilation of cerebral vessels, and reduced vessel branching, and prevents normal vascular development</td>
<td></td>
</tr>
<tr>
<td>Polyamine pools Head</td>
<td>HPLC</td>
<td>Putrescine ↓ Spermine ↓</td>
<td></td>
</tr>
<tr>
<td>Polyamine pools Trunk</td>
<td>HPLC</td>
<td>Putrescine ↓ Spermine ↓ Spermine ↑</td>
<td></td>
</tr>
</tbody>
</table>

5.7 **DFMO and alcohol have a common target molecular pathway but in different manners (I, IV)**

The DFMO-treated Rat-2 embryonic fibroblast cells irreversibly inhibited ornithine decarboxylase (ODC), which is a rate-limiting enzyme in polyamine synthesis, and consequently abolished polyamine pools (Table 1). The same phenomenon was also true for alcohol. Alcohol in brain tissue abolished the polyamine pool, but the defect
was not irreversible. Under DFMO treatment, the uptake of polyamines from extracellular sources was not totally inhibited because DFMO only affects putrescine and spermidine pools, but not the spermine pool. On the contrary, alcohol caused total abolishment of polyamines but the perturbation was not irreversible, as the polyamine pool was regained back to the level of the controls at the developmental stage 12.5 dpc (Table 1 and 5) (Fig. 3).

Fig. 3. Molecular pathways targeted by alcohol and DFMO. Both alcohol and DFMO depleted polyamine pools in the in vitro and in vivo model. The ODC is the primary target for both of them, simultaneously also targeting AceSA1 gene expression. Acetaldehyde, the alcohol metabolite targeting glycolysis, which leads to reduction of the ATP pool. ATP is a substrate for AceSA1 and other biological functions, including acetyl-CoA, are required for histone acetylation. The methionine molecular pathway also interacts with polyamine synthesis by providing deacetylated SAM and also acts as a methyl donor for DNA methylation. Polyamines, acetyl-CoA and methionine pools interact in regulating cellular growth and energy, which is perturbed in alcohol-exposed animals.
6 Discussion

The aim of this investigation was to identify novel pathomechanisms that cause intrauterine growth restriction and facial malformation in the FAS mouse model. The FAS is a sum of major and minor defects appearing in some fetuses exposed to alcohol during critical stages (7-9 dpc) of embryo development. The symptoms include intrauterine growth restriction, facial abnormalities and defects in the central nervous system. Three major defects, i.e., facial malformation, intrauterine growth restriction and mental retardation, have received more attention than any other FAS abnormalities. The head of the ethanol-treated embryo is smaller, which is a typical phenotype of intrauterine growth restriction. In FAS model, the smaller body size is accompanied by an altered embryo turning (Miller & Wells 2011).

Intrauterine growth restriction (IUGR) is the outcome of several defects. As an acute factor, abolished polyamine pools halt growth and development while other defects impose a long-lasting abnormal condition on the embryo. In addition to growth halt due to polyamine abolishment, structural abnormality in the nutrient-supplying and up-taking networks may cause long-lasting defects such as IUGR. Reduced size of the labyrinth zone may limit the embryo’s access to nutrients. Abnormal dilation of cerebral vessels, reduced vessel branching and limited vascular development all cause insufficient supply of nutrients to the embryo.

In the FAS mouse model, neural crest cell migration was perturbed. The defect was verified by β-galactosidase-staining of control and alcohol-exposed embryos at 10.5 dpc. A previously published paper indicated that neural crest cell migration is regulated by several molecular pathways including the gap junction, which has a central regulatory role in it (Huang et al. 1998). In the same line, the mouse neural crest motility could be modulated by a connexin 43 gap and polyamines. The alcohol-exposed embryos also have a significantly reduced number of cranial neural crest cells (Flentke et al. 2011, Garic et al. 2011) which is detectable in their derived facial bone and cartilage, cranial nerve, tooth structure and cardiac outflow tract. Small palpebral fissures, mid-facial flattening and reduction, and an elongated upper lip with a deficient philtrum are facial changes observed in FAS. Migration of the neural crest normally has a pattern, and ethanol exposure may cause rapid cytoskeletal remodeling within the neural crest. The actin bundle is reorganized and thickened to define a linear, bipolar axis (Hassler & Moran 1986).

