Interferon-Gamma Directly Mediates Developmental Biliary Defects

Shuang Cui,¹ Steven F. EauClaire,¹ and Randolph P. Matthews^{1,2}

Abstract

Biliary atresia (BA) is the most common identifiable hepatobiliary disease affecting infants, in which there are defects in intra- and extrahepatic bile ducts and progressive fibrosis. Activation of interferon-gamma (IFN γ) appears to be critical in both patients with BA and in rodent models of BA. We have recently reported a zebrafish model of biliary disease that shares features with BA, in which inhibition of DNA methylation leads to intrahepatic biliary defects and activation of IFN γ target genes. Here we report that *ifng* genes are hypomethylated and upregulated in zebrafish larvae treated with azacytidine (azaC), an inhibitor of DNA methylation. Injection of IFN γ protein into developing zebrafish larvae leads to biliary defects, suggesting that activation of the IFN γ pathway is sufficient to cause developmental biliary defects. These defects are associated with decreased cholangiocyte proliferation and with a decrease in the expression of *vhnf1* (*hnf1b*, *tcf2*), which encodes a homeodomain protein with previously reported roles in biliary development in multiple models. These results support an importance of IFN γ in mediating biliary defects, and also demonstrate the feasibility of direct injection of intact protein into developing zebrafish larvae.

Introduction

DEFECTS IN THE DEVELOPING biliary system can lead to severe liver disease in infants. The most common identifiable disease affecting bile ducts in infants is biliary atresia (BA), a progressive fibro-inflammatory disorder for which the etiology is unclear¹. Studies in patients and in the mouse model of BA demonstrate a central importance of interferon-gamma (IFN γ) activation.^{2,3} Recently, we reported activation of IFN γ in a zebrafish model of liver disease resembling BA, in which DNA methylation is inhibited.⁴ In this previous study, we observed severe developmental intrahepatic biliary defects in several zebrafish models in which DNA methylation is inhibited. In addition, we observed decreased DNA methylation in cholangiocytes from patients with BA, suggesting that inhibition of DNA methylation may play an important role in the pathogenesis of BA. Others have reported similar results in lymphocytes of patients with BA, and have also shown that the *IFNG* gene is hypomethylated in lymphocytes from BA patients.⁵

In several studies, we and others have demonstrated the ability of zebrafish to model human hepatobiliary diseases, including BA.^{4,6} Other zebrafish models of hepatobiliary diseases support a relatively strong conservation of molecular

pathways such as *hnf6/hnf1b*⁷, *jagged/notch*⁸, and intracellular trafficking pathways.⁹

Our zebrafish models of BA in which DNA methylation is inhibited include genetic models (*ntp*, *dnmt1*-morpholino injected larvae) and chemically-treated models (azacytidine, azaC).⁴ Here we report further analysis of the azaC model showing increased expression and decreased methylation of *ifng* genes. To determine the potential role of IFN γ in mediating biliary defects, we injected human recombinant IFN γ protein into developing zebrafish larvae.

Injection of protein into developing zebrafish larvae is a relatively novel technique. Stoll and colleagues¹⁰ previously injected IL-8 into the developing heart of 24 hpf larvae,¹⁰ while Shibata et al. injected recombinant Musashi1 into embryos.¹¹ These studies demonstrate the feasibility of injecting protein into developing zebrafish, and as our phenotype appears late (3–5 dpf), we were concerned that earlier effects of increased *ifng* expression could have deleterious effects on earlier developmental processes. Thus, we injected recombinant human IFN γ protein into 2 dpf larvae to determine the effect on hepatobiliary development.

Here we report that injection of IFN γ into developing zebrafish larvae does in fact lead to defects in biliary development, similar to defects seen after injection of azaC. Also

¹Division of Gastroenterology, Hepatology, and Nutrition, The Children's Hospital of Philadelphia Research Institute, Philadelphia, Pennsylvania.

