Null cyp1b1 Activity in Zebrafish

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CYP1B1 is a cytochrome P450 monooxygenase involved in oxidative metabolism of different endogenous lipids and drugs. The loss of function (LoF) of this gene underlies many cases of recessive primary congenital glaucoma (PCG), an infrequent disease and a common cause of infantile loss of vision in children. CYP1B1 loss of function (LoF) is the main known genetic alteration present in recessive primary congenital glaucoma (PCG), an infrequent disease characterized by delayed embryonic development of the ocular iridocorneal angle; however, the underlying molecular mechanisms are poorly understood.

CYP1B1 craniofacial development CRISPR/Cas9 congenital glaucoma cyp1b1-KO zebrafish

1. Introduction

CYP1B1 is a cytochrome P450 monooxygenase that participates in the oxidative metabolism of different endogenous lipids including steroids [1], arachidonic acid [2] (the primary source of fatty acids) and retinoids [2][3], and it is also involved in drug metabolism [4]. The human *CYP1B1* gene is located on chromosome 2p22-21 and comprises three exons, with the coding region starting in the second exon and ending in the last exon [5]. This gene encodes an approximately 50-kDa transmembrane protein that is anchored to the endoplasmic reticulum membrane and the inner mitochondrial membrane by a transmembrane amino terminus domain [6]. Structurally, the protein consists of several domains such as a hydrophobic amino-terminal region, a proline-rich region (hinge region) and a carboxyl-terminal portion. This last region contains a set of conserved core structures and a substrate-binding region, including an iron protoporphyrin IX (heme) prosthetic group ligated to cysteine thiolate [7].

Loss-of-function (LoF) variants in the human *CYP1B1* gene [8] are the main known genetic cause of autosomal recessive congenital glaucoma (CG) in different populations [9][10][11][12]. Although CG is an infrequent disease, it is the most common glaucoma in the neonatal and infant period and it is also a major cause of visual loss in children [13]. Abnormal development of the embryonic iridocorneal angle underlies CG through poorly understood mechanisms, although CYP1B1 is hypothesized to metabolize a yet unidentified compound required for normal formation of iridocorneal structures [14]. An altered ECM of the TM, a general feature of PCG [15][16][17], is also present in patients carrying null and hypomorphic *CYP1B1* genotypes [18]. In addition to *CYP1B1*, other genes such as *LTBP2* [19][20], *MYOC* [21], *TEK* [22], *FOXC1* [23] and *CPAMD8* [24][25] are involved in a few congenital glaucoma cases. Genes such as *GPATCH3* [26] and *GUCA1C* [27] have been identified as candidate CG genes, although their role in the disease remains to be confirmed. Remarkable phenotypic variability is also present in

CYP1B1-associated glaucoma, ranging from mild adult-onset goniodysgenesis to agenesis of the Schlemm canal [18][28] and complete aniridia [29]. This phenomenon suggests the existence of modifier factors in the phenotypic outcome. In fact, rare variants of *FOXC2* and *PITX2* associated with mild functional alterations have been identified as possible modifiers in congenital glaucoma [30]. Previously, we reported that approximately 30% of Spanish CG patients carry either homozygous or compound heterozygous *CYP1B1* LoF variants, often resulting in null genotypes [12]. Even among the cases with null CYP1B1 enzymatic activity which can be considered natural human knockouts, remarkable phenotypic variation is present [12][31]. These facts, along with the existence of incomplete penetrance and the discovery of a significant proportion of patients who carry nondominant heterozygous *CYP1B1* mutations [12], support the importance of genetic and/or environmental modifier factors in CG pathogenesis.

The function of *CYP1B1* has been explored in different animal models. *Cyp1b*-KO mice have ocular drainage structure abnormalities resembling those reported in human PCG patients, and in this animal model, tyrosinase gene (*Tyr*) deficiency increases the magnitude of dysgenesis, indicating that *Tyr* is a modifier of the ocular drainage structure phenotype, although no intraocular pressure increase was detected in these animals [32]. Further studies have reported modest elevation of the intraocular pressure in *Cyp1b1*-KO mice [33] and altered distribution of TM collagen [33][34] associated with decreased levels of periostin [33], as well as TM endothelial dysfunction [34]. Oxidative stress [33][35][36][37][38][39], cell adhesion and migration [37][40] and lipid metabolism [41][42][43] are also altered in *Cyp1b1*-KO mice, suggesting a multifunctional role of this gene in development and homeostasis. *Cyp1b1* LoF has been explored in zebrafish mainly by morpholino (MO)-mediated knockdown [44][45][46][47]. This approach, which inhibits protein expression only in early developmental stages, results in heart malformations and pericardial edema and also affects the development of neural crest cell-derived tissues [47], indicating the role of *cyp1b1* in early embryo development. Overexpression of *cyp1b1* leads to craniofacial and ocular defects, inhibited ocular fissure closure via an RA-independent pathway and disruption of ocular neural crest cell migration. Interestingly, these studies support the existence of functional conservation between the human and zebrafish *cyp1b1* genes [45].

