

Myriam Baes, Simon Verheijden, and Paul P. Van Veldhoven

Abstract

Peroxisomes are ubiquitous organelles in mammalian cells but it is still unclear how they contribute to normal development and tissue homeostasis. To address this question, gene targeting techniques have been applied on several peroxins to interfere with peroxisome biogenesis in mice. Both peroxins involved in peroxisomal matrix import and peroxins necessary for peroxisome division were inactivated. Besides generalized knockouts, mice were created with conditional inactivation of *PEX* genes either in certain cell types or induced in adulthood. Defective matrix import generates empty peroxisomal ghosts and metabolic derangements that are a direct consequence of peroxisome inactivity. In addition, ablation of functional peroxisomes from hepatocytes affects other cellular compartments such as mitochondria and the endoplasmic reticulum. Peroxisome inactivity in the central nervous system causes both developmental and degenerative pathologies. The impairment of peroxisome division in mice also results in cerebral and hepatic pathologies although peroxisomal metabolites are unaffected.

Keywords

Peroxisomes • Mouse models • Peroxisome biogenesis • Peroxisome proliferation

M. Baes (✉) • S. Verheijden
Laboratory of Cell Metabolism, Department of Pharmaceutical and Pharmacological Sciences,
KU Leuven, Leuven, Belgium
e-mail: Myriam.Baes@pharm.kuleuven.be

P.P. Van Veldhoven
Laboratory of Lipid Biochemistry and Protein Interactions, Department of Cellular and Molecular
Medicine, KU Leuven, Leuven, Belgium

2.1 Introduction

The insights in the processes of peroxisomal matrix import, membrane formation, and proliferation in vertebrates have been greatly advanced by using diverse cell lines in which a peroxin was eliminated. Hereto, fibroblast cells from peroxisome biogenesis disorder (PBD) patients (Steinberg et al. 2006) and CHO cells in which *PEX* genes were inactivated have been used (Fujiki et al. 2006). From the pathological point of view, it is more informative to hinder the peroxisome biogenesis process in other cell types, e.g., hepatocytes or neural cells. To this end, several mouse models were generated in which *PEX* genes were targeted. In this chapter the cellular and tissue phenotypes will be discussed that result from the inactivation of peroxins either involved in peroxisomal matrix import (Pex2p, Pex5p, Pex13p, and Pex7p) or in peroxisomal proliferation (Pex11 α p and Pex11 β p). In view of the perinatal lethality of most mouse models with generalized gene inactivation, and with the purpose to explore peroxisome function in specific cell types and tissues, several cell-type selective (also denoted as conditional) *Pex5*- and *Pex13*-knockouts were created using Cre-loxP technology (Baes et al. 2002; Bjorkman et al. 2002; summarized in Table 2.1).

2.2 Mouse Models with Defects in Peroxisomal Matrix Import

2.2.1 Role of Pex2p, Pex5p, Pex7p, and Pex13p in Matrix Import

The four peroxins that were inactivated in mice take part in the early steps of peroxisomal matrix import (Girzalsky et al. 2010). Pex5p is the receptor recognizing newly synthesized proteins carrying a C-terminal peroxisome-targeting signal 1 (PTS1) consisting of serine–lysine–leucine or a conserved variant. The amino acids preceding this tripeptide may modulate the efficiency of peroxisomal import (Brocard and Hartig 2006). Pex5p occurs in two isoforms in vertebrates, a short (Pex5Sp) and a long form (Pex5Lp), the latter containing an additional stretch of 37 amino acids encoded by an extra exon, that allows it to bind Pex7p. The latter is the receptor for an N-terminal PTS2 sequence, consisting of a nonapeptide that occurs in a minority of peroxisomal matrix proteins. In mammals, these include enzymes of the α -oxidation (phytanoyl-CoA α -hydroxylase), β -oxidation (thiolase) and ether lipid synthesis pathways (alkyl-dihydroxyacetone-phosphate synthase). The cargo–Pex5p or cargo–Pex7p–Pex5Lp complexes dock at the peroxisomal membrane and bind Pex14p complexed with Pex13p. In contrast to yeast, Pex5p is thus not only essential for the import of PTS1 but also of PTS2 proteins. The precise role of Pex13p in the docking and translocation process is not elucidated, but it is an indispensable link in the import chain. According to recent evidence Pex5p and Pex14p form a flexible pore in the membrane through which the cargo is translocated (Meinecke et al. 2010). Subsequently, Pex5p is either monoubiquitinated and recovered to the cytosol for a subsequent import cycle or polyubiquitinated and targeted for degradation (Rucktaschel et al. 2011). It was

