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Conservation and Early Expression of Zebrafish Tyrosine Kinases Support the Utility of Zebrafish as a Model for Tyrosine Kinase Biology

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Abstract

Tyrosine kinases have significant roles in cell growth, apoptosis, development, and disease. To explore the use of zebrafish as a vertebrate model for tyrosine kinase signaling and to better understand their roles, we have identified all of the tyrosine kinases encoded in the zebrafish genome and quantified RNA expression of selected tyrosine kinases during early development. Using profile hidden Markov model analysis, we identified 122 zebrafish tyrosine kinase genes and proposed unambiguous gene names where needed. We found them to be organized into 39 nonreceptor and 83 receptor type, and 30 families consistent with human tyrosine kinase family assignments. We found five human tyrosine kinase genes (epha1, bmx, fgr, srm, and insrr) with no identifiable zebrafish ortholog, and one zebrafish gene (yrk) with no identifiable human ortholog. We also found that receptor tyrosine kinase genes were duplicated more often than nonreceptor tyrosine kinase genes in zebrafish. We profiled expression levels of 30 tyrosine kinases representing all families using direct digital detection at different stages during the first 24 hours of development. The profiling experiments clearly indicate regulated expression of tyrosine kinases in the zebrafish, suggesting their role during early embryonic development. In summary, our study has resulted in the first comprehensive description of the zebrafish tyrosine kinome.

Introduction

Tyrosine kinases

Phosphorylation of the amino acid tyrosine was discovered 33 years ago in mouse cells infected with the polyoma virus.[1,2] The viral enzyme v-SRC was shown to phosphorylate tyrosine[3,4] and to be essential for Rous sarcoma virus-mediated transformation of cells.[5,6] Several other cellular tyrosine kinases were discovered subsequently and were found to act by catalytically transferring the γ-phosphate of ATP to tyrosine residues on proteins. The significance of tyrosine phosphorylation in signaling pathways and physiology has been the subject of several studies, and tyrosine kinases are well-validated targets for cancer therapy.[7–9] Tyrosine kinases are also being studied in the context of a growing number of pathological conditions, including neurodegeneration, autoimmunity, inflammation, and infectious diseases. Research during the last 30 years has helped illuminate crucial features of tyrosine kinase protein sequences and structure–function relationships.[10–14] Current tyrosine kinase inhibitors (TKIs) function by interfering with ATP and/or substrate binding.[15,16] Toxicity, nonselectivity, resistance, redundancy, and idiosyncratic clinical response are some issues facing TKI development. There have been few studies on *in vivo* mechanisms and physiological effects of tyrosine kinase dysregulation, and several tyrosine kinases are yet uninvestigated. The evolution of tyrosine kinase activity and its regulation in metazoans and premetozoans, and its relation to the evolution of multicellularity has been the subject of intriguing recent studies,[17, 18] and such research is facilitated by the availability of suitable animal models. Further, studies on tyrosine kinase biology and development of the next generation of small molecule modulators will be significantly strengthened by *in vivo* studies. In such contexts, the zebrafish has significant potential to be an accessible *in vivo* model to study tyrosine kinases.

Tyrosine kinases in zebrafish

Developmental roles of some tyrosine kinases in zebrafish have been investigated by studying genetic mutants, gene expression patterns, and the effects of experimentally altering gene expression.

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Owing to their significant cellular functions, it is not surprising that past studies show essential roles of both receptor and nonreceptor tyrosine kinase families in several aspects of embryonic development in zebrafish. They include a wide spectrum beginning from very early events in fertilization and gastrulation to later events in organogenesis and tissue regeneration. The EGFR receptor tyrosine kinase family influences neural crest development, cardiovascular development, and regeneration after injury.[19–21] The EPHR family is crucial for gastrulation, mesenchymal-to-epithelial transition, retinotectal system patterning, and extracellular matrix assembly.[22–25] The nonreceptor tyrosine kinase SRC is involved in egg activation during fertilization,[26] and FAK has a role in cell adhesion and exhibits noncanonical regulatory phosphorylation.[27,28] The MET receptor tyrosine kinase regulates liver and cerebellum development;[29–31] the TEC tyrosine kinase is expressed in the zebrafish kidney;[32] the TRKB receptor tyrosine kinase is expressed throughout embryonic development and lacks a kinase domain until neurogenesis;[33] MuSK functions in neuromuscular synapse formation;[34] and PTK7 knockdown results in convergent extension defects.[35] The zebrafish cardiovascular system is profoundly influenced by tyrosine kinases. PDGFRβ[36] and VEGFR have striking effects on zebrafish angiogenesis[37] and analogues of Vadimezan, a multi-kinase inhibitor with effects on VEGFR disrupt angiogenesis in zebrafish.[38] The tyrosine kinase TIE1 regulates endothelial cell contact junctions,[39] while TIE2 mediates vessel stability and may be the target for statin-induced haemorrhage.[40] The cytoplasmic tyrosine kinase JAK2 is encoded by duplicate genes which have distinct expression patterns and functions in hematopoesis,[41] and SYK and ZAP70 function redundantly in angioblast migration.[42] Development of unique skin features of zebrafish are also influenced by tyrosine kinases. The tyrosine kinases KIT and FMS play essential roles in melanocyte and xanthophore generation in zebrafish stripes,[43] and the tyrosine kinase LTK functions in iridophore specification.[44] As with TIE and JAK2, duplicate IGF1R tyrosine kinases have overlapping but distinct functional roles in development and physiology,[45] highlighting the effects of zebrafish genome duplication events on tyrosine kinase evolution in teleosts. Finally, the use of proteomics has revealed the prevalence and importance of tyrosine phosphorylation pathways in zebrafish and the possibility of studying phosphotyrosine signaling *in vivo*.[46,47]