²Department of Pediatrics, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania.

similar to azaC-injected larvae, we observe decreased expression of *vhnf1* (*hnf1b*, *tcf2*), which encodes a homeodomain protein previously shown to be important in biliary development in mammals and zebrafish.^{7,12} These results support a central role of IFN γ in mediating biliary defects in developing vertebrates, and further support a role for IFN γ in the pathogenesis of disorders such as biliary atresia.

Materials and Methods

Fish lines and injections

Zebrafish were TL strain in all studies, and were housed in the laboratory animal facility at The Children's Hospital of Philadelphia Research Institute, in accordance with protocols approved by the institutional animal care and use committee (IACUC).

For the azaC studies, azaC (1 mg/mL) was injected into 2 dpf larvae, identical to previous studies.⁴ For the IFN γ injection studies, recombinant human IFN γ was kindly provided by Dr. Jordan Orange (Children's Hospital of Philadelphia Research Institute). The protein was diluted to 40 μ g/mL in sterile water and injected into the yolk of 2 dpf larvae, similar to our previous studies injecting morpholino or drug at that stage. As a control, IFN γ protein was inactivated by adding dithiothreitol (0.1 M final concentration) and boiling for 5 min.

Morpholino injections were performed similar to previous studies. The sequence of the *crfb17* MOs are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/zeb). Confirmation of knockdown by the splice-blocking *crfb17* MO is shown in Supplementary Figure 1. Rescue to demonstrate specificity is shown by the studies of IFN γ and *crfb17* MO co-injection (see below).

In situ hybridizations

The *in situ* hybridization studies were performed essentially as described previously¹³, in accordance to standard protocols. PCR primers to generate riboprobes for *ifng1-1* and *ifng1-2* are shown in Supplementary Table S1.

PED-6 and cytokeratin staining

These studies were performed identically to previous studies.^{4,14,15} Briefly, 5 dpf larvae were given the fluorescent phospholipid PED-6, and gallbladder uptake was assayed 3 h later. Intensity was classified as "normal," "weak," or "absent," and the relative number in each category was scored and compared using chi square analysis. For cytokeratin staining, 5 dpf larvae were fixed in methanol/DMSO, stained with anti-keratin (Ks 18.04) and appropriate secondary. Livers were then examined by confocal microscopy.

Quantitative PCR

For the quantitative PCR (QPCR) studies shown here, livers were isolated from 4 or 5 dpf larvae. Ten livers per condition were pooled in RNeasy lysis buffer, and RNA extraction and cDNA synthesis were then performed in accordance with standard protocols. Expression QPCR studies were essentially performed as per previous studies, with primers (*vhnf1*, *hprt*) also used in previous studies.¹³ For the methylation-specific QPCR (MSP), *in silico* analysis of sequence surrounding the transcription start site (−2.5 to +0.5 kb) was performed using MethPrimer ([http://](http://www.urogene.org/methprimer/)

www.urogene.org/methprimer/), which identified putative CpG islands and methylated and unmethylated primers. MSP primers are also shown in Supplementary Table S1. In the MSP studies, genomic DNA was isolated from 5 dpf larvae using standard procedures, and then treated with sodium bisulfite using EpiTect (Qiagen). The bisulfite-treated DNA was then used as template for MSP, with detection by SYBR Green using a StepOne Plus. For MSP, primers are designed that target the same region as methylated or unmethylated sequence, taking advantage of bisulfite treatment converting unmethylated cytosine to uracil. Because of the sequence differences, amplification of both the unmethylated and methylated versions can be quantified differentially.

PCNA and apoptosis detection

For proliferating cell nuclear antigen (PCNA) detection, larvae at 5 dpf were fixed in 4% paraformaldehyde. The skin was removed, similar to the larvae prepared for cytokeratin staining, and the larvae were then stained with anti-PCNA antibody (FL-261, Santa Cruz Biotechnologies, Santa Cruz, CA) and appropriate secondary antibody. The larvae were also stained with 2F11 antibody¹⁶ and appropriate secondary. The larvae were counterstained with DAPI and examined by confocal microscopy. Cells in the liver stained with 2F11 and 2F11 plus PCNA were quantified in six samples from both control and IFN γ -injected larvae. Statistical comparisons utilized Student's *t*-test.