Table 2.1 Phenotype of mice with peroxisome biogenesis defects

Targeted peroxin gene	Model	Life span	Growth	Nervous tissue	Other pathologies	Biochemical parameters	References
<i>Pex2</i>	General	Embryonic lethality ^a <24 h– 5 weeks ^b	Retarded	Cortex malformation Cerebellar malformation	Liver (cholestasis, steatosis, steatohepatitis) Hypotonia	Plasmalogens ↓↓ VLCFA ↑ Bile acids ↓↓	Faust and Hatten (1997), Faust (2003), Keane et al. (2007), Kovacs et al. (2009, 2012)
<i>Pex13</i>	General	<12 h	Retarded	Cortex malformation	Hypotonia	Plasmalogens ↓↓ VLCFA ↑	Maxwell et al. (2003), Nguyen et al. (2006)
<i>Pex13-loxP</i> <i>Nextin-cre</i>	Conditional neural cells	10–150 days	Retarded	Cerebellar malformation		Plasmalogens ↓↓ VLCFA normal	Muller et al. (2011)
<i>Pex5</i>	General	<24 h	Retarded	Cortex malformation	Hypotonia	Plasmalogens ↓↓ VLCFA ↑	Baes et al. (1997), Janssen et al. (2000), Baumgart et al. (2001), Janssen et al. (2003)
<i>Pex5-loxP</i> <i>Alfp-cre</i>	Conditional fetal liver	8–16 days	Postnatally retarded	Cortex malformation	Liver (steatosis)	Plasmalogens normal	Krysko et al. (2007)
<i>Pex5-loxP</i> <i>Alb-cre</i>	Conditional adult liver	>12 months	Postnatally retarded	Normal	Liver (hepatomegaly, steatosis, fibrosis, tumors from 12 months)	Plasmalogens normal VLCFA normal Phytanic/pristanic acid ↑ C27/C24 bile acids ↑ Mitochondrial ATP production ↓	Dirkx et al. (2005), Peeters et al. (2011a, b)

(continued)

Table 2.1 (continued)

Targeted peroxin gene	Model	Life span	Growth	Nervous tissue	Other pathologies	Biochemical parameters	References
<i>Pex5-loxP</i> <i>loxP</i> <i>ap2-cre</i>	Conditional adipose ^c	Normal	Lower body weight after weaning	Normal	Increased fat mass Shivering thermogenesis↓ Motor performance↓ Insulin resistance↑	Plasmalogens ↓ Lipolysis ↓ Plasma adrenaline ↓↓	Martens et al. (2010, 2012)
<i>Pex5-loxP</i> <i>Nestin-cre</i>	Conditional neural cells	3 weeks–6 months	Postnatally retarded	Cerebellar malformation Demyelination, inflammation, axon loss Decline in motor and cognitive abilities	Male and female infertility	VLCFA ↑ PUFA ↓ (at young age) Plasmalogens ↓↓ Neutral lipid accumulations in astroglia and in ependymal cells	Krysko et al. (2007), Hulshagen et al. (2008), Bottelbergs et al. (2012)
<i>Pex5-loxP</i> <i>Nes-cre</i>	Conditional forebrain neurons	Normal	Normal	Normal	None	No abnormalities	Bottelbergs et al. (2010)
<i>Pex5-loxP</i> <i>Gfap-cre</i>	Conditional astrocytes	Normal	Normal	Normal	None	Plasmalogens ↓ VLCFA ↑ Neutral lipid accumulations in astroglia and in ependymal cells	Bottelbergs et al. (2010)
<i>Pex5-loxP</i> <i>Cnp-cre</i>	Conditional oligodendrocytes	6–12 months	Normal	Axonal degeneration; subcortical demyelination; neuroinflammation; impaired motor nerve conductance	None	Plasmalogens (in myelin) ↓ VLCFA ↑	Kassmann et al. (2007)

<i>Pex5- loxP Wnt-cre</i>	Conditional peripheral nervous system	Normal	Normal	Normal	None	ND	Martens et al. (2012)
<i>Pex5- loxP Amh-cre</i>	Conditional Sertoli cells	ND	Normal	Normal	Male infertility	Lipid droplets in Sertoli cells	Huyghe et al. (2006)
<i>Pex5- loxP Cmv-cre- ER</i>	Inducible ^d all cells	8 months	ND	Demyelination, neuroinflammation, axon loss	ND	ND	Bottelbergs et al. (2012)
<i>Pex7</i>	General	<24 h->1 year	Retarded	Cortex malformation mild astrocytosis	Testis (infertile) Eyes (cataracts) Adipose (reduced) Harderian gland (atrophy)	Plasmalogens ↓↓ VLCFA↑ (certain organs)	Brites et al. (2003, 2011)
	Hypomorphic allele	Normal	Retarded	ND	Eyes (cataracts)	Plasmalogens ↓ DHA in RBC↓	Braverman et al. (2010)
<i>Pex1/α</i>	General	Normal	Normal (Li) increased BW (Weng)	Normal	None Hepatic steatosis	Plasmalogens and VLCFA normal (Li) Liver triglycerides↑ (Weng)	Li et al. (2002a), Weng et al. (2013)
<i>Pex1/β</i>	General	<24 h	Retarded	Cortex malformation	Hypotonia	Plasmalogens and VLCFA normal	Li et al. (2002b), Ahlemeyer et al. (2012)

(continued)

Table 2.1 (continued)

Targeted peroxin gene	Model	Life span	Growth	Nervous tissue	Other pathologies	Biochemical parameters	References
<i>Pex11α</i>	General	<24 h	Retarded	Cortex malformation	Hypotonia	Normal	Li et al. (2002a)
+							
<i>Pex11β</i>							

General: refers to whole animal knockout, conditional: refers to cell type selective knockouts. In the latter, the biochemical parameters were determined in the targeted tissue; *alb* albumin, *BW* body weight, *ND* not determined, *RBC* red blood cells

^aIn 129SV background

^bIn 129SV/Swiss Webster background

^cAlso in ganglia of the peripheral nervous system (PNS), in adrenal medulla, and in neurons throughout the central nervous system (CNS)

^d*PEX5* was deleted after tamoxifen administration at 1–2 months

shown in yeast that Pex2p is a ubiquitin ligase that is crucial for polyubiquitination of Pex5p (Rucktaschel et al. 2011). In all organisms ranging from yeast to plants and mammals, Pex2p, Pex5p, Pex7p, and Pex13p are indispensable for peroxisomal matrix protein import.