The above studies on zebrafish tyrosine kinases provide insights in the context of a whole organism, and can potentially address important questions regarding physiological roles of tyrosine kinases and tyrosine phosphorylation pathways. While correlating results from the above studies with those in humans, we found that the available literature was not informative with respect to the number of zebrafish tyrosine kinases, their nomenclature, and orthology. We found that sequence databases and other resources were also incomplete in the above respect. An accurate understanding of the developmental and physiological roles of zebrafish tyrosine kinases will ultimately require the comprehensive identification, nomenclature, and orthology analysis of all the tyrosine kinases encoded in the zebrafish genome. Such knowledge will also provide impetus to the use of zebrafish to model human diseases related to tyrosine kinase dysfunction.

Availability of the complete genome sequence of zebrafish allows detailed computational sequence analysis. We created profile hidden Markov models[48] of known human and other known tyrosine kinase domain protein sequences, and used the models to search zebrafish protein databases to identify all zebrafish tyrosine kinase genes. We used sequence alignments and phylogenetic analysis with human tyrosine kinases to confirm our findings and perform unambiguous orthology assignments. In order to experimentally validate our computational results, we detected the expression of a set of unreported tyrosine kinases representative of all zebrafish tyrosine kinase families during the first 24 h of development using nCounter direct digital detection technology. Our findings and their implications are presented here. The goal of this article is to provide the first comprehensive resource of the zebrafish tyrosine kinome. We anticipate that this information will be useful both for research communities using zebrafish as a developmental and genetic model organism, and for those interested in zebrafish as an *in vivo* model for tyrosine kinase biology.

Methods

Profile hidden Markov models

The curated RefSeq[49] full release collection of zebrafish protein sequences in FASTA format was obtained via ftp from the National Center for Biotechnology Information (NCBI) ftp://ftp.ncbi.nih.gov/refseq/D_rerio/mRNA_Prot/zebrafish.protein.faa.gz. This database is updated regularly and contains all the protein sequences encoded in the zebrafish genome. The latest version of the freely distributable implementation of the profile hidden Markov model (HMM) software HMMER3[50] was obtained as source code via ftp from the HMMER website ftp://selab.janelia.org/pub/software/hmmer3/3.0/hmmer-3.0-linux-intel-ia32.tar.gz. The HMMER3 source code was compiled on a system equipped with a dual core Intel(R) Pentium(R) 4 3.06 Ghz CPU and 1.0 Ghz RAM, running Ubuntu Linux 9.04 kernel version i686. The documentation and instructions on the website http://hmmer.janelia.org/ were followed for use of the software. A collection of one hundred most diverse tyrosine kinase domain sequences in FASTA format was obtained from the conserved domain database (CDD) of the NCBI.[51] The protein tyrosine kinase (PTK) domain family is curated as a conserved domain family cd00192, belonging to the protein kinase superfamily cl09925. The top 148 tyrosine kinase domain sequences (rated according to their similarity to the tyrosine kinase domain consensus) were also obtained similarly. These sequence sets are available on the conserved domain database as multiple sequence alignments in several formats. The tyrosine kinase domain boundary is defined on the basis of three-dimensional structures of the proteins and multiple sequence alignment of the various sequences. The tyrosine kinase domain sequences were aligned and refined using the program MUSCLE[52] and reformatted to the 'Stockholm' format using the sreformat command of the package Biosquid http://manpages.ubuntu.com/manpages/lucid/man1/sreformat.1.html. Using HMMER3, profile HMMs were built from the multiple sequence alignments of tyrosine kinase domain sequences.

Identification and orthology of zebrafish tyrosine kinases

Tyrosine kinase domain profile HMMs constructed as described above, were used to search the RefSeq zebrafish

protein sequence collection. All the protein sequence entries generated by HMMER3 were exported to a spreadsheet and the corresponding cDNA and protein sequences extracted from the ENSEMBL, NCBI, and ZFIN sequence databases. The final protein sequences used in the rest of the study were from ENSEMBL, since this database appeared to have the most complete information. All the entries containing valid tyrosine kinase domain sequences as per the CDD were selected and further verification of their identity and orthology to human tyrosine kinases was done as follows. The gene orthology database of ENSEMBL and the 'homologene' database of NCBI provided a starting point for initial assignment. A combination of local and global sequence alignment-based database searches and pairwise alignments were done using the EBI web-based software FASTA, NCBI BLAST, EMBOSS WATER, and EMBOSS NEEDLE. Multiple sequence alignments were performed using CLUSTALW and dendrograms were constructed as described below. Final orthology assignment was based on all the analyses performed.

Multiple sequence alignments and dendrograms

Full-length protein sequences of zebrafish tyrosine kinases identified as described above were subject to multiple sequence alignment, along with available tyrosine kinase domain sequence alignments from the Conserved Domain Database at NCBI. The final alignments were visually examined to identify zebrafish tyrosine kinase domain boundaries. Tyrosine kinase domain sequences of all zebrafish tyrosine kinases were manually extracted from the full-length sequences and assembled into FASTA format files. Pairwise sequence comparison of full-length and tyrosine kinase domain sequences of zebrafish and humans was performed using BLAST to reveal sequence identity. Multiple sequence alignments were performed and dendrograms constructed using full-length tyrosine kinases from zebrafish and humans with the software CLUSTALW. Dendrograms were edited using the program Dendroscope[39] for improving appearance of fonts and colors, replotting as circular cladograms, and exporting as images.