For apoptosis detection, larvae at 5 dpf were again fixed in 4% paraformaldehyde. We used the Roche *in situ* cell death detection kit with TMR-dUTP as per the manufacturer's instructions. This method involves TUNEL staining to add the labeled dUTP to DNA strand breaks. Larvae were then examined under fluorescence.

Results

Elevation of *ifng* in azaC-treated larvae

We have reported an increase in IFN γ pathway activity in livers from zebrafish larvae treated with azacytidine (azaC),⁴ an inhibitor of DNA methylation.¹⁷ Because our models were based on inhibition of DNA methylation, we hypothesized that decreased methylation of specific genes within the IFN γ pathway would result in increased expression of these genes, thus leading to activation of the IFN γ pathway. The IFN γ signaling pathway is complex, as there are numerous mechanisms by which IFN γ signaling can be activated.¹⁸ While increased expression of numerous genes could thus lead to activation of IFN γ , we initially examined genes explicitly in the IFN γ pathway, such as the *ifng*, *crfb17* (IFN γ receptor), and *irf* (interferon regulatory factor) genes. In zebrafish, there are two *ifng* genes and at least 13 *irf* genes (www.ensembl.org/Zv9); the *irf* genes are specific to the IFN γ pathway but are also target genes¹⁹ and thus would be expected to be elevated in our models regardless of methylation status.

In the azaC-treated larvae, we observed a modest increase in expression of both *ifng1-1* and *ifng1-2* by quantitative PCR and by *in situ* hybridization, with increased liver expression (Fig. 1). Expression of *crfb17* (IFN γ receptor) did not appear to be increased in the azaC-treated larvae, while expression of the IFN γ target *irf1* was increased, as expected (Supplementary Figure S2).

FIG. 1. Elevation of *ifng1-1* and *ifng1-2* in azaC-treated larvae. (A, B) Real-time quantitative PCR of *ifng1-1* and *ifng1-2* in whole 5 dpf azaC-treated larvae, showing a trend towards increase in *ifng1-1* expression (A; $p = \text{N.S.}$) and a significant increase in *ifng1-2* expression (B; $*p \leq 0.05$). (C, D) *In situ* hybridization of 5 dpf control (C, cont) and azaC-treated (D, azaC) larvae with *ifng1-1* showing modestly increased liver (black outline) expression in the azaC-treated larva (D). (E, F) *In situ* hybridization of 5 dpf control (E, cont) and azaC-treated (F, azaC) larvae with *ifng1-2* showing more clearly increased liver (black outline) expression in the azaC-treated larva (F). (G, H) Methylation-specific quantitative PCR showing decreased ratio of methylated to unmethylated *ifng1-1* (G) and *ifng1-2* (H) target in the azaC-treated larvae ($*p \leq 0.05$ for both). Color images available online at www.liebertpub.com/zeb

Interestingly, *ifng1-1* and *ifng1-2* are only 8.5 kb apart on chromosome 4 and thus may be regulated jointly. In addition to recent studies implicating methylation at the *IFNG* locus as potentially important in lymphocytes from patients with BA,⁵ several other studies have demonstrated an importance of promoter methylation in *IFNG* expression.^{20–22} To determine whether the upregulation of *ifng1-1* and *ifng1-2* was due to decreased DNA methylation at the respective loci, we performed methylation-specific qPCR (MSP). We initially performed *in silico* studies on the region immediately around both transcription start sites (from -2.5 kb to $+0.5$ kb) to look for CpG islands and to identify methylation-specific primers. As depicted in Figure 1, there was statistically significant relative hypomethylation in the CpG islands neighboring both the *ifng1-1* and *ifng1-2* genes ($p \leq 0.05$ for both), suggesting that decreased DNA methylation may in fact be leading to the increased expression of these genes. These results are consistent with azaC treatment leading to increased expression of *ifng* genes, and thus we examined whether increased expression of IFN γ was sufficient to cause biliary defects.