2.2.2 Macroscopic Phenotypes of Mice with Peroxisome Biogenesis Defects

Inactivation of both PTS1 and PTS2 import throughout the body (further called generalized) in *Pex2*- (Faust and Hatten 1997), *Pex5*- (Baes et al. 1997), and *Pex13*- (Maxwell et al. 2003) knockout mice causes severe developmental abnormalities with intrauterine growth retardation and brain malformations. Depending on the genetic background of the mice, this results in fetal lethality, neonatal death due to severe hypotonia, or survival for a few weeks. Mice with loss of functional peroxisomes from all neural cells (neurons, astrocytes, oligodendrocytes) (*Nestin-Pex5*^{-/-} and *Nestin-Pex13*^{-/-}) are indistinguishable from wild-type mice at birth but they become growth retarded within the first postnatal week. Several succumb after weaning, whereas the survivors develop motor and cognitive deficits and die before the age of 6 months, proving that peroxisomes are indispensable for the integrity of the central nervous system (CNS) in adulthood (Hulshagen et al. 2008; Bottelbergs et al. 2012). Mice with cell type selective inactivation of Pex5p in hepatocytes, testis, or adipose tissue become adult but develop diverse pathologies as summarized in Table 2.1. *Pex7* knockouts are compromised at birth, and the majority dies before weaning although others survive into adulthood and beyond (Brites et al. 2003). A hypomorphic *PEX7*-deficient mouse model expressing less than 5 % of wild-type transcript levels has a normal life span (Braverman et al. 2010). All *PEX7*-deficient mice show pre- and postnatal growth impairment, but only the full male knockouts are infertile.

2.2.3 Peroxisomal Matrix Import Defects Give Rise to Peroxisomal Ghosts

Peroxisomes can arise by division of preexisting peroxisomes, presumably the major pathway (Huybrechts et al. 2009), or by de novo formation from the ER. In both cases, the process of peroxisomal membrane formation and membrane protein import precedes the import of matrix proteins. It can therefore be expected that inactivation of key components of the matrix import machinery yields empty peroxisomes also called “ghosts.” In *Pex2*^{-/-}, *Pex5*^{-/-}, and *Pex13*^{-/-} cells (Baes et al. 1997; Faust and Hatten 1997; Maxwell et al. 2003), vesicles were indeed identified that contain integral membrane proteins such as PMP70 but are devoid of catalase and other matrix proteins. These are however less numerous and larger in size than regular peroxisomes. In *PEX13*-deficient fibroblasts, neurons, and astrocytes, it was shown that trafficking of peroxisomal ghosts is perturbed

resulting in an altered cytoplasmic distribution as compared to mature peroxisomes in wild-type cells. The ghosts cluster and are not aligned along peripheral microtubules (Nguyen et al. 2006). The impairment of peroxisomal matrix import was also proven by performing Western blots on the enzymes acyl-CoA oxidase (PTS1 containing enzyme) and thiolase (PTS2 containing enzyme). These proteins are normally split by the protease TYSND1 after their import into peroxisomes, but they occur in their unprocessed forms in knockout cells. As can be anticipated, in *PEX7*-deficient cells, only the PTS2 containing proteins are mislocalized to the cytosol (Brites et al 2003; Braverman et al. 2010).

2.2.4 Metabolic Consequences of Peroxisomal Matrix Import Defects

The reported biochemical consequences of import incompetent peroxisomes in mice are related to deficient peroxisomal β -oxidation and ether lipid synthesis. α -oxidation is another pathway that exclusively takes place in peroxisomes but when fed a normal diet, mice are not exposed to the substrate, phytanic acid. Therefore, in order to prove defects in this pathway, mice need to be supplemented with phytol, a precursor of phytanic acid. In the mouse models with generalized inactivation of both PTS1 and PTS2 import (*Pex2*, *Pex5*, and *Pex13* knockouts) plasmalogens are reduced to less than 10 % of normal values in brain and liver (Baes et al. 1997; Faust and Hatten 1997; Maxwell et al. 2003). In mice with conditional *PEX5* gene inactivation in peripheral tissues (liver, adipose) plasmalogens are not or only mildly reduced in the targeted tissues (Dirkx et al. 2005; Martens et al. 2012), whereas they are profoundly depleted in brain of Nestin-*Pex5* and Nestin-*Pex13* knockout mice. These data can be explained by transfer of ether lipids from peroxisome-bearing cells to peroxisome-deficient cells in the periphery but not through the blood brain barrier. This is in line with reports that supplementation of rodents with ether lipids can elevate plasmalogens in the periphery but not or hardly in the CNS (Das and Hajra 1988; Brites et al. 2011; Wood et al. 2011). Levels of C26:0 are three- to tenfold increased in tissues of generalized *Pex* knockouts. Remarkably, in brain of the conditional *Pex5* knockouts, C26:0 accumulate to similar extents whether all neural cells (Nestin-*Pex5*) (Hulshagen et al. 2008) or only oligodendrocytes (Cnp-*Pex5*) (Kassmann et al. 2007) or astrocytes (Gfap-*Pex5*) (Bottelbergs et al. 2010) are targeted. In contrast, loss of peroxisomes from neurons does not affect VLCFA levels (Bottelbergs et al. 2010). Surprisingly, in brain of juvenile Nestin-*Pex13* mice C26:0 levels are normal (Muller et al. 2011), which is in discordance with the findings in Nestin-*Pex5* mice. Docosahexaenoic acid (DHA, C22:6n-3), a polyunsaturated fatty acid that requires peroxisomal β -oxidation for its synthesis, is reduced in brain of newborn *PEX5* knockouts (Janssen et al. 2000) and in cerebellum of Nestin-*Pex5*^{-/-} mice (Kryske et al. 2007).