Genomic and expression features

Chromosomal number, location, strand orientation, and RNASeq information was inferred from ENSEMBL database entries and manual BLAST/BLAT searches as needed. Wherever applicable, multiple RNASeq IDs corresponding to single ENSEMBL IDs are reported together. Known mutations of zebrafish tyrosine kinases were compiled from the ZFIN database.

Zebrafish maintenance and embryo collection

Embryos from the AB/LF strain were used for RNA extractions, and were maintained between 25.5° and 28.5°C. The staging series described previously[53] was used to collect embryos into six different pools (maternal/early cleavage stage, shield stage, 70%–100% epiboly, 3–12 somites, 16–19 hours post-fertilization (h) and 22–26 h; maternal stage embryos were between 2–8 cells).

RNA extraction and NanoString nCounter assay and data analysis

TRI Reagent (Sigma) was used to extract total RNA from frozen embryos at specific developmental stages using the method described by the manufacturer. Gene expression analysis using the nCounter system (NanoString Technologies) was done as described previously.[54] The nCounter system uses molecular (fluorescent) barcodes for direct digital detection of individual target mRNA molecules. In brief, sequence-specific biotinylated (3′) capture probe and fluorescent (5′) reporter probe pairs for each of the representative tyrosine kinase genes (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/zeb) were constructed first. These probe pairs were mixed with 100 ng of total RNA extracted from selected zebrafish developmental stages and the solution was incubated at 65°C for 20 h. After the hybridization of the target mRNA and probe pairs, the excess probes were washed away and the target mRNA-probe pair hybrids purified using the nCounter™ Prep Station followed by detection. Simultaneous detection of all the gene transcripts and controls was performed in multiplexed reactions.[54] The raw data were normalized first to the spike controls provided by the manufacturer, and then to two housekeeping genes (hypoxanthine phosphor ribosyl transferase 1, HPRT1 and ribosomal protein L19, RPL19). Two additional housekeeping genes (β-actin, ACTB, and β-glucuronidase, GUSB) were used in the experiment but their values were excluded due to very low counts at certain stages. The spike controls account for variations in hybridizations and purification efficiency. The experiment was done in duplicate and the average of the expression counts were used to infer trends in levels of gene expression. The data were represented as a heat map generated using GENE-E software version 2.0.32 (http://www.broadinstitute.org/cancer/software/GENE-E/index.html).

Results

Based on a combination of profile HMM searches, sequence database searches, pairwise sequence alignments, multiple sequence alignments, and dendrogram construction, we identified the complete set of tyrosine kinase genes encoded by the zebrafish genome and assigned their orthology to human counterparts. Our initial profile HMM searches of the zebrafish RefSeq protein database yielded over 150 hits using default HMMER search parameters. We then proceeded to verify the hits individually using the ENSEMBL, NCBI, and ZFIN databases. We sought to identify gene names and IDs, chromosomal locations and orientations, existing RNASeq data, known mutants, orthology to human genes, and protein sequence similarity. Approximately half of the hits had consistent and accurate information across the three databases above. Information for the other half was missing, incorrect, or inconsistent in one or more of the databases. We chose to use tyrosine kinase sequences we identified in the ENSEMBL database as reference, and used BLAST searches of the ZFIN database to identify existing tyrosine kinase entries or the lack thereof. Our computational analyses led to the identification of 122 distinct tyrosine kinase genes in the zebrafish, with 83 receptor-type organized into 20 families, and 39 nonreceptor type organized into 10 families. In communication with the Sanger Institute and the ZFIN Gene Nomenclature Committee to seek clarification and confirmation of our results, we have suggested modifications to the ENSEMBL and ZFIN databases based on our findings. In naming zebrafish tyrosine kinase genes, we have aimed to ensure consistency with existing human and mouse gene nomenclature, while avoiding conflicts with zebrafish gene nomenclature conventions.

Our results are graphically summarized in a dendrogram (Fig. 1), which is constructed using full-length zebrafish and human tyrosine kinase protein sequences. The dendrogram provides a basis and confirmation of our orthology assignments and shows the clear clustering of zebrafish and tyrosine kinases into identical families. Zebrafish tyrosine kinases can be categorized into 10 nonreceptor tyrosine kinase families and 20 receptor tyrosine kinase families, identical to those in humans. The dendrogram also reveals the fact that each human tyrosine kinase, with the exception of five, has a corresponding zebrafish ortholog. Zebrafish genes yrk and frkb do not have human orthologs, and the human genes bmx, fgr, srm, insrr, and epha1 do not have zebrafish orthologs. While the current version of ENSEMBL shows that the zebrafish gene CH73-340M8.2 (ENSDARG00000040258) is orthologous to the human srm gene (36.3% sequence identity), we suggest that it is related to the human frk gene showing a slightly higher sequence identity score (39.3%) (Supplementary Table S2).

A list of zebrafish tyrosine kinases, their orthologies, and percentage identities to human counterparts is shown in Table 1. The table is classified according to the presence or absence of duplicate zebrafish orthologs. We found that, of the 90 human tyrosine kinases, 55 have an unambiguous zebrafish ortholog, 30 have multiple (66) zebrafish orthologs, and the remaining 5 do not have a zebrafish ortholog. Our study did not find distinct zebrafish orthologs for the human receptor tyrosine kinases EPHA1 and INSRR, and the nonreceptor tyrosine kinases BMX, FGR, and SRM. We also found that the zebrafish tyrosine kinase zYRK which has distinct orthologs in chick and stickleback does not have a human ortholog (Table 1).