The data indicated that alcohol exposure stormed the organism by several waves of insults that targeted embryonic and extraembryonic tissues in different manner. The embryonal stages from 7 dpc to 9 dpc are critically sensitive to alcohol.
exposure. So the question is, what makes the embryogenesis and placentation so vulnerable to teratogen at the critical stages of development? The embryonic and extraembryonic tissues may be targeted by alcohol directly or through its metabolites at several gestational stages and in different manner. In our mice model, the defects were divided into short term, 12 hours after the alcohol at 9.5 dpc, and long term, 12.5 up to 14.5 dpc.

Fig. 4. Presentation of embryonic shift which is targeted by alcohol administration. At gestation 8.5 dpc, the embryo uses anaerobic metabolism and is gradually shifting into aerobic metabolism. Due to the shift from organogenesis into proliferation stage, the demand for nutrients and energy sources such as ATP increases. Thus, metabolism shifts from glycolysis into an oxidative phosphorylation. Ethanol administration at this crucial and vulnerable gestational stage imposes a more lasting hypoxia condition; thus, the transition is halted, and the hypoxia condition may be prolonged on the fetal side. FAS and hypoxia in pregnancy seem to have partially similar facial malformation phenotypes. The graph is completely modified from Baker & Ebert 2013.
6.1 Direct effects of ethanol

Alcohol perturbs the structural modification of chromatin, depending on the gestational stage of embryo tissue, and particular chromosomes under alcohol administration (Liu et al. 2009). Our unpublished microarray data indicated that ethanol administration (3 g/kg) at 10.5 dpc altered significantly the expression of only 350 genes, but the same exposure at 8.75 dpc altered significantly the expression of several thousands of genes. In this study we used a pool of four whole embryos, not specific tissue, to study the intrauterine growth restriction. In other words, we were looking for a common pathomechanism for all kinds of tissues that could have altered global gene expression together with a symmetrical intrauterine growth restriction, a widely reported phenotype in alcohol-exposed fetuses.

Polyamine pools in the embryonic head were abolished shortly after the ethanol treatment. Inhibition of ODC, a rate-limiting enzyme of polyamine synthesis, by ethanol is well-established (Shibley et al. 1995). In this mouse model, an acute alcohol exposure abolished the polyamine pools in the embryos’ brain with a less extensive and different pattern in trunks and in extraembryonic tissues, placenta and yolk sac. Abolishment of the polyamines to an undetectable level in the brains of embryos may be due to inhibition of de novo synthesis or to inhibited uptake from exogenous sources. In mice, demand for polyamine synthesis is met by maternal sources during the early stage of development (Pendeville et al. 2001). It is well established that ethanol targets cell cycles at G1 phase and prolonged S phase (Siegenthaler & Miller 2005). In the same way, polyamine synthesis inhibitors such as DFMO also halt the cell cycle at G1 with a similar pattern. Polyamines at physiological concentrations have an impact on the cell and organism. They can condense chromatin and promote B to Z DNA transition (Howell et al. 1996). A minimal level of polyamines is required for the cell to move from G1 to S phase. The contribution of polyamines to overall chromosome conformation and the accumulation of polyamine-depleted cells in the S-phase indicate that polyamines possess a cationic nature and strong affinity for DNA. Thus, the polyamines are directly involved in the DNA synthesis (Balasundaram & Tyagi 1991) that concerns all dividing and differentiating cell types in the embryo, with symmetric intrauterine growth restriction as an outcome.