In mouse models with *PEX* gene inactivation in liver, bile acid metabolism is severely affected (Dirkx et al. 2005; Keane et al. 2007). The ratio of immature C27/

mature C24 bile acids is increased in liver, bile and plasma, and bile acids appear predominantly in their unconjugated form.

The metabolic derangements in *Pex7* knockout mice pertain to the mislocalization of three enzymes that are part of three different pathways. The defect in ether lipid synthesis results in a profound deficiency of plasmalogens in total *Pex7* knockouts (Brites et al. 2003), whereas they are less drastically reduced in the hypomorphic counterparts (Braverman et al. 2010). As a result of deficient α -oxidation, phytanic acid accumulates in plasma after feeding a phytol-enriched diet (Brites et al. 2003). The consequences of the import defect of the β -oxidation enzyme thiolase, which is involved in breakdown of straight chain substrates, are more variable. C26:0 accumulates in some tissues (brain of newborn mice, spleen, kidney) but not in adult brain, testis, and liver (Brites et al. 2003, 2009). Possibly, the other peroxisomal enzyme with thiolytic activity, SCPx, takes over the function in the tissues with normal VLCFA levels.

2.2.5 Lipid Homeostasis Is Disturbed in Hepatocytes, Sertoli Cells and Astrocytes

Besides the direct consequences of peroxisomal dysfunction, it is remarkable that in certain cell types with peroxisome biogenesis defects lipid droplets accumulate. In *Pex2*^{-/-} and *Pex5*^{-/-} hepatocytes triglyceride concentrations are increased and microvesicular steatosis develops (Dirkx et al. 2005; Keane et al. 2007, Fig. 2.1). This is unexpected since *Pex5*^{-/-} hepatocytes exhibit enhanced mitochondrial β -oxidation (Dirkx et al. 2007). In these peroxisome-deficient livers, PPAR α is significantly activated which is likely due to the accumulation of ligands of this nuclear receptor that cannot be degraded (Peeters et al. 2011b). Possibly, the marked upregulation of the PPAR α target CD36 causes an enhanced influx of fatty acids that may contribute to increased lipid stores. Interestingly, lipid droplets were also seen in the brain of Nestin-*Pex5*^{-/-} mice, in which peroxisome inactivation is restricted to neural cells (Hulshagen et al. 2008). Lipids were particularly found in Bergmann glia astrocytes in the cerebellum and in ependymal cells lining the ventricles, also a glial cell type. Lipid storage was also found in an astrocyte selective (*Gfap-Pex5*^{-/-}; Bottelbergs et al. 2010) but not in neuron (*Nes-Pex5*^{-/-}; Bottelbergs et al. 2010) nor in oligodendrocyte selective (*Cnp-Pex5*^{-/-}; Kassmann et al. 2007) *Pex5* knockout mice, confirming the cell type specificity. In view of the normal life span and absence of major neurological problems of *Gfap-Pex5*^{-/-} mice, it seems that the accumulating lipids are rather harmless. The situation is different in the testis, where similar increased stores of neutral lipids were noticed when functional peroxisomes were deleted from Sertoli cells (Huyghe et al. 2006). This was accompanied with degeneration of the tubuli seminiferi and complete loss of spermatogenesis. It was later shown that Cre expression in Sertoli cells has a widespread impact on immunological signaling, oxidative stress, peroxisomal protein expression and on other features (Xiao et al. 2012). It can however be excluded that Sertoli cell lipid storage and testicular degeneration are artefacts of