Sequence identities between zebrafish and human tyrosine kinase domains ranged between 95% (yes1) and 35% (styk1b) with an average of 71.5%. Sequence identities for full-length sequences ranged between 90% (fynb) and 32% (styk1b) with an average of 62.6%. A detailed list showing current zebrafish tyrosine kinase gene names and those proposed by us, their ENSEMBL, ZFIN, and VEGA gene IDs, genomic locations (chromosome number and orientation), and mutant alleles is shown in Supplementary Table S3.

In total, the zebrafish genome encodes 122 tyrosine kinases (containing kinase domains), of which 39 are nonreceptor type and 83 are receptor type. In comparison, the human genome encodes 90 tyrosine kinases with 32 nonreceptor tyrosine kinases and 58 receptor tyrosine kinases. Of the 55 human tyrosine kinases that have single zebrafish orthologs, 21 are nonreceptor and 34 are receptor type, whereas of the 30 human tyrosine kinases with multiple zebrafish orthologs, only 8 are nonreceptor and 22 are receptor tyrosine kinases

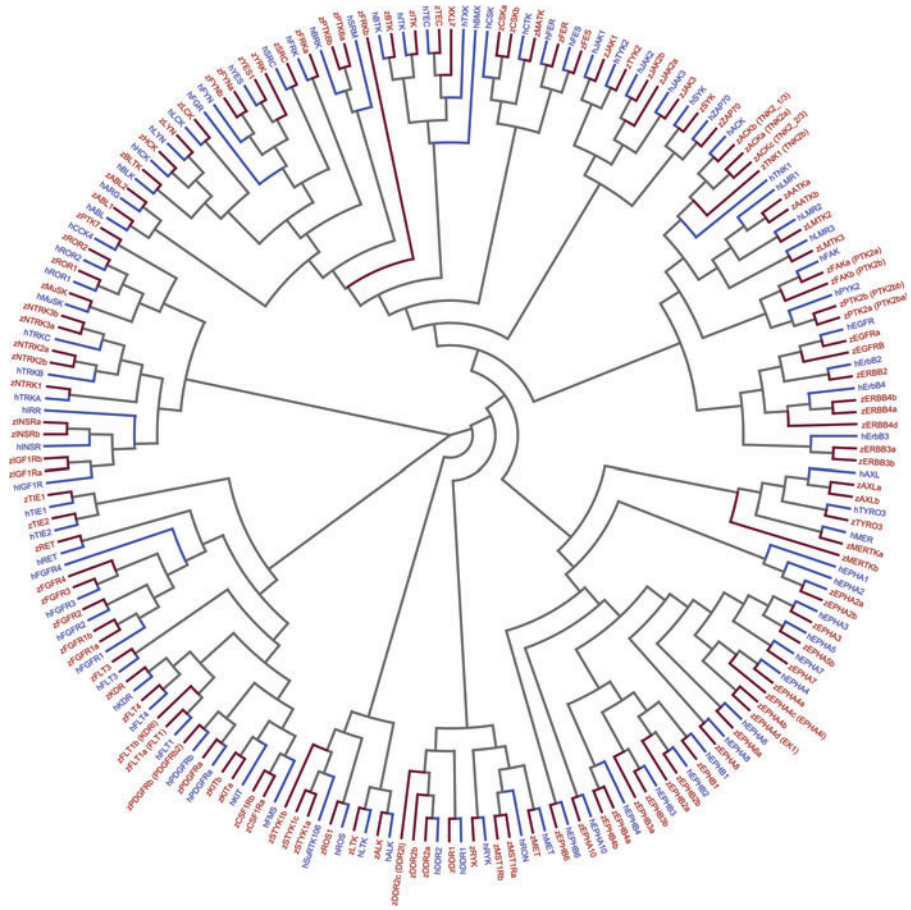

FIG. 1. Dendrogram representing orthologous relationships between the human and zebrafish protein tyrosine kinases. The human genes are prefixed with "h" and the zebrafish genes are prefixed with "z." Color images available online at www.liebertpub.com/zeb

Table 1. Orthology of Zebrafish and Human Tyrosine Kinases with Names, Protein Accession Numbers, and Percentage Sequence Identity of Full-Length Sequences and Kinase Domains