Polyamines may also play a major role in ionic and osmotic stress in many organisms (Tabor & Tabor 1976). Acidification due to polyamine depletion has far-ranging effects on almost all aspects of cell physiology, including protein synthesis, which is a Ca2+/ calmodulin-dependent process. Administration of exogenous
putrescine restores the intracellular pH (pHi) in DFMO-treated cells and reverses the growth inhibition (Poulin & Pegg 1995). Polyamine depletion thus targets intracellular pHi directly. Therefore, it is plausible to assume that the abolishment of polyamines in the fetal brain shortly after ethanol administration causes a global change in the intracellular pH and leads to growth inhibition. Due to their polycationic properties, polyamines are capable of interacting with all sorts of micro and macro molecules that are negatively charged, such as DNA, mRNA, proteins etc. Thus, the main fundamental functions of the organism including DNA, mRNA and protein synthesis experience a growth halt shortly after the alcohol administration and depletion of polyamine pools. This defect may be more escalated by simultaneous reduction of cell energy production (Fig. 3). An in vitro model (I) with polyamine-depleted cells showed simultaneous reduction of expression of AceSA1 which is engaged in the production of acetyl-CoA as a master metabolite in the organism. The nuclear-cytosolic AceSA1 plays a role in post-modification of proteins, especially histone acetylation (Fig. 3). Our unpublished data indicated that AceSA1 expression was sharply reduced in the brains of mice embryos exposed to ethanol. Polyamine pools induce histone acetylation (Pasini et al. 2014). Therefore, it is reasonable that polyamine pools and AceSA1 gene expression moved side by side. Polyamines also have a regulatory role for the gap junction (Shore et al. 2001) which functions as a sieve to allow the passage of substances such as ions and glucose.

In the FAS brain, abolishment of polyamines might have caused functional perturbation of the gap junction, and might thus have allowed the passage of glucose and other small molecules into brain tissue. The data presented here indicated that polyamine depletion in the embryo brain in the short term, at 9.5 dpc, was associated with abnormal dilation of cerebral vessels and reduced vessel branching, and prevention of normal vascular development. This is in agreement with the data of Kucharzewska (Kucharzewska et al. 2010) suggesting that ornithine decarboxylase and extracellular polyamines regulate microvascular sprouting. Ornithine is an amino acid needed for the synthesis of polyamines during embryogenesis.

Amino acid uptake by the yolk sac is a major nutrient supply pathway in the early stage of development. In rodents, pinocytosis occurs by parietal cells in the yolk sac. The role of parietal endoderm as well as visceral endoderm as mediators of nutrient-waste exchange for the developing embryo was suggested by Moerkamp (Moerkamp et al. 2013). Our results indicate that in the CD-1 FAS mice model a reduced number of aligned cells on the thinner Reichert’s membrane
structure may have an effect on nutrient uptake by parietal cells. An investigation by Xu (Xu et al. 2005) indicated that in alcohol-exposed mouse visceral yolk sac endodermal cells, pinocytotic invaginations and storage vesicle were all decreased in number compared with the control group. In addition, abnormality of Reichert’s membrane structure in the yolk sac caused reduction of barrier thickness, making the barrier more vulnerable to toxicity. Brent and Fawcett (Brent & Fawcett 1998) suggested that the process of pinocytosis and the delivery of amino acids to the developing embryo may be altered due to teratogenic effects by antiserum.

In alcohol exposure, the neural tube may suffer from glucose insufficiency from the amniotic fluid source. In FAS model there is a similar situation due to structural abnormalities in the yolk sac. Our unpublished data indicated that reduced lengthening of microvilli was localized on the neural tube. An association of low glucose concentration with very short microvilli has been reported by Shepard (Shepard et al. 1997). Thus, the shortened microvillus alters the uptake of glucose molecules into the neural tube, which may in turn restrict normal growth and development in the tissue and development of neural tube defects. Shorter microvilli absorb less glucose, which escalates the situation-limited glucose uptake and consequently restricts energy production through glycolysis.

Polyamines also modulate biological processes responsible for conversion of EMT or vice versa. During embryonic and fetal development the transition is engaged in gastrulation, mesoderm formation, neural crest development, heart valve development and craniofacial structure formation (Compagnone et al. 2012), which is a critical issue in the development of facial malformation in the FAS model.