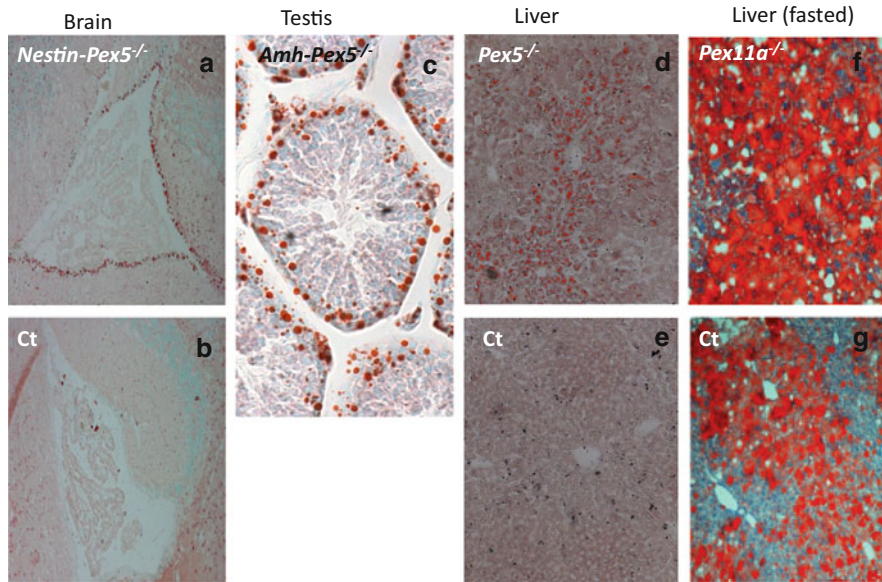


Fig. 2.1 Lipid accumulations in peroxin-deficient cells. Neutral lipids were visualized by Oil Red O staining on frozen sections. Lipid droplets accumulate in ependymal cells lining the fourth ventricle in *Nestin-Pex5*^{-/-} mice (a, b), in Sertoli cells of *Amh-Pex5*^{-/-} mice (selective Sertoli cell knockout) (c), and in hepatocytes depleted of *PEX5* (d, e). Furthermore, *Pex11a*^{-/-} hepatocytes store more lipids than control mice (shown in f, g after a 48-h fasting period; reproduced from Weng et al. (2013) with permission from the American Physiological Society). Ct = sections from control mice

Cre expression because a similar pathology is observed in mice with a generalized peroxisomal β -oxidation defect (*Mfp2*^{-/-} mice; Huyghe et al. 2006). This also indicates that the neutral lipid accumulation in peroxisome-deficient cells is a consequence of impaired β -oxidation although the precise origin and nature of the lipids needs to be determined.

2.2.6 Other Compartments Are Affected in Peroxisome-Deficient Hepatocytes

The absence of mature peroxisomes has also repercussions on other subcellular compartments. Most obvious are alterations in *PEX5*- and *PEX2*-deficient hepatocytes in which mitochondrial and ER structure and function are severely compromised (Baumgart et al. 2001; Dirkx et al. 2005; Kovacs et al. 2009). By ultrastructural analysis mitochondria appear swollen and the cristae of the inner mitochondrial membrane are rare and malformed (Baumgart et al. 2001; Dirkx et al. 2005). The function of the electron transport chain is severely affected, whereby in particular the activities of complex I, III and V are reduced. This results

in impaired synthesis of mitochondrial ATP and, despite an upregulation of glycolysis, to a reduced AMP/ATP ratio and the activation of AMPK (Peeters et al. 2011a). Through the latter kinase, the activity of catabolic processes (such as glycolysis) is increased and of anabolic processes (gluconeogenesis, glycogen synthesis and fatty acid synthesis) is decreased in *Pex5* knockout hepatocytes (Peeters et al. 2011a, b). An additional compensatory mechanism is the proliferation of mitochondria as shown by increased numbers of mitochondria and elevated matrix enzyme activities. Of interest, not all mitochondria within a cell are affected and the mechanism is cell autonomous. Indeed, the few hepatocytes that escape the recombination process in hepatocyte selective *Pex5* knockouts, show normal mitochondria.

Both in *PEX2*- and *PEX5*-deficient liver, mediators of ER stress signaling pathways (PERK, ATF4) are upregulated (Kovacs et al. 2009; Peeters et al. 2011a). Similar to the mitochondrial alterations, the ER stress is a very early event as it already occurs in newborn mice (Baumgart et al. 2001; Kovacs et al. 2012). Microscopic investigations showed ER dilation and proliferation. It was proposed that these ER perturbations cause oxidative stress (see below) and deregulation of SREBP controlled cholesterol homeostasis.

At present, the mechanisms through which peroxisome inactivity affect mitochondria and ER are still obscure. It is also unclear whether these cellular compartments are altered in other cell types in which peroxisomes are inactive.

2.2.7 Does Peroxisome Deficiency Affect the Cellular and Organellar Redox State?

Peroxisomes play a pivotal role in cellular redox metabolism as they harbor both several oxygen radical generating and degrading enzymes (Fransen et al. 2012). When both categories are unable to enter the organelle, it is not unequivocal to predict how this affects the cellular redox homeostasis. Importantly, it has become clear that under normal conditions, peroxisomes are not an isolated compartment with regard to ROS generation and degradation. Peroxide generated in peroxisomes leaks into other cellular compartments and vice versa peroxisomal catalase degrades peroxide that is generated elsewhere in the cell (Fransen et al. 2012). In fact, diverse results were obtained with regard to the oxidative stress state depending on the cell types and *Pex* knockouts investigated. Different approaches were used including evaluation of oxidative damage to proteins, lipids or DNA, transcript levels of anti-oxidant enzymes or direct measurement of reactive oxygen species (ROS)(H_2O_2 , $\text{O}_2^{\cdot-}$) in cultured cells.

