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A New School in Liver Development: Lessons from Zebrafish

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Abstract

There is significant overlap in the genes and pathways that control liver development and those that regulate liver regeneration, hepatic progenitor cell expansion, response to injury and cancer. Additionally, defects in liver development may underlie some congenital and perinatal liver diseases. Thus, studying hepatogenesis is important for understanding not only how the liver forms, but also how it functions. Elegant work in mice has uncovered a host of transcription factors and signaling molecules that govern the early steps of hepatic specification, however the inherent difficulty of studying embryogenesis *in utero* has driven developmental biologists to seek new systems. The rapidly developing vertebrate zebrafish is a favorite model for embryology. The power of forward genetic screens combined with live real-time imaging of development in transparent zebrafish embryos has highlighted conserved processes essential for hepatogenesis and has uncovered some exciting new players. This review will present the advantages of zebrafish for studying liver development, underscoring how studies in zebrafish and mice complement each other. In addition to their value for studying development, zebrafish models of hepatic and biliary diseases are expanding, and using these small, inexpensive embryos for drug screening has become *de rigueur*. Zebrafish provide a shared platform for developmental biology and translational research, offering innovative methods for studying liver development and disease.

The story of hepatogenesis has something for everyone. It involves transcriptional regulation, cell-cell interaction, signaling pathways, control of cell proliferation and apoptosis plus morphogenic processes that sculpt vasculature, parenchymal cells and mesenchyme to form the multi-faceted liver. Decades of research on liver development in mice and other vertebrates offer valuable lessons in how the multi-potent endoderm is programmed to form a functional liver. Of equal importance are insights that have illuminated the mechanisms by which hepatic progenitors are activated in a damaged liver, how the adult liver regenerates and, possibly, the basis for engineering liver cells *in vitro* for cell transplantation to sustain patients with liver failure. Moreover, processes that are key to liver development are often co-opted during pathogenesis. Therefore, reviewing hepatogenesis is informative for both basic and translational researchers.

In this review, we bring to light the many advantages offered by the tropical freshwater vertebrate zebrafish (*Danio rerio*) in studying hepatogenesis. By comparing zebrafish and mice, we highlight how work in each system complements the other and emphasize novel paradigms that have been uncovered using zebrafish. Finally, we highlight exciting efforts using zebrafish to model hepatobiliary diseases.

Developmental Genetics in Zebrafish

Zebrafish are the darling of developmental biologists because they produce hundreds of offspring with every mating, and their transparent embryos develop outside of the mother, allowing constant visualization (see Fig. 1) and easy manipulation. Additionally, since they are sustained by nutrients in the yolk, the extra-embryonic structures that are essential for supporting the mammalian embryo do not form in zebrafish.

Only a standard microscope is needed to observe developmental events such as a beating heart, twitching muscle and a growing liver. Organogenesis is underway in nearly all systems by 24 hours post fertilization (hpf) and the embryo is swimming by 2 days post fertilization (dpf). By 5 dpf, all major organ systems are established (Fig. 2) and larvae are ready to feed.

The array of genetic tools and large number of progeny make zebrafish ideal for developmental genetic studies. Assembly of the zebrafish genome is nearly complete, and despite the genome duplication that occurred during teleost evolution, the high genetic conservation among vertebrates makes it straightforward to identify zebrafish orthologs of human genes. Moreover, each embryo is provided with mRNA and proteins from the mother to sustain early development. These maternal stores allow mutant embryos that lack a cell-essential gene to survive longer than their mammalian counterparts.

The ability to carry out forward genetic screens in zebrafish is among their most powerful attributes. Forward screening allows the investigator to take an unbiased approach to identify genes essential for a process of interest by identifying embryonic mutants that are defective in that process. For instance, our interest in hepatic outgrowth led us to screen for mutants that fail to expand their liver bud (1). Liver mutants have been identified in several large-scale forward screens which used either chemical or retroviral mutagens (Table 1). Their analysis has illuminated aspects of hepatogenesis that are conserved between mice and zebrafish and led to the discovery of new players.