Unambiguous Orthology

Zebrafish gene name	*Zebrafish protein accession*	*Human gene name*	*Human protein accession*	*% Sequence identity (full length sequences)*	*% Sequence identity (kinase domain)*
erbb2	ENSDARP00000010252	erbb2	ENSP00000269571	54.0	56.0
pdgfra	ENSDARP00000124752	pdgfra	ENSP00000257290	61.0	80.0
pdgfrb (pdgfrb2)	ENSDARP00000028652	pdgfrb	ENSP00000261799	50.0	71.0
flt3	ENSDARP00000075800	flt3	ENSP00000241453	42.0	58.0
fgfr2	ENSDARP00000124761	fgfr2	ENSP00000351276	77.0	91.0
fgfr3	ENSDARP00000011898	fgfr3	ENSP00000260795	77.0	88.0
fgfr4	ENSDARP00000091059	fgfr4	ENSP00000292408	65.0	75.8
flt4	ENSDARP00000112456	flt4	ENSP00000261937	54.0	64.0
kdr	ENSDARP00000049203	kdr	ENSP00000263923	50.0	62.0
met	ENSDARP00000123904	met	ENSP00000317272	51.0	81.0
ntrk1	ENSDARP00000106635	ntrk1	ENSP00000357179	54.0	79.0
epha3 (epha3l)	ENSDARP00000021706	epha3	ENSP00000337451	80.0	87.0
epha5	ENSDARP00000075583	epha5	ENSP00000273854	79.0	83.0
epha6	ENSDARP00000121462	epha6	ENSP00000374323	74.0	77.0
epha7	ENSDARP00000093563	epha7	ENSP00000358309	86.0	94.0
epha8	ENSDARP00000041799	epha8	ENSP00000166244	69.0	71.0
epha10	ENSDARP00000064387	epha10	ENSP00000362139	57.0	57.0
ephb1	ENSDARP00000111570	ephb1	ENSP00000381097	85.0	90.0
ephb6	ENSDARP00000074926	ephb6	ENSP00000410789	48.0	50.4
tyro3	ENSDARP00000116623	tyro3	ENSP00000263798	51.0	61.0
alk	ENSDARP00000124055	alk	ENSP00000373700	54.0	60.0
ltk	ENSDARP00000062906	ltk	ENSP00000263800	55.0	62.0
tie1	ENSDARP00000124224	tie1	ENSP00000361554	57.0	90.0
tie2	ENSDARP00000055680	tie2	ENSP00000369375	51.0	90.0
ror1	ENSDARP00000118145	ror1	ENSP00000360120	74.0	77.0
ror2	ENSDARP00000101065	ror2	ENSP00000364860	73.0	77.0
ddr1	ENSDARP00000102505	ddr1	ENSP00000427552	57.0	73.0
ret	ENSDARP00000072093	ret	ENSP00000347942	59.0	85.0
ryk	ENSDARP00000106622	ryk	ENSP00000296084	79.0	92.0
musk	ENSDARP00000064921	musk	ENSP00000189978	58.0	85.0
ros1	ENSDARP00000118327	ros1	ENSP00000357494	54.0	57.0
lmtk2	ENSDARP00000098121	lmtk2	ENSP00000297293	44.0	43.0
lmtk3	ENSDARP00000116726	lmtk3	ENSP00000270238	56.0	57.0
ptk7	ENSDARP00000089232	ptk7	ENSP00000230419	64.0	72.0
jak1	ENSDARP00000114784	jak1	ENSP00000343204	63.0	72.0
jak3	ENSDARP00000049468	jak3	ENSP00000432511	50.0	56.0
tyk2	ENSDARP00000055123	tyk2	ENSP00000431885	50.0	62.0
abl1	ENSDARP00000017269	abl1	ENSP00000361423	69.0	62.0
abl2	ENSDARP00000041696	abl2	ENSP00000427562	69.0	63.0
fer	ENSDARP00000124066	fer	ENSP00000281092	77.0	84.0
fes	ENSDARP00000006235	fes	ENSP00000331504	58.0	68.0
bltk	ENSDARP00000120039	blk	ENSP00000406620	68.0	77.0
lck	ENSDARP00000124901	lck	ENSP00000328213	69.0	81.0
lyn	ENSDARP00000040554	lyn	ENSP00000428924	75.0	83.0
src	ENSDARP00000093618	src	ENSP00000362659	82.0	94.0
yes1	ENSDARP00000009659	yes1	ENSP00000352892	85.0	95.0
hck	ENSDARP00000115909	hck	ENSP00000365012	75.0	86.0
matk	ENSDARP00000104189	matk	ENSP00000378485	63.0	67.6
btk	ENSDARP00000124430	btk	ENSP00000308176	63.0	72.0
itk	ENSDARP00000022015	itk	ENSP00000398655	59.0	65.0
tec	ENSDARP00000106254	tec	ENSP00000370912	68.0	77.0
txk	ENSDARP00000092779	txk	ENSP00000264316	55.0	66.0
syk	ENSDARP00000122788	syk	ENSP00000364907	65.0	76.0
zap70	ENSDARP00000006727	zap70	ENSP00000264972	65.0	68.0
tnk1 (tnk2b)	ENSDARP00000118604	tnk1	ENSP00000312309	38.0	38.0

(*continued*)

Table 1. (Continued)