In addition to polyamines at 9.5 dpc, 12 hours after alcohol administration, several protein pools were changed. PECAM-1 expression was reduced in the cephalic region. The PECAM reduction might cause both altered morphology of microvascular formation and perturbation in migration across the microvasculature in the brain (Akers et al. 2010). Thus, reduced distribution of nutrients and excretion of waste could be a consequence. Dilation of cerebral vessels, reduced vessel branching, and prevention of normal vascular development were detected.

At later stages, due to dilation of vasculature in the brain, nutrient and waste transportation in FAS animals may be perturbed. In rodents at early stages of development, the nutrient supply to the embryo is totally dependent on the yolk sac.

The barrier passively filters nutrients (Richardson et al. 2000). Alcohol targets the barrier that surrounds the embryo. The structure consists of trophoblast, Reichert’s membrane and visceral endoderm. The disappearance of decidua capsular and Reichert’s membrane is developmentally regulated. Reichert’s
membrane remains intact but may break at any time around 19 dpc (Dickson 1979). The thickness of the alcohol-exposed Reichert’s membrane was markedly reduced and lost tight interaction with the connected cells, being close to rupturing in some areas. The malformed structure somehow showed to have similarity with collagen IV deficiency which is involved in immunological embryo toxicity (Leung et al. 1977). In another study, Pöschi et al., (2003) showed that Reichert’s membrane of collagen IV-deficient embryo appeared to be thinner and ruptured.

Alcohol in the short term (9.5 dpc) caused upregulation of two proteins, PIGF/VEGF, which in the placenta have been associated with hypoxia, angiogenesis and permeability. The organism may implement a compensatory pathway to increase angiogenesis and expand the labyrinth zone in the alcohol-exposed conceptus, or increase the permeability to meet the demand for nutrients. The PIGF/VEGF ratio was increased during 9.5-14.5 dpc stages and consequently increased the permeability of the placenta-yolk sac, resulting in the barrier to become thinner and consequently, more vulnerable to immunological embryo toxicity.

The upregulated VEGF may not able to induce angiogenesis; on the other hand, extracellular PIGF is known to enhance the synthesis and secretion of VEGF (Bottomley et al. 2000). Also PIGF-1 can antagonize the VEGF angiogenesis capability by locating in the same cell (Björndahl et al. 2004), which leads to formation of intracellular heterodimer. Our results also indicated that although alcohol increased the PIGF expression it was unable to alter the VEGF localization and heterodimer formation. Perturbation of PIGF/VEGF may cause stormy changes in the FAS conceptus, which affects embryo and placenta growth and development. The PIGF/VEGF is an indicator of three distinct types of hypoxia. The hypoxia condition causes dramatic challenge in the placental unit and influences fetoplacental angiogenesis (Kingdom & Kaufmann 1997). In alcohol-exposed mice placenta at 9.5 dpc, a lower ratio of PIGF/VEGF was detectable at a relatively limited time (12 hours). The situation started to change by a shift in PIGF/VEGF at 11.5 dpc with a peak level during 9.5–14.5 dpc. The upregulated PIGF/VEGF may indicate a normoxic situation in the placenta. Upregulation of PIGF may have deleterious effects on placenta growth and development (Ahmed et al. 2000). Of three distinct types of hypoxia, it seems that the FAS condition is not pre-placental hypoxia, because according to Kerbs (Krebs et al. 1997) peripheral placental villi showed increased branching angiogenesis with rich branch but shorter terminal capillary loops. Furthermore, the change in the PIGF/VEGF ratio is not uteroplacental hypoxia (placenta and fetus are hypoxic) because we should detect
an increased expression of VEGF and reduced expression of PI GF, which lead to changes in angiogenesis. The situation in the FAS placenta seems to be post-placental hypoxia at 14.5 dpc, (the fetus is hypoxic, whereas the mother is normoxic) because in this situation PI GF was increased while VEGF was decreased. Although the labyrinth zone is reduced, the situation could resemble decreased branching angiogenesis and failure of terminal villi formation (Ahmed et al. 2000).