In hepatocytes from newborn *Pex5* and *Pex2* knockout mice, increased expression of anti-oxidant proteins and of genes related to oxidative stress generation such as NADP oxidase were reported (Baumgart et al. 2001; Kovacs et al. 2012). As a potential mechanism, it was proposed that induction of the ER stress-related protein PERK phosphorylates the transcription factor NRF2 that further activates oxidative stress genes (Kovacs et al. 2012). However, studies on liver and on cultured

hepatocytes from adult conditional hepatocyte selective *Pex5* knockouts did neither reveal upregulation of antioxidative enzymes nor oxidative damage to proteins, nor increased cytosolic peroxide (Dirkx et al. 2005). A complicating matter is that mitochondria are also severely affected at the level of the electron transport chain in peroxisome-deficient hepatocytes, as mentioned above, which could also affect the redox state of the cell.

In cerebella and in cultured cerebellar neurons of *Nestin-Pex13* mice, MnSOD2 levels are elevated (Muller et al. 2011). Furthermore, in the latter neurons, increased levels of superoxide but not of H_2O_2 were detected, rather compatible with a mitochondrial origin of oxidative stress. This was accompanied by increased apoptotic death of these neurons. In the equivalent *Nestin-Pex5* knockout mice, signs of oxidative stress were exclusively found in cerebellar Purkinje cells based on immunohistochemical detection of nitrotyrosine and 4-hydroxynonenal (Bottelbergs et al. 2012). Catalase was also markedly increased but this was confined to astrocytes including Bergmann glia in the cerebellum. When using a biochemical approach on several brain regions, there was no evidence for lipid peroxidation (Bottelbergs et al. 2012). Because anti-oxidative treatment could not prevent the severe neurodegeneration of *Nestin-Pex5* knockout mice (see below), it is improbable that excessive ROS underlies the brain phenotype (Bottelbergs et al. 2012).

Taken together, it is likely that the diverse cellular circumstances (in vivo versus in vitro, culture media (M. Fransen, personal communication), antioxidative capacity of different cell types) have a major impact on the redox state of cells lacking functional peroxisomes. When also mitochondria or the ER are affected in peroxisome-deficient cells, it is difficult to distinguish between primary and secondary consequences of peroxisome deficiency on the redox state.

2.2.8 Peroxisome Ablation Causes Developmental and Degenerative Neuropathologies

The CNS seems to be particularly vulnerable to peroxisome dysfunction, whereby both developmental and degenerative pathologies arise. Full deletion of peroxisomal function in *Pex2*, *Pex5*, and *Pex13* knockouts invariably causes cortical neuronal migration defects during fetal development resulting in abnormal lamination of the cortex at birth (Faust and Hatten 1997; Baes et al. 1997; Maxwell et al. 2003). It is not clear yet whether the severe neonatal hypotonia in these mice is related to these CNS or to peripheral anomalies. By using liver and brain selective *Pex5* rescue and knockout mice, it was shown that both peroxisome dysfunction in the liver and in the brain contribute to the hampered cortical development in *Pex5*^{−/−} mice (Janssen et al. 2003; Krysko et al. 2007). The cerebellum that matures between birth and weaning, is also affected as shown in *Pex2* knockouts (Faust 2003) and in mice with neural inactivation of *PEX5* (*Nestin-Pex5*; Krysko et al. 2007) or *PEX13* (*Nestin-Pex13*; Muller et al. 2011). Multiple

processes are disorganized including migration of granular cells, Purkinje cell arborization, and cerebellar foliation.

Additional pathologies in mice with selective deletion of functional peroxisomes from neural cells (*Nestin-Pex5*) are dysmyelination in cerebellum in juvenile mice and demyelination at later ages throughout the brain (Hulshagen et al. 2008; Bottelbergs et al. 2012). This is accompanied with severe inflammation, whereby microgliosis precedes astroglia activation. Axons degenerate and are lost but there is no evidence for neural cell death.

The question whether this detrimental neuropathology originates from a particular cell type was addressed by creating cell type selective (neurons, astrocytes, oligodendrocytes) *Pex5* knockouts. The oligodendrocyte (*Cnp-Pex5*) knockout clearly showed the most severe phenotype with similar pathologies as in *Nestin-Pex5*^{−/−} mice but with later onset and slower progression (Kassmann et al. 2007). This suggests that besides cell autonomous functions of peroxisomes in oligodendrocytes, other mechanisms cause the early lethality of *Nestin-Pex5*^{−/−} knockouts. In contrast, ablation of peroxisomal function in forebrain neurons (*Nex-Pex5*^{−/−}) or in astrocytes does not cause obvious neurological symptoms nor impaired life span. Nevertheless, anomalies occurred in brains of *Gfap-Pex5*^{−/−} mice (Bottelbergs et al. 2010) such as increased levels of C26:0 and overexpression of catalase in astrocytes, as previously mentioned. The same neurodegenerative sequence could also be provoked by deletion of *PEX5* in postnatal life excluding that the neurodegenerative phenotype is merely a result of developmental problems (Bottelbergs et al. 2012).

Pex7^{−/−} mice exhibit more moderate developmental and degenerative brain pathology including anomalies in neuronal migration and mild astrocytosis in adulthood (Brites et al. 2003, 2009). Myelination in different brain regions seemed normal and no microgliosis was observed throughout the brain of *Pex7* knockouts.

