Biochemical and Biophysical Research Communications xxx (2015) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Analysis of mutants from a genetic screening reveals the control of intestine and liver development by many common genes in zebrafish

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ARTICLE INFO

Article history:
Received 19 March 2015
Available online xxx

Keywords:
Zebrafish
Genetic screen
The intestine
The liver
Organogenesis

ABSTRACT

Both the intestine and liver develop from the endoderm, yet little is known how these two digestive organs share and differ in their developmental programs, at the molecular level. A classical forward genetic screen, with no gene bias, is an effective way to address this question by examining the defects of the intestine and liver in obtained mutants to assess mutated genes responsible for the development of either organ or both. We report here such a screen in zebrafish. ENU was used as the mutagen because of its high mutagenic efficiency and no site preference. Embryos were collected at 3.5dpf for RNA whole mount *in situ* hybridization with a cocktail probe of the intestine marker *ifabp* and the liver marker *lfabp* to check phenotypes and determine their parental heterozygosis. A total of 52 F2 putative mutants were identified, and those with general developmental defects were aborted. To rule out non-inheritable phenotypes caused by high mutation background, F2 putative mutants were outcrossed with wild type fish and a re-screen in F3 generations was performed. After complementation tests between F3 mutants with similar phenotypes originating from the same F2 families, a total of 37 F3 mutant lines originated from 22 F2 families were identified after screening 78 mutagenized genomes. Classification of mutant phenotypes indicated that 31 out of the 37 mutants showed defects in both the intestine and liver. In addition, four “intestine specific mutants” and two “liver specific mutants” showed selectively more severe phenotype in the intestine and liver respectively. These results suggested that the intestine and liver share a substantial number of essential genes during both organs development in zebrafish. Further studies of the mutants are likely to shed more insights into the molecular basis of the digestive system development in the zebrafish and vertebrate.

1. Introduction

Both the intestine and liver are derived from the endoderm, playing fundamental roles in vertebrate physiology [1]. Much efforts have been paid to uncover their developmental mechanisms in animal models. The TGFβ/Nodal signaling pathway is at the top of the molecular hierarchy and essential for endodermal cell fate determination [2]. The Zebrafish Cdx1b, a functional equivalent of mammalian Cdx2, regulates the expression of downstream factors of Nodal signaling during early endoderm formation [3,4]. The pan-endoderm factors such as members of the Gata family are essential for the intestine and liver organogenesis [5–7], while Foxa factors are required for the initiation of liver development [8]. It has been reported that the mesodermal signals FGF, WNT, BMP and RA pathways play very important roles in the anterior-posterior patterning of the gut [9–13] and the initiation and differentiation of hepatocyte [14–16]. Given that both the intestine and liver are derived from the endoderm, it is of no surprise that endodermal determination factors and mesodermal induction signals are essential for the development of both organs. On the other hand, some specific regulatory mechanisms were also reported. TOR signaling controls epithelial morphogenesis in the vertebrate intestine [17]. The Delta-Notch signaling needs to be blocked for the intestinal epithelial cells to differentiate along a secretory pathway [18], and the repression of secretory cell fate by Notch signaling is mediated by the inhibition of *ascl1a* expression [19]. Seiler discovered that smooth muscle tension induces invasive

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http://dx.doi.org/10.1016/j.bbrc.2015.03.119
0006-291X/

remodeling of the zebrafish intestine [20]. Some factors have been identified to affect liver budding and growth. For instance, Hex is crucial for the initiation and budding of the liver primordium [21], while Prox1 controls hepatocyte migration from the endodermal epithelium into the septum transversum [22].

Despite of the numerous reports on genes function in the regulation of intestine or liver development, there is no comprehensive comparison of the similarities and differences of the developmental molecular mechanisms between these two close yet distinct organs. A forward genetic screen randomly surveying the whole genome without gene predisposition may offer us an answer by studying organogenesis of the intestine and liver via genetic mutants.

The zebrafish, *Danio rerio*, has proven to be a powerful model system to study vertebrate organogenesis due to its unique characteristics and conserved molecular mechanisms [23,24]. Zebrafish are easy to raise and reach sexual maturity at around three months. Each female lays several hundred of eggs per week. The embryos are small, transparent and develop outside of the mother body, which are ideal for large-scale genetic screen [25,26]. A lot of mutagenesis screens have been conducted in the organism since the first two large-scale screens in 1996 [27,28], including efforts in searching for either the intestine [29] or liver mutants [14,30]. However, there is no report that examines both digestive organs at the same time.