Reverse genetics approaches, whereby a specific gene is targeted and the resulting phenotype is analyzed, are feasible in zebrafish in a limited fashion. Targeted gene mutation is being developed, but is not yet standardized. Instead, antisense morpholino oligonucleotides are routinely used for transient gene knock-down in embryos. Morpholinos injected into the yolk of the newly fertilized embryo diffuse quickly and uniformly into the cells, which are in syncytia with the yolk. As development progresses, the morpholinos are diluted with each cell division so that the degree of knock-down decreases as the embryo ages (2). Thus, morpholino knock-down is effective for examining early embryonic events, such as endodermal patterning, but less useful for affecting events later in development. The future of the field awaits improvement and expansion on the initial promising results provided by technologies such as TILLING (3) and zinc finger nucleases (4, 5) to carry out targeted gene mutation in zebrafish.

Creating transgenic zebrafish is easy, rapid and inexpensive. Fluorescent transgenes controlled by ubiquitous, inducible or tissue-specific promoters can be visualized in real-time as they proceed through development, enabling detailed fate mapping and spatio-temporal analysis of gene expression. For instance, the *fabp10* promoter drives high transgene expression in hepatocytes (6) (Fig. 1) and the *Tg(gutGFP)^{s854}* line expresses GFP in endodermally-derived organs (7), allowing exquisite analysis of hepatogenesis. Additionally, crossing transgenic lines generates multi-labeled organs. For instance, fish expressing RFP in the liver and pancreatic islet cells, and GFP in the exocrine pancreas (*Tg(fabp10:dsred;elas:GFP;ins:dsred)*) allow evaluation of multiple organs at a glance (8).

The zebrafish community is well organized and open access. The growing attendance at the biennial International Meeting on Zebrafish Development and Genetics reflects the expanding popularity of this model, both for biomedical scientists and educators. A comprehensive catalog of zebrafish mutants, morphants, transgenics as well as images of the developmental expression pattern of thousands of transcripts are maintained on the community website, zfin.org. Most published mutants and transgenics are maintained and distributed by the stock center (<http://zebrafish.org/zirc/home/guide>). The Trans-NIH Zebrafish Initiative supports grants and other resources for improving and expanding the use, mutagenesis and analysis of this organism.

Of Mice and Zebrafish

Decades of outstanding studies using reverse genetics, embryo explants and tissue culture have uncovered a network of transcription factors and signaling pathways required for forming the mammalian liver (9–11). Hepatogenesis in mice begins approximately 1/3 of the way through gestation and concludes near birth, whereas in zebrafish, the liver is fully formed in five days. In mice, members of the FoxA, Gata and Hnf families of transcription factors combine with signaling pathways initiated by members of the fibroblast growth factor (Fgf) and bone morphogenic protein (Bmp) families to activate genes important for driving hepatic lineage and to suppress pancreas formation (10, 11). The Hhex and Prox1 transcription factors are activated in hepatic progenitors and are required for hepatoblast specification. Many of the same genes and pathways are also essential for zebrafish hepatogenesis (8, 12–17).

Hepatic outgrowth begins in mice when hepatoblasts invade the surrounding septum transversum mesenchyme (STM) and continues through late gestation. Moreover, the hepatic vasculature co-develops with hepatoblasts and is required for liver development (18) and fetal hematopoiesis. Thus, vasculogenesis, hematopoiesis and hepatogenesis are intertwined in mice: dysfunction of one of these processes prevents the proper development of the others. In contrast, embryonic hematopoiesis does not take place in the zebrafish liver; therefore abnormalities in the embryonic liver will not lead to early lethality associated with hematopoietic dysfunction. Early liver development in zebrafish is independent of vasculogenesis – the embryo can develop for several days without circulation (7, 19, 20). An illustrative example is provided by our analysis of *Klf6*, a gene hypothesized to be important for liver growth and function. In previous work, loss of *Klf6* in mice caused early embryonic lethality due to defective hematopoiesis (21), precluding analysis of the liver. However, our analysis of *klf6* zebrafish morphants revealed a role in development of endodermally-derived organs, especially the liver, distinct from its role in hematopoiesis (Zhao, Sadler and Friedman, unpublished). This demonstrates how zebrafish can complement and extend findings in mice.