Injection of IFN γ leads to biliary defects

To determine whether increased IFN γ activity was sufficient to mediate biliary defects in zebrafish larvae, we injected recombinant IFN γ protein into 2 dpf larvae. Human recombinant IFN γ was injected into the yolk of 2 dpf larvae, similar to our previous studies injecting morpholino antisense oligonucleotide (MO) or drug into the yolk at that stage. We examined biliary function and anatomy of the IFN γ -injected larvae, similar to previous studies. To examine biliary function, we administered the fluorescent phospholipid PED-6 to 5 dpf larvae and assayed gallbladder uptake. PED-6 is swallowed and absorbed through the intestine into the liver, and excreted into the bile, concentrating in the gallbladder. There was diminished gallbladder PED-6 uptake in larvae injected with IFN γ when compared to control larvae (Fig. 2;

$p \leq 0.0001$). This decreased gallbladder function was attenuated if the IFN γ protein was treated by boiling and denaturing, and was also attenuated by co-injection with a MO directed against the IFN γ receptor-1 gene (*crfb17*) (Fig. 2; $p = \text{N.S.}$ between control vs. inactive IFN γ and for control vs. IFN γ + *crfb17* MO), suggesting that the injected IFN γ is biologically functional in the larvae.

Similar to previous studies, we examined intrahepatic biliary anatomy by cytokeratin immunostaining. Confocal projections of livers from 5 dpf IFN γ -injected larvae demonstrated severe defects in the intrahepatic bile ducts (Fig. 2), similar to treatment with azaC reported previously. These results demonstrate that IFN γ is sufficient to cause disruption of biliary development in zebrafish, and support an importance of IFN γ in mediating the biliary defects noted after azaC treatment.

Gene expression changes in IFN γ -injected larvae

We have previously reported a downregulation of Hnf6 target genes in azacytidine-treated larvae, which is consistent with the known role of HNF6 in biliary development in zebrafish and mammals.^{7,23} We examined expression of *vhnf1*, an Hnf6 target gene, in the IFN γ -injected larvae. There is decreased expression of the Hnf6 target gene in livers isolated from 5 dpf IFN γ -injected larvae when compared to control (Fig. 3). A similar decrease in *vhnf1* expression was also seen in livers isolated from 4 dpf IFN γ -injected larvae (data not shown). These results suggest a possible downstream effect of IFN γ on Hnf6 and consequently *vhnf1*. We were not able to rescue the IFN γ -mediated defects by co-injection with *vhnf1* mRNA, however (not shown), suggesting that IFN γ may also affect other critical pathways.

Cell proliferation/death in IFN γ -injected larvae

In order to more fully understand the effects of IFN γ injection in mediating biliary defects, we examined cell

FIG. 2. Biliary defects in IFN γ -injected larvae. **(A)** Gallbladder PED-6 uptake, showing decreased uptake in the IFN γ -injected larvae ($p \leq 0.0001$ relative to control by chi square). Uptake in larvae injected with inactivated IFN γ is also shown, as is uptake in larvae injected with IFN γ and morpholino antisense oligonucleotides directed against *crfb17* (IFN γ receptor). There is no significant difference in uptake between these conditions and control ($p = \text{N.S.}$). **(B–E)** Confocal projections of cytokeratin immunostaining in whole-mount 5 dpf control **(B)**, azaC-treated **(C)**, and IFN γ -injected larvae **(D, E)**. Note that the IFN γ -injected liver demonstrates only short, isolated ducts, similar to bile ducts noted in larvae injected in azaC reported previously⁴ and in **(C)**. Scale bar, 25 μm . Color images available online at www.liebertpub.com/zeb