To the best of our knowledge, herein we report the first *cyp1b1*-KO zebrafish model for exploring the pathogenic mechanisms involved in *cyp1b1* LoF. We show that *cyp1b1* inactivation does not mimic congenital glaucoma but leads to adult-onset and variable craniofacial alterations. Transcriptomic analysis reveals alteration of genes participating in extracellular matrix (ECM) and cell adhesion, developmental signaling pathways, lipid metabolism and inflammation. The established *cyp1b1*-KO zebrafish line provides a new model with which to investigate the biological function of this gene and opens new avenues for studying the molecular mechanisms underlying *cyp1b1* LoF-associated pathogenesis.

2. Current Insights

CYP1B1 LoF mutations are the main identified genetic cause of CG; however, the pathogenic mechanisms are not clear. To the best of our knowledge, this is the first *cyp1b1*-KO zebrafish model generated to analyze the mechanisms underlying *cyp1b1* LoF. The CRISPR/Cas9 *cyp1b1*-KO zebrafish line carried the c.535_667del133

deletion. RT-qPCR demonstrated a remarkable reduction in *cyp1b1* mRNA. In addition, this mutation was predicted to lead to a frameshift (p.(His179Glyfs*6)) and to a truncated cyp1b1 enzyme translated from residual mutant mRNA. The truncated protein lacks important functional domains, including the enzyme active center, which is located downstream of the premature termination codon. Altogether, these data support that the obtained mutation results in a complete *cyp1b1* LoF.

Approximately 25% of F0 *cyp1b1* crispant larvae presented variable microphthalmia and lower jaw underdevelopment at 144 hpf. These early defects might have been due to disrupted migration of neural crest-derived cells, which are involved in cranial and jaw morphogenesis [48]. Consistent with this idea and with our results, cyp1b1 has been described to be expressed in the developing eye and pharyngeal arches both in zebrafish [45] and in chicken [49] embryos, and zebrafish *cyp1b1* knockdown affects the development of neural crest cell-derived tissues in zebrafish, resulting in early mild ocular defects [47]. In contrast, the established *cyp1b1*-KO zebrafish line did not manifest these early phenotypes, although at 24 hpf, all the embryos presented two new features: egg volume reduction and transitory developmental delay that completely recovered at 48 hpf. Accordingly, craniofacial and ocular developmental delay observed in zebrafish *cyp1b1*-knockdown in the first 48 hpf also recovers by 96 hpf [45]. Interestingly, the egg and growth abnormalities in the *cyp1b1*-KO zebrafish line were exclusively observed in the offspring of *cyp1b1*-KO females and correlated with *cyp1b1* mRNA levels during early embryonic development, demonstrating their maternal inheritance and suggesting the participation of maternal *cyp1b1* mRNA in early embryo development. Remarkably, the early morphological phenotypes were absent in the established *cyp1b1*-KO zebrafish line, which might be explained by lethality and/or compensating mechanisms. *Cyp1b1* LoF may be lethal in F0 zebrafish with susceptible genetic backgrounds, leading to selection of animals with compensating genetic backgrounds. Consistent with this hypothesis, we did not observe morphological defects among adult F0 crispants (>one year), suggesting that phenotypically affected larvae probably died due to feeding limitations associated with craniofacial defects. In addition, phenotypic differences between F0 crispants and established KO zebrafish lines are not uncommon [50][51][52] and may result from functional replacement of the deactivated gene by functionally related paralog or non-paralog compensatory genes [51]. These compensatory genes may be more easily upregulated in stable genetically engineered KOs than in microinjected F0 mosaic KOs [51]. Moreover, mutations that activate NMD mechanisms, such as those present in our *cyp1b1*-KO zebrafish line, are more prone to triggering compensatory mechanisms [52][53] than posttranscriptional interferences, such as those produced by MO knockdown.