There are obvious differences between fish and mammals, yet evolution has conserved many players essential for hepatogenesis, tailoring the specifics to match the unique attributes of each species. For example, the hepatic vasculature and hematopoiesis are all essential for hepatogenesis in mammals, but not in zebrafish. Moreover, secretion hepatic specification depends on Fgfs secreted from the mammalian heart (22), which is nestled against the hepatic bud as the embryo curls into the fetal position. In contrast, the early zebrafish embryo stretches along the top of their yolk (Fig. 1), creating distance between the liver and the heart and making it unlikely that these organs communicate. Interestingly, however, it is clear that Fgfs play an essential role in hepatic development in zebrafish (8, 15) implying that the signal has been conserved, but the source can change. Another difference is the role of the STM, which provides important inductive signals to the mammalian hepatic bud. The existence of the STM in zebrafish is questionable; instead, the lateral plate mesoderm (LPM)

appears to serve an analogous function in zebrafish (13, 23). Third, the cellular and anatomical architecture of the zebrafish liver differs from mammals: instead of a portal triad, plates of hepatocytes lined by sinusoids and biliary ductules (24–26) compose the teleost liver, while maintaining the same functions. This has been comprehensively reviewed for Medaka (27). Finally, as in mammals, the biliary system consists of extra and intrahepatic ducts and ductules. However, the preductular epithelial cell is an extra branch on the teleost biliary tree (25, 26), analogous to the Canal of Hering. These cells form junctions with canaliculi to collect the bile, which they transport to the ductules through intracellular lumen (25, 26, 27). These unusual cells may be the fish version of hepatic progenitors (28) and warrant further study.

The challenges in using zebrafish for studying liver biology are primarily technical: the lack of an easy technology for knocking out genes and the relatively few useful antibodies. Moreover, as the field is still young, stellate, Kupffer and oval cells have not yet been identified in zebrafish. Since much of the power of the zebrafish model rests in the attributes of their embryos, little has been done to understand adult hepatic biology, although it is clear that liver regeneration (1) and hepatocarcinogenesis are similar to mammals (29).

Overview of Zebrafish Hepatogenesis

As in their terrestrial vertebrate relatives, the zebrafish liver arises from endoderm. Here, we focus on events in hepatogenesis that occur after endoderm is formed, as zebrafish endoderm development has been reviewed elsewhere (30). There are three main stages of hepatogenesis: (i) specification (as part of endoderm patterning), (ii) differentiation and (iii) hepatic outgrowth, accompanied by morphogenesis. The zebrafish liver develops from anterior endodermal progenitor cells (7), identifiable by 22 hpf through expression of *hhx* and *prox1* (15). The liver primordium is clearly distinguishable by 48 hpf as a prominent bud extending left of the midline over the yolk. Expression of genes indicative of hepatocyte function, such as ceruloplasmin, are first detectable by 32 hpf (31) and other markers of differentiation follow (Fig. 1). As hepatocytes differentiate, proliferation accelerates. Hepatic outgrowth begins between 60–72 hpf and continues until the liver achieves its appropriate proportional size. By the end of outgrowth on 5 dpf, the bi-lobed, boomerang-shaped liver consists of a larger left lobe which crosses the midline under the anterior gut and the smaller right lobe which extends ventrally towards the head of the pancreas (Fig. 2). Few studies thus far have focused on genes required for outgrowth and little is known about morphogenesis.

Transcriptional Control

The same transcriptional networks control hepatogenesis in mice and in zebrafish (Fig. 1). The earliest transcription factors expressed in the zebrafish hepatic bud are *prox1* and *hhx* (12, 15, 30). *prox1* is first detected at 22 hpf and persists well through differentiation (15, 23). *hhx* is expressed in the hepatic bud from 22–50 hpf (32–34) and *hhx* morphants have a small liver (12). Hepatic nuclear factors (*hnf1*, 3, and 4) have been extensively studied for their roles in mammalian hepatogenesis (11). *hnf1b* (also called *vhnf1*) is essential for several key developmental decisions (35), including hepatic specification in zebrafish (32). A genome-wide scan in zebrafish identified a long list of genes specifically expressed in the liver, nearly 50 of which are targets of an Hnf family member (17).