6.2 Acetaldehyde, the first metabolite of ethanol, as a teratogen

In a dam exposed to ethanol, intoxication by alcohol proceeds by metabolizing it in the liver. Acetaldehyde is the primary hepatic oxidative metabolite of ethanol and it reaches the fetus either by placenta production or by placental transferase, which in turn could affect fetal growth and development (Bosco & Diaz 2012). Increased placenta-yolk sac permeability may allow more acetaldehyde to pass to the embryo side. Alcohol administration during embryogenesis and organogenesis, the most sensitive periods of pregnancy, impairs the homeostasis of glucose (Murdoch & Simm 1992). Acetaldehyde, the primary metabolite of alcohol, may also impair the pyruvate dehydrogenase activity (Hard et al. 2001) which catalyzes the breakdown of glucose molecule into two molecules of acetyl-CoA. Thus, the homeostasis of anaerobic and aerobic metabolism pathways for energy production might suffer under alcohol metabolism.

The impaired development of mitochondria plays a role in central nervous system defects in FAS. The mitochondria play an essential role in cell metabolism, and poorly developed mitochondria lead to abnormality of the whole cell, including proliferation (Xu et al. 2005). Due to alcohol treatment, production of ATP as a main source of energy and acetyl-CoA as a master molecule to be utilized in many biological pathways is restricted. Under normal development at gestation 8.5 dpc, there is a shift from anaerobic to aerobic metabolism known as the embryonic shift. Biologically it is a shift from glycolysis into oxidative phosphorylation (Baker & Ebert, 2013).

In animals, ATP synthesis by oxidative phosphorylation is the fundamental means of cell energy production (Senior et al. 2002). However, under alcohol administration, the organism might be forced back to a more hypoxic condition or halt at that situation. Ethanol metabolism induces cellular oxidative stress by suppression of oxidative phosphorylation and NADH accumulation (Cunningham & Van Horn 2003). Hypoxia occurs when oxygen demand exceeds delivery (Metzen 2007). At normal development, metabolism shifts from anaerobic to
aerobic at 8.5dpc and consequently (Fig. 4), the demand for oxygen is enhanced, but due to perturbation caused by alcohol exposure the transition may face difficulty. The hypoxic state is assayed by measuring the protein pool of VEGF and PI GF markers. Upregulation of VEGF and PIGF in the placenta (9.5 dpc–11.5 dpc) may indicate an imposed hypoxic condition on the conceptus.

Conceptus tissues respond to alcohol exposure in different manner. Immunostaining of brain tissue by PECAM indicated reduced angiogenesis in the brain with no changes in the placenta. Access to nutrients seems not to be a crucial factor to prevent growth restriction. In chicken, inhibition of polyamine synthesis blocks embryo development even if the embryo has continuous access to nutrients. In fact, the alcohol-exposed gestational stage is crucial for the development of vitelline circulation. The embryo allows the shift from reliance on diffusion-dependent nutrition delivery to a more efficient system of vascular conduits (Jollie 1990). In our FAS-model, the labyrinth was smaller, the gap junctions were damaged and the polyamine pools that play a role as gap junction regulators were abolished. On the other hand, we showed that also the structure and function of yolk sac were perturbed due to alcohol administration. In mice, the yolk sac has a crucial role in the early embryo development, and exposure to teratogen has consequences for embryo development.

It seems that under normal conditions, by shifting from anaerobic into aerobic metabolism, the AceSA1 gene expression was also increased in the cephalic region. Developmentally regulated AceSA1 gene expression might also be in agreement with shifting from glycolysis to oxidative phosphorylation, due to demand for ATP as a substrate of AceSA1. In fact, during organogenesis and fetal proliferation, the nuclear-cytosolic localization of AceSA1 might be crucial in dynamic histone acetylation and lipogenesis (Fig. 3).

Growth restriction defects due to FAS might retard embryo growth and development by imposing a hypoxic condition. Thus, polyamine deficiency caused either by polyamine synthesis inhibitors (DFMO) or alcohol exposure share a common pathomechanism (Fig. 3) resulting in craniofacial malformation and defects in the labyrinth. Similar craniofacial defects and neuronal deficits have also been detected due to hypoxia and ethanol administration (Cudd et al. 2001).