The main phenotype detected in the *cyp1b1*-KO zebrafish line comprised variable adult-onset jaw and craniofacial alterations (increased head height and reduced jaw length), suggesting that disrupted ECM alterations may underlie these defects. Consistent with this hypothesis, defects in ECM remodeling, more than deposition failures, have been proposed to cause progressive TM atrophy associated with fragmentation and irregular distribution of collagen fibers present in aging *Cyp1b1*-KO mice and absent in young animals (< two weeks old) [34]. We were not able to determine the exact age onset of the craniofacial phenotype. Further work is required to determine when these defects start to manifest. The adult craniofacial alterations observed in our *cyp1b1*-KO zebrafish line also presented incomplete penetrance and variable expressivity characterized by uni- (Ph1) or bilateral (Ph2) jaw shortening. Inbreeding increased the penetrance from 26.6% to 86.6%, indicating that the phenotype is strongly

influenced by the genetic background. The typical human phenotype associated with *CYP1B1* LoF, i.e., PCG, also presents phenotypic variability [54] and incomplete penetrance [55], illustrating that although the phenotypes are different in these two species, they are also highly influenced by the genetic background. Another interesting parallelism between this *cyp1b1* LoF zebrafish model and human CG [12] is the unexpected presence of abnormal phenotypes in some heterozygotes, which again indicate the role of modifiers in these phenotypes. In contrast to humans, we did not observe ocular glaucoma-related histological defects associated with complete *cyp1b1* LoF in zebrafish, which might be due to developmental species differences and shows that zebrafish are not adequate to model *cyp1b1*-associated glaucoma. In accordance with our results, 48-hpf zebrafish embryos with MO *cyp1b1* knockdown did not present glaucoma; they only manifested mild ocular phenotypes that recovered by the larval stage [47] and presented minimal effects on zebrafish craniofacial development at 96 hpf [45]. Nevertheless, microinjection of human wildtype *CYP1B1* mRNA but not of LoF mutant versions reproduces phenotypes resulting from *cyp1b1* overexpression in zebrafish larvae [45], showing the functional equivalence between the human and zebrafish ortholog proteins. Mammalian species such as mice or even other species with ocular developmental pathways phylogenetically closer to those of humans may be needed to develop appropriate CG models. In this regard, *Cyp1b1*-KO mouse models show subtle iridocorneal angle abnormalities also dependent on modifier factors such as *Tyr* deficiency, but these defects result in undetectable [32] or modest intraocular pressure elevation [33]. Interestingly, *Tyr* is not a modifier of the PCG phenotype in humans [56], supporting that *CYP1B1*-associated phenotypes are species-specific. Keeping in mind these limitations, the zebrafish may provide valuable information to determine the precise biological functions of *cyp1b1* as well as to understand the general pathogenic processes underlying *cyp1b1* LoF.

To characterize the molecular basis of the phenotypes associated with *cyp1b1* LoF, we performed a transcriptomic analysis in the offspring (seven dpf) of *cyp1b1*-KO zebrafish with craniofacial defects. The functional enrichment analysis of DEGs identified a consistent alteration of genes involved in three biological processes that could be directly related to the observed phenotypes: (i) the ECM and cell adhesion, (ii) the regulation of cell proliferation and (iii) lipid metabolism (retinol, steroids and fatty acids). In addition, metabolic-related oxidation–reduction processes, which included many cytochrome P450 genes, and immune response and inflammation were also significantly enriched in our analysis.

In the first group, we found altered expression of a repertoire of matrix metalloproteinase (MMP)-encoding genes that may disrupt ECM assembly and remodeling, playing a direct role in adult and early craniofacial phenotypes observed in *cyp1b1*-KO zebrafish. Some of these MMPs participate in neural crest-derived cell migration (*ADAMTS20A* or *LOC101886654*) [57], regulate fibronectin levels in zebrafish (*mmp11b*) [58] or break down elastin and other proteins (*cela1.3*, a serine-type endopeptidase orthologous to the human chymotrypsin-like elastase 1 or *CELA1*) [59]. Similarly, the identification of cell adhesion DEGs, such as those encoding protocadherins (*Pcdh1g30*, *Pcdh1g3*, *Pcdh1gb9*, *Pcdh1g2* and *Pcdh1g26*), desmosomal proteins (desmoglein (*Dsg2.1*) and desmocollin (*Dsc2l*)) and periostin (*Postna*) indicate possible dysregulation of developmental signaling and developmental processes, including morphogenesis [60][61]. In fact, *Postna* modulates ECM organization [62] and is involved in ocular developmental defects observed in the *Cyp1b1*-KO mice [33], and MO-mediated *dsg2.1* knockdown is associated with head development disruption [63].