Some transcription factors required for endoderm formation may also function later in liver development. For instance, *gata* family members (15, 16, 36, 37), *sox17* (7), *foxa2* and *foxa3* (7, 15, 30) are expressed in the early endoderm and their loss impairs proper endoderm development. These same genes are expressed again during hepatic budding (30), where they also might play a role. The difficulty in addressing this lies in the ability to distinguish

which phenotypes are due to the loss of a particular gene versus those secondary effects due to the global, early abnormalities observed in some mutants and morphants, including those with defects in endoderm formation. We addressed this by devising a scoring system that evaluated overall health, size and achievement of developmental milestones so that a finding of disrupted events late in hepatogenesis is less likely to be attributed as a secondary consequence of severe global defects or developmental delay.

Conserved Signaling Pathways Drive Hepatic Specification

Fgfs, Bmps, and the Wnt/ β -catenin pathway are the primary signaling pathways required for mammalian hepatogenesis (22, 38–40). These three pathways also control early hepatic development in zebrafish, albeit with some differences.

Fgf signaling is essential for hepatic specification in mice and zebrafish, even though the heart does not seem to be the likely source of Fgfs in zebrafish. Over-expressing dominant negative Fgf-receptor in zebrafish embryos between 18 and 26 hpf, decreases the later expression of *hhex*, *prox1*, *gata4*, *gata6* and *ceruloplasmin* (15). While it is unclear which Fgf family member is most important for specification, *fgf10* released from the mesenchyme prevents cells fated to be pancreas, hepatopancreatic duct or intestine from becoming hepatocytes (8). It is not yet clear whether the mesenchyme is the only source of Fgfs required for zebrafish hepatic specification.

Several studies highlight the utility of zebrafish to undertake detailed analysis of developmental processes on a fine-scale time course and the ability to identify new genes regulating these common pathways. For example, Bmps are required to induce hepatic differentiation in mammalian embryo explants (22, 38, 41) and hepatic progenitors *in vitro* (42). The STM is the likely source of Bmp4 and potentially other Bmps in mammals. Although zebrafish lack the STM, strong data demonstrates that Bmps are essential for zebrafish hepatic specification: *lost-a-fin* mutants lack a bmp/activin receptor and display reduced expression of many hepatic specification genes, as do embryos that over-express a dominant negative Bmp receptor (15). Interestingly, the requirement for Bmps decreases as the embryo ages, with the susceptible window between 18–26 hpf, overlapping with the time frame in which Fgfs are required (15) (Fig. 1). A forward genetic screen revealed that proper positioning and morphology of the LPM and subsequent hepatic induction by Bmp2a both rely on an unforeseen player, *myosin phosphatase targeting subunit 1* (14). *bmp2b* is also expressed in the LPM (13), yet it is not clear exactly which Bmps contribute to hepatic specification.

In the canonical Wnt signaling pathway, Wnt binds to its cell surface receptor, causing dissociation of β -catenin from the APC complex and preventing degradation (40). This pathway drives hepatic outgrowth in mice (43–46) and is also essential for hepatoblast maturation (46). This complex pathway also plays multiple roles in zebrafish liver development. Inducing *wnt* expression in early somitogenesis blocks liver specification, whereas inducing *wnt* just a few hours later creates an enlarged liver. Expanding the liver at the expense of pancreatic cells also occurs when β -catenin is stabilized due to loss of one copy of *apc* (47). *wnt2bb* is expressed in the LPM from 18–52 hpf and a forward genetic screen identified *prometheus* mutants which display delayed hepatic specification due to mutation of *wnt2bb* (23). These data combined with studies from other vertebrates suggest a model whereby Wnt signaling acts in several phases of hepatogenesis: (i) during early somitogenesis, Wnt signaling to the anterior endoderm suppresses hepatic fate (48) and (ii) just a short time later, Wnt secretion from the LPM promotes hepatic formation (23, 44, 47). (iii) Later, β -catenin activation drives hepatic differentiation and outgrowth and reigning in

this pathway is essential to achieve correct liver size and morphogenesis (44, 46, 47). It will be of interest to investigate the interplay between Wnt, Bmp and Fgf signaling.

Much of the zebrafish work on signaling pathways controlling hepatogenesis conform to the framework provided by reverse genetic studies in other vertebrates, however forward genetic screens are often most powerful in uncovering novel paradigms. This is exemplified by the identification of mutants with a putative “developmental checkpoint” where endodermal organs fail to expand. For instance, mutation of the novel *digestive-organ expansion factor (def)* gene (49) and the RNA binding gene in *nil per os (npo)* (50), as well as embryos with a block in the TOR pathway (51), undergo proper specification of endodermal organs but then fail to mature and expand. *def* mutants have activation of a novel p53 isoform causing cell cycle arrest (49). A lack of apoptosis is a key distinction between these putative “checkpoint” mutants and other outgrowth mutants, such as *uhrfl* (1), suggesting multiple mechanisms control liver outgrowth and maturation.