Duplicates/Ambiguous Orthology

Zebrafish gene name	*Zebrafish protein accession*	*Human gene name*	*Human protein accession*	*% Sequence identity (full length sequences)*	*% Sequence identity (kinase domain)*
egfra	ENSDARP00000121251			63.0	67.0
egfrb	ENSDARP00000110107	egfr	ENSP00000275493	46.0	46.0
erbb3a	ENSDARP00000124007			49.0	47.0
erbb3b	ENSDARP00000116216	erbb3	ENSP00000267101	54.0	53.0
erbb4 (5/5)	ENSDARP00000106313			71.0	71.0
erbb4a	ENSDARP00000086551			73.0	69.0
erbb4b	ENSDARP00000116813	erbb4	ENSP00000342235	83.0	79.0
igf1ra	ENSDARP00000017066			65.0	77.0
igf1rb	ENSDARP00000046541	igf1r	ENSP00000268035	63.0	79.0
insra	ENSDARP00000023951			68.0	82.0
insrb	ENSDARP00000096601	insr	ENSP00000303830	65.0	84.0
csf1ra	ENSDARP00000089365			45.0	63.0
csf1rb	ENSDARP00000121694	csf1r	ENSP00000286301	38.0	56.0
kita	ENSDARP00000099069			48.0	68.0
kitb	ENSDARP00000044029	kit	ENSP00000288135	45.0	63.0
fgfr1a	ENSDARP00000116533			73.0	89.0
fgfr1b	ENSDARP00000067688	fgfr1	ENSP00000380280	78.0	84.0
flt1a (flt1)	ENSDARP00000002466			51.0	61.0
flt1b (kdrl)	ENSDARP00000007209	flt1	ENSP00000282397	44.0	57.0
mst1ra	ENSDARP00000123874			40.0	64.0
mst1rb	ENSDARP00000124745	mst1r	ENSP00000296474	41.0	67.0
ntrk2a	ENSDARP00000078225			63.0	91.0
ntrk2b	ENSDARP00000116694	ntrk2	ENSP00000314586	60.0	86.0
ntrk3a	ENSDARP00000124017			73.0	89.0
ntrk3b	ENSDARP00000086161	ntrk3	ENSP00000377990	87.0	87.0
epha2a	ENSDARP00000011069			55.0	72.0
epha2b	ENSDARP00000044917	epha2	ENSP00000351209	60.0	77.0
epha4a	ENSDARP00000123962			82.0	88.0
epha4b	ENSDARP00000030606			66.0	77.0
epha4c (epha4l)	ENSDARP00000003161			74.0	77.0
epha4d (ek1)	ENSDARP00000096552	epha4	ENSP00000386829	64.0	73.0
mertkb (CU570987.1-201)	ENSDARP00000110210			45.0	57.0
mertka	ENSDARP00000101815	mer	ENSP00000295408	45.0	64.0
ephb2a (ephb2)	ENSDARP00000112928			86.0	90.0
ephb2b (ephb2)	ENSDARP00000043755	ephb2	ENSP00000383053	88.0	92.0
ephb3a	ENSDARP00000040208			69.0	70.0
ephb3b	ENSDARP00000050394	ephb3	ENSP00000332118	81.0	91.0
ephb4a	ENSDARP00000111946			63.0	82.0
ephb4b	ENSDARP00000088414	ephb4	ENSP00000350896	60.0	82.0
axla	ENSDARP00000087680			53.0	54.0
axlb	ENSDARP00000087681	axl	ENSP00000301178	59.0	59.0
ddr2a	ENSDARP00000124159			78.0	85.0
ddr2b	ENSDARP00000123523			66.0	73.0
ddr2c (ddr2l)	ENSDARP00000124904	ddr2	ENSP00000356899	58.0	69.0
aatka	ENSDARP00000103704			60.0	58.0
aatkb	ENSDARP00000125077	aatk	ENSP00000324196	59.0	55.0
styk1a	ENSDARP00000095367			44.0	44.0
styk1b	ENSDARP00000115394			32.0	35.0
styk1c	ENSDARP00000112925	styk1	ENSP00000075503	36.0	41.0
jak2a	ENSDARP00000107572			65.0	78.0
jak2b	ENSDARP00000105218	jak2	ENSP00000371067	68.0	82.0
fyna	ENSDARP00000123888			89.0	89.0
fynb	ENSDARP00000037198	fyn	ENSP00000346671	90.0	94.0
cska	ENSDARP00000095046			85.0	85.0
cskb	ENSDARP00000088849	csk	ENSP00000220003	86.0	85.0
acka (tnk2a)	ENSDARP00000115160			56.0	55.0
ackb (tnk2(1/3))	ENSDARP00000109337			59.0	59.0
ackc (tnk2(2/3)	ENSDARP00000084221	tnk2	ENSP00000329425	56.0	55.0

(*continued*)

Table 1. (Continued)

Duplicates/Ambiguous Orthology

Zebrafish gene name	*Zebrafish protein accession*	*Human gene name*	*Human protein accession*	*% Sequence identity (full length sequences)*	*% Sequence identity (kinase domain)*
faka (ptk2a)	ENSDARP00000042715			81.0	84.0
fakb (ptk2b)	ENSDARP00000124099	ptk2	ENSP00000429911	79.0	82.0
ptk2a (ptk2ba)	ENSDARP00000036466			60.0	61.0
ptk2b (ptk2bb)	ENSDARP00000057818	ptk2b	ENSP00000332816	60.0	63.0
ptk6a	ENSDARP00000063046			47.0	53.0
ptk6b	ENSDARP00000078382	ptk6	ENSP00000217185	48.0	51.0
frka (frk)	ENSDARP00000124324			64.0	70.0
frkb (CH73-340M8.3)	ENSDARP00000058893	frk	ENSP00000357615	39.0	45.0

Unique to Zebrafish

Zebrafish gene name	*Zebrafish protein accession*	*Ortholog gene name*	*Ortholog protein accession*	*% Sequence identity (full length sequences)*	*% Sequence identity (kinase domain)*
yrk	ENSDARP00000121344	yrk (chicken)	NP_001103257.1	77.0	89.0

Unique to Humans

Human Gene Name	*Human Protein Accession*
epha1	ENSP00000275815
srm	ENSP00000217188
fgr	ENSP00000363117
bmx	ENSP00000340082
insrr	ENSP00000357178

Classification according to the presence or absence of duplicate zebrafish orthologs. Names proposed by us are shown with current names in parentheses.

