Zebrafish Models of Fibrotic Disease

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Zebrafish models of fibrotic disease include, among others, cardiovascular disease models, liver disease models (categorized into Alcoholic Liver Diseases (ALD) and Non-Alcoholic Liver Disease (NALD)), and chronic pancreatitis models.

Keywords: zebrafish ; animal models ; fibrotic disease ; chemical induction ; genetic manipulation ; ECM accumulation

1. Introduction

Fibrotic disorders, including various cardiovascular diseases (CVD), liver cirrhosis and chronic kidney disease (CKD), are characterized by a progressive and irreversible accumulation of extracellular matrix in the organs concerned. Eventually, this excess of fibrotic tissue impairs the function of the organ, potentially resulting in a life-threatening situation [1][2][3]. For instance, according to WHO global health estimates, around 31% of all deaths worldwide are attributed to CVD each year, and cardiac fibrosis (CF) is implicated in almost all forms of CVD [4]. In fact, it is difficult to accurately estimate the incidence of each fibrotic disease, because most of these disorders are concealed at early stages and manifest themselves across multiple organ systems.

2. Fibrotic Diseases and Underlying Mechanisms

From a mechanistic perspective, fibrosis is the result of an abnormal tissue repair that eludes homeostatic regulatory mechanisms, and then becomes a progressive fibrotic process that ultimately results in organ dysfunction and failure [5]. It is characterized by a massive net accumulation of extracellular matrix (ECM) that is composed of macromolecules, including collagens (e.g., COL1 and COL3), elastins, fibronectins, etc. Myofibroblast cells are considered to be responsible for the overproduction of ECM and fibrosis progression [6][7].

As a range of related disorders [6], fibrotic diseases have been classified into systemic fibrotic diseases (such as systemic sclerosis [8], and IgG4-associated tissue fibrosis [9]), organ-specific fibrotic diseases (such as cardiac, kidney, pulmonary, and liver fibrosis) [10], and other organ-specific fibrotic diseases (such as intestinal and bladder fibrosis) [11][12][13][14].

The progression of chronic diseases eventually resulting in fibrosis is the pathological outcome of a complex cross-talk between several key players like epithelial, endothelial and inflammatory cells that elicit and sustain fibrosis, and myofibroblasts that are the primary ECM-secreting cell type executing fibrosis.

It is generally accepted that repetitive injury to the epithelial compartment is a pivotal event in the development of fibrotic diseases. Indeed, epithelial cells can dedifferentiate upon continued stress into simplified and flattened cells that secrete paracrine factors like hedgehog and Wnt ligands, thereby stimulating myofibroblast differentiation. When cellular injury persists, these cells might even become senescent typically exhibiting apoptosis-resistance and displaying cell-cycle arrest without further proliferation and repair. Significantly, senescent cells also secrete numerous proinflammatory and profibrotic paracrine mediators (including TGF-b) that further drive the activation of myofibroblasts and amplify inflammatory processes [15][16][17].

During interstitial fibrosis, injury to the endothelial cells (together with the basal membrane delineating peritubular capillaries) can also result in capillary rarefaction, interstitial inflammation and fibroblast activation. The subsequent microvascular dysfunction can then further lead to local hypoxia, one of the forces causing fibrosis [18][19].

Inflammatory immune cells like macrophages are present in all types of diseases and fibrosis. There are numerous indications that fibroblasts play an essential role in mediating fibrosis in organs. As terminally differentiated cells, activated myofibroblasts are special fibroblasts that are rarely observed in non-pathological situation, but they are frequently observed in the process of wound healing. These cells share some characteristics of smooth muscle cells and secrete α-smooth muscle actin (α-SMA), fibronectin ED-A, CD31, adhesion molecules and other mesenchymal cells markers [20].

In normal wound healing, most myofibroblasts undergo apoptosis and disappear following the completion of re-epithelialization [21][22][23]. However, persistent myofibroblast activation is a shared feature in fibrotic diseases. Therefore, overproduction of their hallmark, alpha-smooth muscle actin (α-SMA), is prevalently used as a marker of fibrosis [24].

Regarding the origin of fibrogenic myofibroblasts, most of them originate from resident cells, although it varies depending on the organ involved [25][26][27]. For instance, hepatic myofibroblasts in fibrotic liver are mostly derived from liver-resident hepatic stellate cells [26][28], mesothelial cells [29] and portal fibroblasts (PFs) [26]. A small contribution is made by bone marrow (BM)-derived cells (mesenchymal stem cells and fibrocytes) [26][28]. In the case of cardiac fibrosis, the source of myofibroblasts are resident fibroblasts and perivascular cells [19], while the contribution of other cell types such as endothelial cells, fibrocytes, epicardial cells, haematopoietic bone marrow-derived cells, is still controversial [25].