In an in vitro model in Rat-2 embryonic fibroblast cells, inhibition of polyamine synthesis by DFMO simultaneously reduced expression of nuclear-cytosolic AceSA1. It seems that a global shift in polyamine pools immediately targets expression of AceSA1, which might be engaged in several biological functions, including lipogenesis and histone acetylation (Fig. 3). Cells produce
acetyl-CoA from different sources and have a baseline for the acetyl-CoA pool. Under abnormal conditions, AceSA1 may contribute efficiently to the homeostasis of the acetyl-CoA pool. Thus, teratogens that cause intrauterine growth restriction may have a common molecular pathomechanism, viz. reduced polyamine synthesis and reduced nuclear-cytosolic AceSA1 synthesis. Highly expressed AceSA1 in tissues such as the kidney, which is functionally not engaged in lipogenesis, may indicate a dynamic and vital role for the tissue with specific functions. This is true especially for kidney where the AceSA1 expression is high. After a binge acetate due to acetaldehyde metabolism, AceSA1 under critical stages, homeostasis of histone acetylation and deacetylation, the organism may try to save metabolite homeostasis to dispose of the released acetate in the nucleus by recycling it back to acetyl-CoA, to be used for histone acetylation. The produced acetyl-CoA might selectively acetylate a group of histone chromatin of the gene clusters that are able to maintain the organism under intoxication. We have observed that alcohol administration inhibits neural crest migration. Previously we suggested that the nutrient uptake could be targeted due to defective pyruvate dehydrogenase activity. In addition, dynamic AceSA1 expression in the nuclear cytoplasm may also play an essential role in de novo synthesis of cholesterol in the brain. In mouse embryos, the cholesterol required by the brain mostly comes from de novo synthesis (Yoshida & Wada 2005). Cholesterol is a prerequisite for activating sonic hedgehog signal molecules, which are engaged in the regulation of neural crest migration. Our unpublished data indicated that cholesterol pools were significantly reduced under alcohol administration. Therefore, the neural tube defect in the FAS embryo may be caused by altered growth and development of the neural tube cells. The neural crest migration defect might be a sum of several factors including polyamine deficiency, perturbation of glucose uptake and metabolism, destruction of the gap junction and Reichert's membrane malformation and malfunction.

In rodents, de novo synthesis of cholesterol is suggested to play a crucial role in the supply of lipids to the embryo. Alcohol may halt the organism at G1 cell cycle, in the same way as DFMO, a rate limiter of polyamine synthesis that also halts cells at G0 cycle. In agreement with the results, AceSA1 expression pattern and polyamine pools reduction move hand in hand. Acetyl-CoA is a master molecule donating two carbon atoms and connecting several metabolic pathways. It is generated from the breakdown of carbohydrate, lipids, or amino acids, or synthetized from acetate. Alcohol administration roughly targets all of them with different pathomechanisms.
A study by Tokutake (Tokutake et al. 2010) indicated that the size of acetyl-CoA pools appears to be larger during feeding in the liver, heart, and brown adipose tissue, whereas total CoA pools were markedly smaller in the perirenal, epididymal and ovarian adipose tissue. On the other hand, in adult mice we found the highest expression of AceSA1 in the kidneys, ovaries and testes. In other tissues such as liver, lung, heart, brain and muscles, AceSA1 was expressed but at a clearly lower level than in the kidney. On the other hand, during embryogenesis the expression was developmentally regulated and the transcript was detected in the cephalic region at 9.5 dpc, which was intensified at 10.5 dpc. At a later stage of development, 11.5 dpc, AceSA1 was expressed in the liver. In kidneys the transcript of AceSA1 appeared in a subset of the renal tubules at 16.5 dpc and remained in these structures in the newborn. At gestational stage of 16.5 dpc the nephric mesenchyme undergoes mesenchymal-to-epithelial transition.