(Fig. 2). The 8 human nonreceptor tyrosine kinases are represented by 17 zebrafish orthologs, whereas the 22 human receptor tyrosine kinases are represented by 49 zebrafish orthologs. Such gene duplication is in accordance with genome duplication during teleost radiation, but intriguingly, almost three times more duplicate genes appear to be present in receptor tyrosine kinases compared to nonreceptor tyrosine kinases (22 vs. 8 and 49 vs. 17, Fig. 2) in zebrafish.

Despite the difference in the actual number of genes, we found further that every human tyrosine kinase family is represented in the zebrafish, suggesting conserved tyrosine kinase evolution in vertebrates. Of all the zebrafish tyrosine kinase genes, only 15 have reported genetic (mutant) alleles (Supplementary Table S2). Thirteen genes do not currently have assigned ZFIN IDs, whereas 16 genes do not currently have assigned VEGA IDs. RNASeq information based on models generated using data from the Wellcome Trust Sanger

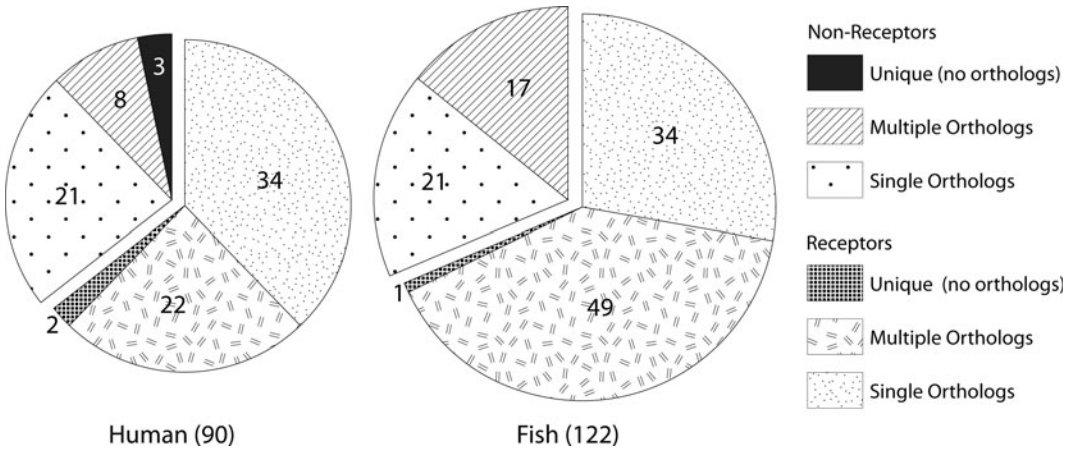

FIG. 2. Pie charts showing the numbers of receptor and nonreceptor tyrosine kinases in humans and zebrafish with single and multiple orthologs.

Institute zebrafish transcriptome sequencing project (http://www.ensembl.org/info/docs/genebuild/ rnaseq_annotation.html) predicts that almost all zebrafish tyrosine kinases are expressed as early as 1–5 days post-fertilization.

Early expression of zebrafish tyrosine kinases

With the exception of two genes (csf1rb and erbb3a), all other zebrafish tyrosine kinase genes are predicted to be expressed based on the available RNASeq data. In order to generate experimental data to support our computational analysis, we selected a set of 30 tyrosine kinase genes from our list that were either previously unannotated or not reported during the first 24 h of zebrafish development. Direct digital detection using specific probes with the nCounter technology platform allowed us to measure the expression of the selected 30 zebrafish tyrosine kinases representing all families, normalized to that of three housekeeping genes. We found that at specific time points within the first 24 h (24 hpf, hours post fertilization) of development (post-fertilization), 22 (of the 30) tyrosine kinase genes we selected were expressed above the baseline. While 8 genes show minimal expression, PTK7 (CCK4) shows the highest level of expression (Fig. 3). Considering our study and available RNASeq and EST data, csf1rb happens to emerge as the only gene whose expression is not currently reported in zebrafish, and these data are likely to be generated in due course. Our results, in conjuction with Sanger's RNASeq data, strongly suggest that all zebrafish tyrosine kinase families are dynamically expressed and have roles in early embryonic development. Sequences of the specific probes used and normalized expression measurements are shown in Supplementary Tables S1 and S4, respectively.