Functionally enriched DEGs playing a role in cell proliferation pathways and craniofacial morphogenesis suggested an alteration in development signaling in the *cyp1b1*-KO zebrafish that might also contribute to the craniofacial phenotypes observed in adult mutant zebrafish and maybe in F0 crispant larvae. Among these genes, we found members of the c-Jun/AP-1 (*junba* and *junbb*) canonical Wnt (*wnt9b*) signaling pathways, indicating that those members were altered. Interestingly, *wnt9b* knockdown produces jaw and craniofacial defects in zebrafish larvae [64]. On the other hand, downregulation of some genes of this group (*grhl3*, *furina*, *ahrra* and *cdk6)* leads to craniofacial maldevelopment in different animal models [65][66][67]. Three of these genes (*grhl3*, *furina* and *ahrra*) were upregulated in our animal model, suggesting they might participate in possible genetic compensation of *cyp1b1* LoF. Additional downregulated genes such as *fosl1a* and *relb* participate in bone matrix remodeling [68] and osteoclast differentiation [69], respectively.

Regarding lipid metabolism, we identified four DEGs (*rbp1*, *rbp2b*, *ugt2a2* and *ugt1ab*) involved in retinol transport and metabolism [70], suggesting that retinol metabolism alteration might be an additional mechanism contributing to the observed phenotypes. Retinoid signaling plays a key role in embryonic development of different organs, including the eye [71], and alteration of this pathway may disrupt migration of cranial neural crest cells, leading to ocular and craniofacial defects [72][73][74][75], similar to those observed in our *cyp1b1*-KO zebrafish line. In addition, and consistently with this idea, cyp1b1 has been described to metabolize retinol to retinaldehyde and then to retinoic acid (RA) in vitro [3][49], and treatment of zebrafish with exogenous RA results in prognathic jaw development, while inhibition of endogenous RA decreases head height [76], resembling the phenotypes observed in the *cyp1b1*-KO zebrafish. Further investigations are necessary to elucidate the involvement of retinoids in our *cyp1b1*-KO zebrafish model. Genes involved in steroid hormone biosynthesis and functionally related with *cyp1b1* were also differentially expressed in the *cyp1b1*-KO zebrafish, although only three of them (i.e., *cyp24a1*, *ugt2a2* and *hsd11b2*) were upregulated, indicating their possible participation in *cyp1b1* LoF compensation. *Cyp24a1* participates in vitamin D hydroxylation and fatty acid omega oxidation and it is associated with hyperlipidemia in rats [77]. Alteration in lipid metabolism is further supported by the identification of several DEGs of the lipid metabolism-modulating PPAR signaling pathway [78], including, for instance, *cyp7a1* and *cyp8b1*, which are involved in bile acid biosynthesis [79]. In line with our findings, *Cyp1b1*-KO mice present PPAR pathway dysregulation [41], although some key genes followed different trends in our study. For instance, *igfbp1*, a regulator of liver fatty acid homeostasis, was overexpressed in our study and downregulated in KO mice. *Igfbp1* expression is affected by diet and sex [41][43], therefore, differences in these variables may explain the discrepancy. The finding of altered expression of lipid metabolism genes and lipid composition in *Cyp1b1*-KO mice is also consistent with our results [41][43][80]. Similarly interesting is the identification of differentially expressed redox genes, including several upregulated cytochrome P450 family members (e.g., *cyp24a1*), suggesting that they may compensate, at least partially, *cyp1b1* LoF. Finally, inflammation pathways were also affected in *cyp1b1*-KO zebrafish, which is in line with the inflammatory response inhibition reported in *Cyp1b1*-KO mice [39]. Alteration in inflammatory pathways in the *cyp1b1*-KO zebrafish is supported by the reported roles of this cytochrome in inflammation. In fact, *cyp1b1* is induced in response to inflammation [81] and, along with *Cyp1a1* and *Cyp1a2*, it participates in lipid mediator pathways that regulate neutrophilic inflammation in mice [42]. Further work is required to confirm the status of inflammatory pathways in the zebrafish *cyp1b1* mutant.

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