A growing body of evidence suggests that numerous molecules, such as transforming growth factor-beta (TGF-b), Twist, Snai1, Wnt, Hedgehog and Notch are involved in regulating the various pathways of fibrogenesis. The most common outcome of their activity is an increasing population of activated myofibroblasts and the progressive development of fibrosis.

Among these molecules, TGF-b has been considered to be a primary mediator in the regulation of fibrosis, especially TGF-b1 that has been accepted as a master of myofibroblasts activation, transformation and differentiation in fibrosis [7][30]. It has been documented that TGF-b stimulates the EMT program in tubular epithelial cells, promoting the proliferation and activation of myofibroblasts, upregulated by increased Twist and Snai1 gene levels [31][32].

This protraction of β-catenin activation results in epithelial dedifferentiation and interstitial fibrosis Wnts have been established as playing an important role in myofibroblast activation and interstitial fibrosis which might be due to the essential role that Wnt ligands play in paracrine signalling between injured epithelial cells and interstitial myofibroblasts. Similarly, Hh ligands are also upregulated during fibrogenesis, accompanied by Hh pathway activation shown by increased Gli1 gene expression [33]. This provides evidence that Hh ligands also stimulate myofibroblast activation through paracrine secretion in the signalling loop.

In parallel to the Wnt and Hh pathways, the Notch pathway also plays a pivotal role in organs development and fibrogenesis. For instance, Notch induction was observed in cardiac fibrosis formation and regeneration along with the dedifferentiation of epithelial cells [34]. In addition, expression of Notch in cultured epithelial cells results in activation of their EMT program through regulating the gene expression ofSnail, which is a key regulator of EMT [35]. Epithelial Notch may then be assumed to promote fibrosis in vivo, via activation of the EMT program.

3. Zebrafish Models of Fibrotic Disease

Cardiovascular disease (CD) is one of the leading causes of morbidity and mortality worldwide. Ischemic and many non-ischemic cardiomyopathies commonly involve major cardiac muscle loss and fibrosis, and are the primary causes of heart failure [36]. In adult mammals, dead cardiomyocytes, caused by myocardial infarction, are replaced with irreversible fibrotic scars rather than being regenerated [37]. To better understand the pathogenesis of CF, how regeneration affects fibrogenesis, and eventually seek a cure, some zebrafish models of CDs have been developed [38][39][40] in adult zebrafish, namely through ventricular apex resection and cryoinjury (**Table 1**).

Table 1. Overview of reported zebrafish models of fibrotic disease, differentiating the larval and adult stage, and techniques used for induction and validation.

	Larvae			Adults		
Organs	**Induction**	**Detection**	**References**	**Induction**	**Detection**	**References**
Heart	**Not known**	**Not known**	**Not known**	**Ventricular apex resection (20%)**	**AFOG staining**	**Poss 2002 [63]**
				Cryoinjury	**Aniline Blue or MT staining**	**Chablais et al., 2011 [21]**
				Multiple cryoinjuries	**AFOG staining**	**Bise et al., 2020 [65]**

	Larvae			Adults		
Organs	Induction	Detection	References	Induction	Detection	References
Liver	TAA induction	Sirius Red staining & qPCR	van der Helm et al., 2018 [23]	EtOH induction	Picrosirius Red staining	Park and Kim 2019 [66]
	EtOH & NTR/MTZ ablation	IHC staining	Huang et al., 2016 [67]	EtOH & NTR/MTZ ablation	IHC staining	Huang et al., 2016 [67]
	EtOH & *mpi* knockdown	qPCR	DeRossi et al., 2019 [68]	EtOH & *mpi* knockdown	MT staining	DeRossi et al., 2019 [68]
				Ovarian senescence & obesity	Sirius Red staining	Turola et al., 2015 [69]
				Overexpressed tgfbβ1α induction	Sirius Red & IHC staining	Yan et al., 2019 [13]
Pancreas	Not known	Not known	Not known	Hedgehog (Hh)-induction	MT staining	Jung et al., 2011 [22]
				Transgene *KRAS*^{G12D} expression	MT staining	Oh and Park 2019 [14]
Other organs (tissues)	Not known	Not known	Not known	PHMG-P induced gill fibrosis	MT staining	Oh et al., 2018 [70]
				Ionizing irradiation caused muscle fibrosis	MT staining	Epperly et al., 2012 [71]

NTR, nitroreductase; MTZ, metronidazole; AFOG, acid fuchsin orange G; MT staining, Masson's trichrome staining; TAA, thioacetamide; IHC, immunochemistry; *mpi*, mannose phosphate isomerase; PHMG-P, polyhexamethylene guanidine phosphate.