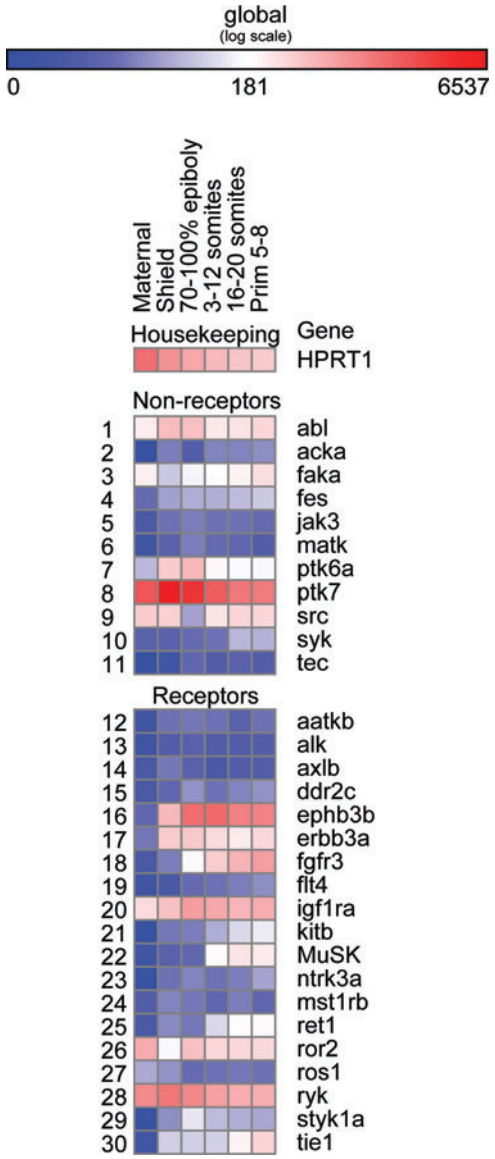

FIG. 3. Gene expression levels of selected tyrosine kinase genes. Nanostring digital readouts for expression levels of nonreceptor and receptor genes are represented by heat map. (HPRT gene is a reference housekeeping gene). The *numbered columns* represent distinct time windows during the first 24 hours of embryonic development (1 = 2-cell stage/maternal; 2 = shield stage; 3 = 70% epiboly– bud stage; 4 = 3–12 somites; 5 = 16–20 somites; 6 = 26 somites–Prim 8 stage). Color images available online at www.liebertpub.com/zeb

Discussion

This study was conducted with the intention of creating an unambiguous resource providing detailed information on zebrafish tyrosine kinases and their relationship to human tyrosine kinases. The information we report is crucial for developmental and genetic studies involving zebrafish tyrosine kinase genes, and for *in vivo* studies of tyrosine kinase biology using zebrafish. Because of the fact that no such reported study can be found, and that the zebrafish genome annotation is ongoing, our study generated useful annotation data for most of the zebrafish tyrosine kinase genes.

Every human tyrosine kinase family is represented in the zebrafish genome, and mRNA detected in 24 hpf embryos shows that tyrosine kinases from all families are indeed expressed and appear to be regulated. Our analysis further shows that gene duplication, which is known to be common in zebrafish due to whole genome duplication in the teleost lineage, is seen more in receptor tyrosine kinase genes than nonreceptor tyrosine kinase genes. This is consistent with the theory that following genome duplication, gene duplicates which acquired novel functions and contributed to diversity were retained more frequently than others.[55] Receptor tyrosine kinases are longer and contain more domains with greater variation than nonreceptor tyrosine kinases that contain fewer and less variant domains. This suggests a greater probability of evolution of novel combinations and functions and therefore greater duplicate gene retention in receptor tyrosine kinases. This explanation is also consistent with the recent discovery of higher divergence among receptor tyrosine kinases during metazoan evolution, which may have facilitated cell-to-cell communication and allowed responses to a variety of extracellular cues during the evolution of multicellularity.[56] The same rationale would help explain the retention of a larger tyrosine kinase repertoire compared to that of nonreceptor tyrosine kinases in zebrafish. Future studies to assess these features in other vertebrate lineages will provide a more conclusive explanation of our observation.

A detailed understanding of *in vivo* effects of tyrosine kinase modulation is needed to achieve a complete mechanistic comprehension of tyrosine kinase biology, and to address current and future issues in tyrosine kinase inhibitor development. Such understanding has been difficult to achieve with the sole use of *in vitro* biochemical studies, cell culture models, human tissue/clinical studies, or mammalian animal models. It is likely that the probability of success in cancer therapy (or indeed that in any disease) may be enhanced by the use of simpler vertebrate model organisms such as zebrafish[57] to model and study disease. For example, understanding the consequences of altered tyrosine kinase activity (by gene overexpression, knockdown/disruption, or small molecule treatment) in the context of a cancer model[58,59] in zebrafish will contribute effectively to research in cancer and tyrosine kinase biology.

A major issue with tyrosine kinase inhibitor development is the assessment of their toxicity and safety. The data generated from our study (and such future ones) may facilitate decision-making in toxicity and safety assessment of tyrosine kinase inhibitors by employing zebrafish animal models. We envision that future studies exploring the biochemistry and signaling of zebrafish tyrosine kinases will provide a basis to apply existing or novel kinase assay platforms in zebrafish. The use of zebrafish cancer disease models using strategies of nonspecific tumor generation, tumor xenograft, tumor suppressor knockdown, or oncogene overexpression are likely to lead to *in vivo* pharmacology models.

Our results also highlight the need to understand individual roles of the tyrosine kinase orthologs in zebrafish development and physiology. The likelihood of multiple active orthologs should be taken into account while designing studies involving tyrosine kinase gene knockdown and disruption, ectopic expression, or small molecule treatment. It should further be noted that the currently annotated version of the zebrafish genome sequence contains unmapped and partial scaffolds. As the zebrafish genome project continues, the quality of sequence information will be greater and gene annotation will be richer. While we believe that our study includes all of the protein tyrosine kinases, new and refined sequence information may result in modifications to our computational findings, with functionally relevant annotations. Revisiting a complete genome sequence of the zebrafish will help to determine if the five exceptions in the list of human and zebrafish tyrosine kinase orthologs we report will remain.

In summary, our findings represent the first comprehensive identification of all zebrafish tyrosine kinases, in which we found remarkably high sequence identity to human tyrosine kinases, identical family classification, and evidence of early developmental expression. Apart from highlighting the need to investigate mechanistic aspects of zebrafish tyrosine kinase biochemistry and physiology, we believe that our study provides strong rational evidence in support of utilizing the zebrafish as an *in vivo* model organism for future studies on tyrosine kinase biology and pharmacology.

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Disclosure Statement

No competing financial interests exist.

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