proliferation and cell death in the IFN γ -injected larvae. We examined proliferation of cholangiocytes by immunostaining using the cholangiocyte marker 2F11 and the proliferation marker PCNA. At 3 dpf and 4 dpf, during early biliary growth, there were fewer cholangiocytes as well as a lower number of cholangiocytes undergoing cell division in the IFN γ -injected larvae (Table 1 and Figure 4). Of note, this effect seemed to be relatively specific to cholangiocytes, as at 3 dpf there was no difference in hepatocyte number or proliferation between control and IFN γ -injected larvae (Table 2). Moreover, at 4 dpf the relative number of cholangiocytes in the IFN γ -injected larvae is lower, which also supports a more specific effect on cholangiocytes (Table 2). By 5 dpf, there continued to be fewer cholangiocytes, but the number of dividing cholangiocytes was indistinguishable between control and the IFN γ -injected larvae (Fig. 4 and Table 1). This is consistent with an effect of IFN γ on cholangiocytes in the developing liver, possibly affecting the initial differentiation, but more clearly affecting proliferation.

FIG. 3. Decreased expression of *vhnf1* in IFN γ -injected larvae. Real-time quantitative PCR of *vhnf1* in control (inactive IFN γ) and IFN γ -injected larvae showing a 40% decrease in *vhnf1* expression ($*p \leq 0.05$).

We also examined the IFN γ -injected larvae for apoptotic cell death of cholangiocytes in the developing liver. Examination of apoptotic cell death by TUNEL staining did not demonstrate an increase in apoptosis in the IFN γ -injected larvae (Fig. 4). This contrasts with BA and with the mouse model of BA, in which apoptosis appears to be playing a larger role.²⁴

Discussion

Here we have shown that IFN γ is sufficient to elicit biliary defects in developing zebrafish larvae. In addition, in azaC-injected larvae, there is hypomethylation of *ifng1-1* and increased liver expression of this gene as well. These findings

TABLE 1. DECREASED NUMBER OF CHOLANGIOCYTES IN IFN γ -INJECTED LARVAE

	2F11+	PCNA+ and 2F11+	Fraction PCNA/2F11
3 dpf control	10.3 $\pm$ 3.1	8.0 $\pm$ 2.1	0.78 $\pm$ 0.09
3 dpf IFN γ -injected	5.8 $\pm$ 1.5*	4.5 $\pm$ 1.4*	0.75 $\pm$ 0.09
4 dpf control	28.7 $\pm$ 6.2	22.3 $\pm$ 3.2	0.79 $\pm$ 0.09
4 dpf IFN γ -injected	14.3 $\pm$ 6.2**	10.5 $\pm$ 4.5***	0.74 $\pm$ 0.07
5 dpf control	35.3 $\pm$ 10.0	9.8 $\pm$ 2.8	0.28 $\pm$ 0.06
5 dpf IFN γ -injected	20.3 $\pm$ 8.9*	9.2 $\pm$ 3.8	0.47 $\pm$ 0.12*

Larvae were stained with PCNA and 2F11, and positive cells were counted from 6 samples in each condition. At 3 and 4 dpf, there are significantly fewer cholangiocytes in the IFN γ -injected larvae, and significantly fewer proliferating cholangiocytes. At 5 dpf, there continue to be fewer cholangiocytes in the IFN γ -injected larvae, but the number of PCNA-positive cells is similar in both conditions. Note that the relative increase in cholangiocytes is similar in both conditions (ie, the number of cholangiocytes at 4 dpf is approximately 3 $\times$ the number at 3 dpf in both). The fraction of PCNA-positive cells is similar at both 3 and 4 dpf, but there is a higher percentage of PCNA positive cells at 5 dpf. ($*p \leq 0.05$; $**p \leq 0.005$; $***p \leq 0.0005$, all relative to control for the same age; other comparisons not significant; comparisons by Student's *t*-test).