VAR commences with anesthetizing and mounting of zebrafish, followed by incision of the chest wall and transection of the cardiac ventricle apex [41]. Massive ECM accumulation, detected with acid fuchsin orange G (AFOG), is observed in the wound of zebrafish 9 days post injury (dpi). Heart regeneration that is complete at 60 dpi starts with restoration of the ventricular wall in the wound area, followed by proliferation of myocytes [39][42][20].

A robust and fairly reproducible injury to the heart can be generated with this technique, and a certain part of the heart can be ablated specifically. The method is, however, inherently invasive, difficult to operate, low-throughput and sometimes causes lethality. Besides, not only are the cardiomyocytes resected, but also endocardial cells, epicardial cells, and vascular endothelial cells [41].

Histologically, fish hearts that have undergone cryoinjury develop a collagen and fibrin-rich scar from 4 to 14 dpi, progressively dissolving the fibrin as well as contracting the scar area from 14 dpi on and taking around 2 months to heal [38][43]. Additionally, compared to the VAR zebrafish model, collagen deposition in cryoinjury models was more pronounced, and a longer time was required for complete resorption [39][43]. Finally, using a multiple-cryoinjury approach it was demonstrated that the zebrafish heart shows a growing inefficacy in scar resorption related to the number of cryoinjuries applied. For instance, after six applications, the heart presented with uncomplete fibrotic tissue resolution and increased accumulation of collagen at the wound site.

The liver is the largest internal organ in both mammals and lower vertebrates, such as zebrafish and frogs. Chronic liver damage is mainly caused by toxins, viral infections (e.g., hepatitis C virus (HCV)) [44], autoimmune conditions, and metabolic and genetic diseases [45]. Liver fibrosis is the common outcome of chronic or iterative insults.

Physiologically, with the exception of Kupffer cells, the zebrafish liver encompasses the same primary cell types (e.g., hepatocytes, stellate cells, biliary cells, and endothelial cells) [46] performing similar functions as their mammalian counterparts [47]. Therefore, zebrafish are considered to be an important tool for studying liver diseases. Several zebrafish fibrotic liver models have been documented using both larvae and adults, including alcoholic liver disease (ALD) chemically induced models, and genetic models (**Table 1**).

Alcohol abuse is a common cause of liver fibrosis known as ALD. Ethanol (EtOH) immersion has been applied to both zebrafish larvae [48][49] and adults [50][51] for fibrotic liver modelling. To that end, zebrafish are immersed in EtOH-containing water replenished on a daily basis to keep the EtOH concentration stable and the housing environment clean. the animals in both studies also developed steatosis and a hepatocyte ballooning phenotype.

Importantly, ethanol treatment of genetically engineered zebrafish that express a hepatocyte-specific ablation system, can exacerbate and accelerate dramatically fibrogenesis compared to wild type zebrafish [52]. The ablation critically depends on the cell-specific expression of nitroreductase (NTR) that converts the nontoxic metronidazole (MTZ) in which the zebrafish are immersed, into a highly toxic DNA inter-strand cross-linking agent. (2016) detected excessive overproduction and accumulation of the ECM protein COL1A with immunostaining after 25 h and 50 h in larvae. Similarly, COL1A deposition could also be detected in adult fish (1% EtOH) already at 48–72 h [52], much more quickly than the one to several months of exposure time that are typically needed with EtOH treatment alone [50][51].

NAFLD is a much more common chronic liver disease than ALD. Although many of these diseases have been modelled in mice, and a few zebrafish models of NAFLD were generated [53][54][55], only one zebrafish obesity-associated fibrotic liver model is described so far [53]. Investigating whether menopause is associated with the severity of liver fibrosis, Turola et al. Of interest, old female fish with failing ovarian function presented livers with the most severe fibrosis accumulation, and old male fish showed a higher degree of fibrosis in comparison to young male fish.