2.3 Mouse Models with Impairment of Peroxisome Proliferation

2.3.1 Role of Pex11 Proteins in Peroxisome Proliferation

Peroxisins of the Pex11p family are involved in the early steps of peroxisome proliferation (Schrader et al. 2012). As their overexpression elevates and their inactivation lowers peroxisome abundance in cells of species ranging from plants to mammals, it is clear that they regulate peroxisome number. There are three isoforms in mammals that share 40–60 % sequence similarity. Whereas *PEX11α* and *PEX11γ* show tissue specific expression and are primarily found in liver, *PEX11β* is ubiquitously expressed. Another distinguishing feature is that only the *PEX11α* gene is regulated by PPARα (Schrader et al. 2012). It is however less clear whether the isoforms exhibit also functional differences, analogous to the three yeast Pex11p homologues that have partially divergent functions (Huber et al. 2012). All the Pex11 proteins harbor an amphipathic helix that is essential

for integration in and deformation of the peroxisomal membrane (Koch and Brocard 2011). In subsequent steps the Fis1 and Mff1 proteins are attracted that in turn recruit DLP1, the executor of peroxisome fission (Koch and Brocard 2012). The inactivation of *PEX11 α* and *PEX11 β* genes in mice results in strongly differing phenotypes, reflecting the difference in expression pattern and possibly the distinct function.

2.3.2 Pex11 α Knockout Mice Display a Mild Hepatic Phenotype

Two different mouse models lacking *PEX11 α* were generated (Li et al. 2002a; Weng et al. 2013). Although in both models a complete loss of *PEX11 α* mRNA was confirmed, researchers came to different conclusions with regard to peroxisome numbers in cells. As Pex11 α p is primarily expressed in hepatocytes, liver sections were investigated. Gould and coworkers did not detect a reduction in peroxisome abundance but there was a tendency for peroxisome clustering in *Pex11 α —* hepatocytes (Li et al. 2002a). In contrast, according to Weng et al. (2013) immunofluorescently labeled peroxisomes were 20–30 % lower in number in liver sections. By EM analysis they further showed that small and rounded peroxisomes were more prevalent in *Pex11 α —* hepatocytes both under normal diet and after treatment with fibrates, whereas in controls a higher frequency of elongated and irregular peroxisomes were present. As there is no clear explanation for the discrepant findings between the research groups, it remains inconclusive whether for basal maintenance of peroxisome numbers, the function of Pex11 α p in hepatocytes can be taken over by the family members Pex11 β p and/or Pex11 γ p. It was surprising that, although Pex11 α p is the sole isoform inducible by PPAR α , *Pex11 α* knockout mice display normal peroxisome proliferation when treated with classical PPAR α ligands (Li et al. 2002a). It was suggested that the elevation of peroxisome numbers by PPAR α might be mediated by altered metabolite levels. On the other hand, Pex11 α p was required for peroxisome proliferation induced by 4-phenylbutyrate, a compound that acts independently of PPAR α (Li et al. 2002a).

Interestingly, Weng et al. 2013 showed that *Pex11 α* knockout hepatocytes store more triglyceride containing lipid droplets in basal conditions than wild types, which is exacerbated after challenging the mice with high fat diets or by fasting (Fig. 2.1). Body weights of *Pex11 α* knockout mice were significantly increased as compared to controls. This shows that Pex11 α p is not fully redundant with Pex11 β p and Pex11 γ p isoforms. The lipid accumulations are reminiscent of those in *PEX5*-deficient hepatocytes and indicate that lipid homeostasis in hepatocytes can be perturbed even when peroxisomes are only marginally affected. It was proposed that the reduced peroxisomal surface area in *Pex11 α —* hepatocytes impairs the uptake of fatty acids from the cytosol leading to reduced peroxisomal β -oxidation and lipid accumulation. This explanation however implies that hepatic peroxisomes significantly contribute to long chain fatty acid degradation, whereas it is currently believed that this only accounts for less than 10 % (Mannaerts et al. 1979) or 25 % (Grum et al. 1994). It is unfortunate that peroxisomal metabolic activities were not

determined in *Pex11 α* ^{−/−} liver by any of the research groups. Gould and coworkers did analyze C26:0 in plasma of *Pex11 α* ^{−/−} mice and the activity of α - and β -oxidation and plasmalogen synthesis in *Pex11 α* ^{−/−} fibroblasts, none of which were altered compared to wild-type controls (Li et al. 2002a). Weng et al. reported deregulation of transcript levels of enzymes related to peroxisomal β -oxidation in liver (Weng et al. 2013) but as there is no concerted change, this does not clarify the observed lipid accumulations.

2.3.3 Deletion of a Single or Both PEX11 β Alleles in Mice Impairs Neural Function

The ubiquitous expression of *PEX11 β* is indicative of its housekeeping role to maintain peroxisome numbers in all cells in basal conditions. In agreement, in *Pex11 β* ^{−/−} mouse fibroblasts, cultured neurons and astrocytes abundance of peroxisomes was halved as compared to wild-type cells (Li et al. 2002b; Li and Gould 2002). Also in brain sections peroxisomes were reduced by 30–50 %. Unexpectedly, newborn *Pex11 β* ^{−/−} mice show a phenotype that closely resembles the mouse models with peroxisome matrix import defects including a cortical migration defect, hypotonia, a prominent growth retardation and neonatal lethality (Li et al. 2002b).