FIG. 4. Cell proliferation and cell death in IFN γ -injected larvae. (A–F) Cell proliferation in control (A, C, E) and IFN γ -injected (B, D, F) larvae. Livers (white outline) were stained with anti-PCNA (red) to label proliferating cells and 2F11 (green) to label cholangiocytes, and were counterstained with DAPI. Representative livers from 3 dpf (A, B), 4 dpf (C, D), and 5 dpf (E, F) are shown. Note that there are fewer cholangiocytes in the IFN γ -injected larvae and fewer proliferating cholangiocytes in the IFN γ -injected at 3 dpf and 4 dpf. This is quantified in Table 1. (G, H) TUNEL staining of whole-mount 5 dpf larvae show no positive cells (white arrows) in the liver (white outline) in either control (G) and IFN γ -injected (H) larvae. Scale bar 25 μ m for (A–F).

suggest that biliary defects caused by inhibition of DNA methylation in developing zebrafish larvae are at least partially due to activation of IFN γ signaling. This has implications for diseases such as biliary atresia, in which there are biliary defects and activation of IFN γ , as it suggests that the IFN γ may be directly leading to the defects, at least in part.

Possible mechanisms by which IFN γ may lead to biliary defects

Our results above demonstrate that injection of IFN γ leads to fewer cholangiocytes in the developing zebrafish larvae, seemingly as a result of decreased proliferation, and possibly as a result of decreased differentiation of progenitor cells into cholangiocytes. We also observed decreased expression of *vhmf1*, a downstream target of HNF6, in a genetic pathway known to be important in biliary development.^{7,23} While this could be secondary to the decreased number of cholangiocytes, our previous results have demonstrated diffuse expression of *vhmf1* in the developing liver,⁷ suggesting that the decrease in *vhmf1* expression is due to globally decreased liver expression and not simply secondary to fewer cholangiocytes in the IFN γ -injected larvae.

Any effect of activation of IFN γ signaling on biliary development would most likely be mediated via the IFN γ receptor, as our results demonstrate that MO-mediated knockdown of the receptor at least partially reverses the defects. Activation of this pathway leads to increased expression of interferon regulatory factors (IRFs), which in turn activate additional downstream genes.¹⁹ These secondary effects of IFN γ activation govern several cellular processes, including inhibition of cell growth and proliferation, and increased cell death. Of note, the liver appears to be particularly sensitive to excess IFN γ activation, as mice deficient in the IFN γ inhibitor *Socs1* demonstrate liver degeneration,²⁵ and mice overexpressing *Ifng* in their liver demonstrate chronic hepatitis.²⁶ Thus, our results are consistent with previous studies showing an effect on liver cells. We do see inhibition of cell proliferation in our IFN γ -injected larvae, although our effect does appear more pronounced in cholangiocytes than in hepatocytes. We cannot exclude an additional effect of IFN γ on earlier differentiation of progenitor cells into cholangiocytes, but as there is limited evidence for IFN γ directly inhibiting epithelial cell development in other systems, inhibition of proliferation appears the more likely mechanism.

TABLE 2. MODEST DECREASE IN NUMBER OF HEPATOCYTES IN IFN γ -INJECTED LARVAE

	Non-2F11+	PCNA+ non-2F11+	Fraction PCNA/2F11	Ratio 2F11+/non-2F11+
3 dpf control	202.5 $\pm$ 37.4	189.8 $\pm$ 35.9	0.94 $\pm$ 0.02	0.05 $\pm$ 0.02
3 dpf IFN γ -injected	172.2 $\pm$ 62.0	158.5 $\pm$ 56.1	0.92 $\pm$ 0.04	0.04 $\pm$ 0.01
4 dpf control	356.8 $\pm$ 36.1	276.8 $\pm$ 19.6	0.78 $\pm$ 0.07	0.08 $\pm$ 0.01
4 dpf IFN γ -injected	265.8 $\pm$ 48.5**	199.5 $\pm$ 37.3**	0.75 $\pm$ 0.07	0.05 $\pm$ 0.02*