6.3 The effects of acetate, the second metabolite of ethanol

Acetate and acetaldehyde are the products of oxidative metabolism of alcohol in the liver of mothers exposed to ethanol. When co-injected, acetate and alcohol required six times longer to be cleared out from the plasma and uterus (Simm & Murdoch 1990). This indicates that alcohol administration delays acetate utilization or exerts influence on uterine metabolic processes. Thus, under alcohol administration due to imposed restriction on glucose availability and excess level of acetate, AceSA1 is one of the key genes that rescues the embryo by supplying acetyl-CoA to the nucleus for histone acetylation, consequently inducing gene expression, and on the other hand, by consuming more acetate anions it prevents more changes in intracellular pH. In mammalian (HCT116) cells under normal growth condition, ATP-citrate lyase (ACL) is a major source of acetyl-CoA for global histone acetylation although the AceSA1 also participates in histone acetylation, at least in part. The AceSA1 compensates for the loss of ACL function if its substrate, acetate, is available. In the absence of physiologic ACL level, incubation of cells with a supraphysiologic level of acetate, one of the substrates required for AceSA1, may supply histone acetyl-CoA for histone acetylation in a dose-dependent and AceSA1-dependent manner (Wellen et al. 2009).

We found that novel pathomechanisms (Fig. 3 & 4) could cause intrauterine growth restriction and facial malformation in the FAS model. Materno-fetal barriers (Fig. 2), which play an essential role in nutrient supplying and waste excretion, were damaged. In addition, we found that polyamine pools are common targets for
alcohol and DFMO. This finding may be helpful in providing more knowledge of intrauterine growth retardation. The weakness of work is that we did not specify how the premature permeability barrier affects the embryo development. The precise role of barrier malformation in the development of FAS as well as the role of PIGF/VEGF in the development of FAS has to be determined. Polyamines could be used as rescue agents in order to estimate the proportion of direct effects of alcohol and deficiency of polyamines on embryo development. Although all the evidence indicated that the intracellular pH in the alcohol-exposed embryo may become acidic, it could be very interesting to follow intracellular pH changes after alcohol injection, first 4 hours after the treatment and later at stages 9.5, 11.5 and 14.5 dpc. It would be helpful to develop specific rescues to prevent harsh changes in the intracellular pH changes and supply proper amount of exogenous polyamines and acetyl-CoA.

For future research, in addition to the above-mentioned experiments with alcohol, the effects of the alcohol metabolite pool, acetaldehyde and acetate, on the pH would be an interesting issue. In addition, nanoparticles that are able to stick specifically to lipids, polyamines, acetate and acetaldehyde, folic acid, glucose and oxygen will help us to follow the passage of the essential metabolite across the barriers and answer many questions concerning how to prevent development of FAS and other disorders caused by teratogens.
7 Conclusions

By applying different approaches we aimed to search for novel pathomechanisms leading to the development of FAS. Facial changes and intrauterine growth restriction were reproduced in a mouse model which indicated that the novel pathomechanisms might be applicable to humans as well. It must be noted, however, that due to the fast metabolic rate in mice the ethanol dose used here was high compared with the levels observed in pregnant women. Embryonic and extraembryonic tissue responded differently to ethanol treatment. The abolishment of polyamine pools was the most significant targeted molecular pathway in the development of facial malformations. The embryos’ brain growth and development was paralyzed due to polyamine depletion that imposed halt on cell growth. The growth halt may alter neural crest migration in the cephalic region, leading to development of neural tube defect. The neural tube defect alters embryo turning. In addition to polyamine deficiency, the nutrient supply to the embryo was impaired at several stages of development. Together, abnormal dilation of cerebral vessels, reduced vessel branching, and disrupted normal vascular development in the brain on one hand, and reduced labyrinth zone in the placenta on the other may impair normal nutrient supply to the brain.

Malformation of barrier structures, including thinner and irregular vessel endothelium, destructed gap junction, discontinued and lost contact between parietal cells and Reichert’s membrane, unaligned trophoblasts and lost tight connections with adjacent trophoblasts, are all engaged in the nutrient supply to the embryo. Altered PlGF/VEGF ratio is also associated with the barrier malfunction in ethanol-exposed conceptus. In this work, it was also found that alcohol and DFMO both depleted polyamine pools and caused cell growth halting, with the exception that alcohol has a reversible effect on polyamines while the effect of DFMO is irreversible.
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