Fate Choice

The plasticity of the primitive endoderm is well established; these cells can form the liver, foregut or exocrine pancreas (10). Understanding the genetic decisions that drive this choice has implications beyond embryology. The ability to manipulate endodermal cells in culture to form differentiated functioning hepatocyte could lead to stem cell therapy for patients with liver failure.

Zebrafish studies prove that while cells of the anterior endoderm remain multipotent, the fate choices come at a price. For instance, *histone deacetylase 1 (hdac1)* mutants fail to fully form a liver or pancreas; instead they have an enlarged foregut (52). Similarly, enlarging the liver by over-expressing *wnt8* during somitogenesis reduces the size of the pancreatic bud (47). Work by Chung *et al.* (2008) exemplifies how detailed analysis of liver development is feasible in zebrafish. Using embryos expressing tissue-specific transgenes combined with a photoactivatable dye for lineage tracing, they demonstrate that the precise timing of Bmp2b expression directs endoderm to become liver instead of pancreas. Conversely, *loss-a-fin* mutants are in Bmp signaling, and have a smaller liver bud and expanded expression of a pancreatic marker (13). It is interesting that studies with mouse endodermal cells driven to adopt a hepatic fate *in vivo* also require Bmp signaling (42), further verifying the commonalities between zebrafish and mice.

Epigenetic Control: A New Paradigm

In addition to the specific genes targeted by individual transcription factors, recent evidence from zebrafish points to the importance of epigenetic regulation of gene expression in liver development. Histone acetylation and DNA methylation are major means of epigenetic control of gene expression. Analyses of embryos that lack either Hdac or DNA methyltransferase (Dnmt) activities demonstrate that epigenetic controls both hepatic specification and outgrowth.

hdac1 mutants develop small livers attributable to hepatic patterning defects. Moreover, only a subset of *hdac1* embryos express an hepatocyte-specific transgene (52), indicating that *hdac1* is required for both hepatic specification and for differentiation. Treating embryos with an Hdac inhibitor prior to 24 hpf reduces *hhex* and *prox1* expression, resulting in a small liver. Moreover, while *hdac1* and *hdac3* knock-down cause multiple defects in embryos, loss of *hdac3* appears more specific for the liver. *hdac3* morphants have both patterning and hepatic outgrowth defects attributed to the repression of the Tgf β family member, *gdf11* (19).

A direct role for epigenetics is provided by analysis of *dnmt1* mutants. These embryos undergo normal hepatic patterning and differentiation, but fail to undergo outgrowth due to massive hepatocyte apoptosis (Anderson and Stainier, personal communication). This finding is reminiscent of our observations in *uhrf1* mutants, which do not expand the liver bud due to both a block in cell proliferation and a marked increase in hepatocyte apoptosis (1). Uhrf1 is essential for DNA methylation by recruiting Dnmt1 to hemimethylated DNA (53, 54). Thus, loss of either *dnmt1* or *uhrf1* results in aberrant DNA methylation and hepatocyte apoptosis. Understanding epigenetic control of liver development is in its infancy and represents a fertile new direction.

Disease Models

While zebrafish are celebrated for their utility in studying development, recent studies to model hepatobiliary diseases are particularly exciting. Hepatocytes in fish and mammals carry out similar functions, and the histopathology of steatosis, cholestasis, and neoplasia are alike in both organisms. Histologically, hepatocytes are identifiable by 36 hpf, the extrahepatic biliary duct by 52 hpf, and endothelial-lined sinusoids at 3 dpf with blood flow easily observed in 4 dpf embryos. Bile production, serum protein secretion, glycogen storage, lipogenesis and xenobiotic metabolism are fully operational by 5 dpf and various analyses can be carried out on the complex larvae (Fig. 2).