It is most intriguing that this severe pathology is not accompanied with defects in peroxisomal protein import and consequently not with changes in peroxisomal metabolites. Catabolism of C24:0, phytanic and pristanic acid and synthesis of plasmalogens were unaltered in fibroblasts and no significant changes in plasmalogen and C26:0 concentrations were observed in liver and brain. *PEX11 β* deficiency in men likewise has no effect on biochemical parameters, but in contrast to mice, this was associated with a mild neurological phenotype (Ebberink et al. 2012). It was therefore proposed that the pathologies in PBD could be caused by mechanisms independent of metabolic perturbations (Li et al. 2002b). Because the pathogenic metabolites fail to be identified in PBD in men and mice, this is an attractive alternative consideration. On the other hand, we should bear in mind that very similar pathologies as those in PBD occur in patients suffering from peroxisome single enzyme disorders (such as MFP2/D-BP deficiency, ACOX1 deficiency) in which metabolic deficits are expected to be the prime cause (Van Veldhoven 2010).

More in-depth analysis revealed increased oxidative stress in neuronal cultures and in brain sections of *Pex11 β* ^{−/−} mice, which was accompanied with increased neuronal cell death by apoptosis (Ahlemeyer et al. 2012). Neuronal differentiation was impaired as shown by reduced synaptophysin expression. Interestingly, haploinsufficiency of *PEX11 β* caused similar but less extensive neural abnormalities that were easier to detect in vitro than in vivo. Strikingly, these anomalies were not related to effects on proliferation as peroxisome numbers were neither reduced in cultured neurons nor in brain tissue. In view of unchanged metabolism in homozygous knockouts, no biochemical alterations are expected to

occur in the heterozygous mice. The latter however display deregulated expression of several genes at the mRNA and protein level. The cellular changes in *Pex11 β* ^{+/-} mice did not markedly affect CNS functioning because the mice are macroscopically indistinguishable from wild types and they are fertile. This is in sharp contrast with the phenotype of mice with peroxisome import defects in neural cells that show reduced growth, fertility, coordination and motor defects as described above (Hulshagen et al. 2008; Muller et al. 2011).

It remains enigmatic how a 50 % loss of Pex11 β protein levels can affect oxidative stress and survival of neural cells in the absence of functional and structural peroxisome defects. As Pex11 β p is the isoform with the highest expression level in the majority of tissues, it would be interesting to investigate whether its loss from other cell types has similar consequences.

Conclusions and Perspectives

Although peroxisomes are ubiquitous cell organelles, many of their secrets still need to be uncovered. To investigate the necessity of peroxisomes in diverse cell types and tissues, analysis of mouse models with peroxisome deficiencies is a powerful approach. In the first generation, generalized knockouts with a complete *PEX* gene inactivation were created and phenotyped. It is striking that impeding peroxisomal matrix import versus peroxisome proliferation has a different impact on peroxisomal metabolite levels but causes similar developmental problems. These include not only brain malformations but also intrauterine growth retardation. The latter may complicate the investigation of pathologies in newborn mice as they can either be a direct consequence of peroxisome dysfunction or rather a nonspecific result of the developmental delay.

In the second generation of mice, conditional inactivation of *PEX* genes was achieved, allowing the study of the function of peroxisomes in adult tissues. Some pitfalls should be taken in consideration when analyzing cell type selective knockouts, e.g., not all pathologies might be mediated via cell autonomous defects and could therefore be missed in these conditional knockouts. Furthermore, when using Cre loxP technology, one should be aware of potential effects of Cre activity on the functioning of the targeted cells. It was indeed shown that Cre expression in Sertoli cells causes oxidative stress and deregulation of gene expression (Xiao et al. 2012). In addition, the success of this approach relies on the cell type specificity of gene inactivation that fully depends on the promoter that is used to drive Cre expression. There are several examples of promoters that were presumed to be cell type selective but induce Cre expression during fetal development in several other cell types. For example, aP2-Cre mice, which were thought to only drive gene recombination in adipocytes, also show Cre activity in cells sharing a common lineage with adipocytes such as chondrocytes, myocytes, neurons and osteocytes (Martens et al. 2010, 2012). Without any doubt, taking these limitations into account, additional cell type selective peroxin knockouts will reveal the importance of peroxisomes in tissues thus far not investigated.

In the future, besides the consequences of complete peroxisome dysfunction, also the impact of milder peroxisome biogenesis impairments will need to be explored. Indeed, increasing numbers of patients with mild dysfunction are diagnosed with a PBD in adulthood (Steinberg et al. 2009; Regal et al. 2010; Ebberink et al. 2010; Sevin et al. 2011; Mignarri et al. 2012; Matsui et al. 2012). Therefore, mouse models should be generated with point mutations resulting in partial peroxisome dysfunction. Attention should also be paid to the possibility that heterozygous null mutations of certain *PEX* genes could trigger pathologies, as shown for *PEX11 β* (Ahlemeyer et al. 2012).

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