Carbontetrachloride (CCl4) and thioacetamide (TAA) are the most commonly used toxic chemicals to generate fibrotic liver disease through repetitive injection in rodents [56][57][58][59]. However, liver fibrosis was not detected in zebrafish larvae following CCl4 treatment using gene expression as a read-out [60]. In contrast, three days of TAA exposure induced ECM accumulation in the liver as visualized by Sirius-red staining and upregulated fibrosis-related genes (col1a1,acta-2,hand-2,tgfb) in zebrafish larvae [60]. This might be due to the difference in cell organization within the liver of fish and humans, e.g., the lack of lobular architecture, or less organized bile duct hepatocytes and stellate cells [46].

Recently, a zebrafish model of fibrotic liver disease was reported, generated by overexpression of TGF-β1α (the counterpart of TGF-β1 in mammals) in the liver driven by the organ-specific fabp10 promoter [61]. TGF-β1 has a critical role in the epithelial to mesenchymal transition (EMT) process and is involved not only in chronic lung [62] and kidney diseases [63], but also in chronic liver and cardiovascular diseases [64]. Following 3–6 weeks of TGF-β1α overexpression using a 1 µM mifepristone-inducible system, abundant collagen accumulation was revealed in the liver in adult zebrafish by Sirius Red staining. The levels of accumulated collagen were reduced when fish were exposed to higher concentrations of mifepristone (2–3 µM) for 6 weeks.

Besides fibrogenic gene overexpression, mutations in mannose phosphate isomerase (mpi) present in hepatocytes also promotes hepatic fibrosis. [48]mpidepletion in heterozygous adult zebrafish liver resulted in a continuous upregulation of fibrogenic genes (i.e. ,col1a1a,col1a1b,andacta2) and accumulation of collagen as detected with Masson’s trichrome staining. Of interest, mildmpideletion in very early-stage zebrafish embryos (from 96 to 120 hpf) reinforced the fibrosis effects of EtOH administration and elevated the expression ofcol1a1aandacta2within 24 h.

Based on the models developed to date, it can be concluded that advanced fibrosis in adult zebrafish requires prolonged and sustained injury [61][50][53][51]. Although fibrogenesis of the liver has been detected in larval zebrafish, sensitive techniques (e.g., IHC, qPCR, etc.) were required due to the lower abundancy of accumulated ECM [60][52][48]. Future model optimization should be directed towards a balance between the time length of treatment and ECM occurrence.

Common diseases related to injury of the pancreas are diabetes mellitus, pancreatitis, and pancreatic adenocarcinoma. The zebrafish pancreas develops and functions to secrete hormones for energy homeostasis in the early embryo stage (≤48 hpf). Human pancreas- related diseases have been mimicked in zebrafish, namely chronic pancreatitis [33], cystic fibrosis [65], diabetes [66], and pancreatic cancer [67][68].

Fibrosis has been examined in some of these models. in zebrafish (**Table 1**). Both types ofHhoverexpressing transgenic zebrafish exhibited identical phenotypes, i.e., Indian and SonicHhcaused progressive pancreatic fibrosis in older animals. According to histopathologic analysis, Hh-induced progressive pancreatic fibrosis (manifested as the destruction of the histo-architecture) was observed in one-month-old fish.

Contrary toHhtransgenic fish, oncogenic KRASG12D expression in the elastase 3I domain resulted ultimately in pancreatic endocrine tumours [67]. This expression can be placed under the control of the zebrafish ubb promoter by using the construct Tg(ubb-Lox-Nucleus-mCherry-Lox-GFP-KRASG12D). The results show that the invasion of carcinoma stimulated fibrosis, as evidenced by the ECM accumulation visualized by trichrome staining.

Gill fibrosis and muscle fibrosis have also been described [69][70]. As the zebrafish counterparts of mammalian lungs, gills are sites for gas transfer and are important locations for chemoreception or gas sensing. Both lungs and gills are respiratory organs responsible for O2 uptake and CO2 excretion, and they share similar morphological features. Following persistent exposure over 28 days, gill fibrosis was evidenced both at the mRNA level (detected with qPCR) and protein level (detected with Masson's trichrome staining)

In addition, since fibrosis is a major complication of ionizing irradiation exposure [71][72], Epperly et al. [72] modelled irradiation-induced fibrosis in zebrafish to address the current lack of models for screening of novel irradiation mitigators and protectors (**Table 1**). Using 30 Gy irradiation, 25% of fish developed abnormalities in the shape and structure of fin and tail, and massive ECM accumulation was detected in their dorsal musculature. Interestingly, following continuous treatment with a small molecule, ethyl pyruvate, the survival rate improved, and deposited collagen was reduced.

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