The most straightforward disease modeling efforts are focused on understanding congenital disorders that result from defects in embryo morphogenesis. For instance, the genetic basis of bile duct paucity in Alagille Syndrome can be comprehensively investigated in zebrafish (55). We carried out a forward genetic screen for mutants with features of liver disease and identified a model for hepatic steatosis in *foie gras* mutants, choledochal cysts in *nf2* mutants and cholestasis in *vps18* mutants (56). Another interesting model of fatty liver and hepatic injury mediated by *Tnfa* was recently described in *ducttrip* mutants, which are defective in s-adenosylhomocystine hydrolase (57).

A distinct advantage of using fish to study liver disease is the opportunity to test effects of drug and toxin exposure by their simple addition to the water. We used this approach to develop a model of acute alcoholic liver disease. With continuous exposure to 2% ethanol, zebrafish larvae develop hepatomegaly, steatosis, and changes in hepatic gene expression, all indicating that alcohol is metabolized by the zebrafish and provokes oxidative stress (58). In addition to alcohol, the zebrafish system facilitates testing of the liver's response to a plethora of toxins, enabling high-throughput toxicity detection and drug screening and discovery.

Future Directions

In the nearly 30 years since researchers began using zebrafish as a developmental genetic model, work from many model organisms has significantly advanced our understanding of liver development. We now understand that transcriptional and signaling networks guide cellular decision-making and drive hepatic progenitors to create the machinery needed to perform the vast array functions carried out by the liver, to proliferate and to organize multiple cell types into the mature liver. There are, however, a many areas that remain under explored: how do different cell types interact during development? what is the embryological origins of stellate and Kupffer cells? do the pathways regulating cell proliferation in the embryo also control regeneration in adults? Zebrafish are a well established model to address each of these questions. Of particular importance is the link between genes that govern development and those that contribute to pathology: how are they deregulated during liver disease, and can they be manipulated as a means of treatment? Zebrafish provides a powerful system capable of detailed developmental genetic analysis

and is especially suited for the study of hepatogenesis. This combined with the growing number of liver disease models provides an exciting framework for understanding development, disease, and potential new therapies.

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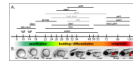


Figure 1. Overview of the genes involved in zebrafish liver development

A. Each gene in this figure has been shown to be either expressed in the liver (listed in grey) or directly regulate liver development (listed in black). **B.** Live images of embryos during development. Outgrowth is imaged by detection of the red fluorescent transgene expressed in hepatocytes in *Tg(fabp10:dsred)* embryos.

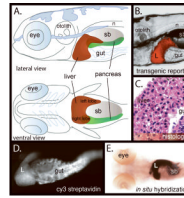


Figure 2. The liver in 5 dpf zebrafish is mature

The liver (L) is analyzed through multiple techniques in the 5 dpf zebrafish. **A.** The bi-lobed zebrafish liver lies ventrally and anterior to the swim bladder (SB) and notochord (N). It can be visualized using a transgenic reporter expressing red fluorescent protein under a hepatocyte specific promoter (Tg(*fabp10*-dsred) (B) hematoxylin and eosin staining (C) by deconvoluted whole mount cy3-streptavidin staining (D) and whole mount *in situ* hybridization using the *fabp10* probe (E).

Table 1

Forward genetic screens identify liver mutants

Laboratory	Location	Mutant	Gene	Reference
Nusslein-Volhard	Max-Planck Institute, Tübingen	<i>lumpazi</i>	unknown	(55)
		<i>gammaler</i>	unknown	
		<i>tramp</i>	unknown	
		<i>tippelbruder</i>	unknown	
Fishman	Harvard Medical School	<i>beefeater</i>	unknown	(56)
		<i>nil per os</i>	<i>rbm19</i>	(46)
Hopkins	Massachusetts Institute of Technology	<i>foie gras</i>	<i>fgr</i>	(52)
		<i>neurofibromatosis 2</i>	<i>nf2</i>	(52)
		<i>vps18</i>	<i>vps18</i>	(52)
		<i>uhrf1</i>	<i>uhrf1</i>	(1)
		<i>digestive-organ expansion</i>	<i>def</i>	(45)
		<i>factor</i>		(57)
Stainier	University of California, San Francisco	<i>prometheus</i>	<i>wnt2bb</i>	(20)
		<i>hdac1</i>	<i>hdac1</i>	(48)
		<i>fgf10</i>	<i>fgf10</i>	(11)