While gene knock-out technologies, such as TALEN and CRISPR/Cas9, are extremely useful in mutating a target gene [31–33], forward genetic screen, though time-consuming, is still the best approach to get specific genetic mutants to study the molecular mechanisms underlying organogenesis unbiasedly. The most effective way of inducing small, intragenic lesions on a genome-wide scale is chemical mutagenesis. For zebrafish, ENU (N-ethyl-N-nitrosourea) is so far the best mutagen to introduce random point mutation into the genome [34]. The mutagenesis efficiency of the viral vector is merely one-ninth of that observed of ENU [35]. After ENU mutagenesis, F2 families can be used to screen for heterozygous mutants carrying recessive mutations based on the phenotypes revealed in F3 progenies, following Mendel's law of segregation, mutants are then proceeded to map-based cloning to identify the genes responsible for specific phenotypes.

We describe here a forward genetic screen for mutants with intestine and/or liver defects, and the mutants were classified into three categories based on their phenotypes. Further interrogations on these genetic mutants may help us better understand the development of these organs and the underlying molecular mechanisms.

2. Materials and methods

2.1. Zebrafish strains and maintenance

Zebrafish strain AB^{tü} was utilized as the wild type line for outcrossing of the mutants in the study. Fishes were raised and maintained according to standard procedures [36].

2.2. Collection of fertilized eggs

Male and female fishes were separated in the crossing tank at the night before the crossing day. On the next day, the separator was removed to allow mating of the two fishes to obtain synchronized fish embryos. Fertilized eggs were collected and raised in 0.03% sea salt water at 28.5 °C. The developmental stages of the embryos were determined according to previous description [37]. Embryos for RNA whole mount *in situ* hybridization (WISH) were raised with 0.03% 1-phenyl-2-thiourea (PTU) at 12 hours post fertilization (hpf) to inhibit pigment formation, and harvested at 3.5 days post fertilization (dpf) when both the intestine and liver are morphological developed in a large part [23,38].

2.3. ENU mutagenesis and breeding of F2 families

Male fishes (AB strain) were treated with standard ENU mutagenesis procedure [39]. Survivors were outcrossed with wild type female fishes to generate F1 families. F2 families were generated by self-crossing fishes from different F1 families.

2.4. Screening procedure and classification of the mutants

Siblings from the same F2 family were randomly crossed to get F3 progeny. Around 40 embryos from each crossing pair were collected at 3.5dpf to perform WISH. The F2 parents are potential candidates for heterozygous mutants if approximately 25% of the F3 embryos show similar defects according to Mendel's law of heredity. Putative F2 mutants were outcrossed with wild type fishes to generate F3 families.

Similarly, the F4 embryos were utilized to determine F3 heterozygous mutants. Complementation tests were performed if several mutant pairs from the same F2 family exhibit similar phenotypes, and they would be assigned into the same complementation group upon positive results. Subsequently, mutants were classified into different categories based on their phenotypic characteristics (Fig. 1).

2.5. Whole-mount in situ hybridization (WISH)

Probe synthesis and whole-mount RNA *in situ* hybridization were carried out as previously described [30]. The 3.5dpf embryos were digested with Proteinase K (1:1,000, Thermo Fisher) at 37 °C for 26 min. Antisense RNA probes were labeled with digoxigenin (DIG, Roche Diagnostics). Images were taken under a Zeiss Stereo & Zoom Microscope.

3. Results

3.1. ENU mutagenesis and generation of F2 screening families

Thirty healthy male zebrafishes (AB strain) around six months old were used for ENU mutagenesis. Nine fishes survived the treatment, which were outcrossed with wild type female fishes to generate F1 families. Each founder yielded around 100 F1 offspring to grow into adulthood, generating a total of 958 F1 fishes. Fishes from different F1 families were crossed with each other rather than with wild type fishes to generate F2 families, greatly improving the screening efficiency. As a part of a large-scale screen, our screen was performed in some F2 families.

3.2. Screening in F2 families and outcrossing of the mutants

Inbreeding is the method used to identify heterozygous mutant parents. F3 embryos for screening were obtained by randomly crossing the siblings within the same F2 family. A total of 203 crossing pairs were screened, which came from 42 F2 families, among which 66 candidate mutant pairs were identified. Twenty nine pairs with ambiguous results were performed for second identification where 14 pairs were found to be false positives and excluded. Nine pairs were discarded due to their general developmental defects which would bring about the intestine and liver phenotypes as a secondary consequence. Forty three putative mutant pairs originated from 27 F2 families were chosen to

Please cite this article in press as: F. Jiang, et al., Analysis of mutants from a genetic screening reveals the control of intestine and liver development by many common genes in zebrafish, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.03.119