In the same 6 larvae shown in Table 1, non-2F11-expressing cells $\pm$ PCNA were counted. Of note, the total number of cells counted in the livers is the sum of the 2F11 + and non-2F11 + cells. At 3 dpf, there is not a statistically significant difference between non-2F11 + cells (mostly hepatocytes) in control and in IFN γ -injected larvae. At 4 dpf, there is a difference between the number of hepatocytes and proliferating hepatocytes in control and in the IFN γ -injected larvae. The last column shows the ratio of cholangiocytes to noncholangiocytes in the livers, showing that at 4 dpf there are proportionally fewer cholangiocytes in the IFN γ -injected larvae. (* $p \leq 0.05$; ** $p \leq 0.005$, all relative to control for the same age; other comparisons not significant; comparisons by Student's *t*-test).

There may be indirect effects of IFN γ on biliary development, or effects of IFN γ not mediated through IRFs that could lead to an inhibition of cholangiocyte differentiation. As noted, the homeodomain gene *vhnf1* has a clear role in biliary development in zebrafish and in mice. The inability of *vhnf1* mRNA injection to rescue the defects caused by IFN γ injection suggests that the downregulation of *vhnf1* by IFN γ is not sufficient to lead to biliary defects, however. Alternative downstream targets of IFN γ that may have effects on biliary development include members of the GTPase family.¹⁹ Although this family of proteins has a wide variety of functions, GTPases play an important role in intracellular trafficking and the establishment of cell polarity—processes clearly important in biliary development and disease pathogenesis.^{6,9,13,27}

IFN γ in biliary atresia

As noted above, IFN γ appears to play a critical role in both the animal model of BA and in patients with BA.^{2,3} Our studies here support the utility of zebrafish as a model to study BA, as we have demonstrated an importance of IFN γ in mediating biliary defects similar to other zebrafish models resembling BA. Moreover, our results support the critical role for IFN γ in mediating biliary defects, and suggest that the defects in patients may be mediated by IFN γ .

In addition, we have demonstrated that in our zebrafish model with features of BA in which DNA methylation is inhibited, there is upregulation and hypomethylation of *ifng* genes. Regulation of *IFNG* and *IRF* genes by DNA methylation has been reported in several diseases, including BA,⁵ and other conditions ranging from ulcerative colitis to various cancers.^{22,28,29} The previous report of *IFNG* hypomethylation in BA focused on lymphocytes, but our studies suggest the possibility that these genes could be hypomethylated in the liver as well, consistent with our previous report showing decreased DNA methylation in cholangiocytes from BA patients.⁴ Thus, our results here and elsewhere are consistent with a model in which decreased DNA methylation in cholangiocytes leads to aberrant activation of *IFNG* and other genes, resulting in biliary defects.

Successful injection of intact protein into larval zebrafish

We have shown that injection of protein into the yolk of 2 dpf larvae leads to an effect on biliary development. This suggests that similar approaches could provide important methods to examine the effects of proteins on development. As noted above, others have recently injected IL-8 into the developing heart of 24 hpf larvae,¹⁰ and Musashi1 into early embryos.¹¹ In our studies, we believe that the effect is relatively limited to the liver, as yolk is initially metabolized in the liver and thus the injected IFN γ likely enters the liver readily. Injection of protein then represents a technique useful in modulating developmental processes locally, and could thus be applied to multiple developing tissues in zebrafish embryos and larvae.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Randolph P. Matthews, MD, PhD

Division of Gastroenterology, Hepatology, and Nutrition

The Children's Hospital of Philadelphia Research Institute

902C Abramson Research Building

3615 Civic Center Boulevard

Philadelphia, PA, 19104

E-mail: matthews@email.chop.edu