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Review

Interrenal Organogenesis in the Zebrafish Model

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ABSTRACT

In recent years, many genes that participate in the specification, differentiation and steroidogenesis of the interrenal organ, the teleostean homologue of the adrenal cortex, have been identified and characterized in zebrafish. In-depth studies of these genes have helped to delineate the morphogenetic steps of interrenal organ formation, as well as some of the molecular and cellular mechanisms that govern these processes. The co-development of interrenal tissue with the embryonic kidney (pronephros), surrounding endothelium and invading chromaffin cells has been analyzed, by virtue of the amenability of zebrafish embryos to a variety of genetic, developmental and histological approaches. Moreover, zebrafish embryos can be subject to molecular as well as biochemical assays for the unraveling of the transcriptional regulation program underlying interrenal development. To this end, the key mechanisms that control organogenesis and steroidogenesis of the zebrafish interrenal gland have been shown to resemble those in mammals, justifying the future utilization of zebrafish model for discovering novel genes associated with adrenal development and disease.

INTRODUCTION

The external fertilization and rapid development of the zebrafish embryo has made it suitable for studying early vertebrate development.[1] Due to the permeability and optic transparency of the zebrafish embryo, developing organ primordia in the whole mount specimen can be readily detected by histochemistry, immunohistochemistry or in situ hybridizations.[2] Various cell types that integrate to constitute specific organs, such as embryonic heart and kidney, can be labeled simultaneously by a combination of histological approaches.[3,4] Hence, the zebrafish is particularly useful for capitulating the dynamic processes of organ formation, especially cell migration events or morphogenetic movements of organ primordia. Furthermore, the establishment of transgenic fluorescent reporter lines has allowed the tracking of specific lineages as well as dissection of cellular processes with high resolution. For example, endothelium-specific transgenic reporter lines have enabled the elucidation of mechanisms underlying vessel formation, angiogenesis and endothelial tube assembly.[5-7]

Zebrafish mutants that are generated from either forward or reverse genetic approaches have offered great opportunity for understanding the interrelationships among gene, development and disease.[8,9] Large-scale chemical mutagenesis screens have identified numerous mutants with a wide spectrum of defects manifested in various organs, helping to elucidate specific signaling pathways leading to organogenesis such as heart and blood formation. The advent of antisense morpholino oligo knockdown approach, proven effective and specific in the zebrafish embryo, has further facilitated the developmental analysis for any candidate genes of interest.[10]

Although the endocrine gland structures of fish and mammals are largely different, their developmental processes in organogenesis appear to share high similarities.[11] Also, endocrine function is well conserved between teleosts and mammals, which could be reflected by the profiles of hormones, hormone receptors as well as transcriptional regulators. Interestingly, some zebrafish mutants or morphants faithfully phenocopy human endocrine disorders such as combined pituitary hormone deficiencies[12] and neonatal diabetes mellitus.[13] Hence, they promise to provide insights into the molecular etiology of their human disease counterparts.

This short review highlights the teleostean counterpart of mammalian adrenal gland, the interrenal gland, based on recent findings in the zebrafish model. The ontogeny,

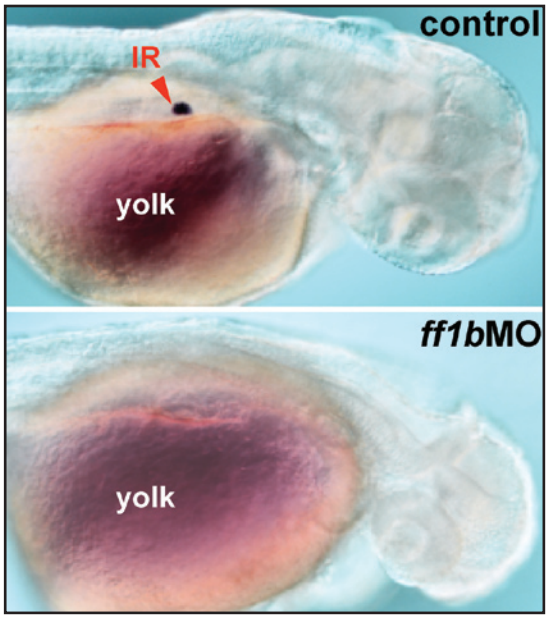

Figure 1. The detection of steroidogenic interrenal tissue by whole-mount chromogenic 3β-Hsd enzymatic activity assay in wild-type control (upper panel) and *ff1b* antisense morpholino (*ff1b*MO) injected (lower panel) embryos. The embryos were treated with 0.003% phenylthiourea from 12 hpf onwards to prevent pigmentation. Dorso-lateral views of embryos at 2 dpf are shown with anterior oriented to the right. The steroidogenic interrenal cells are completely ablated in *ff1b* morphant. Red arrowhead indicates interrenal tissue (IR), lying above yolk sac in the mid-trunk region.

molecular determinants, cell migrations and tissue-tissue interactions are outlined, in order to discuss the relevance of zebrafish interrenal organ to mammalian adrenal gland.

MOLECULLAR AND CELLULAR CONTROLS OF INTERRENAL ORGANOGENESIS

FF1b marks and determines the onset of interrenal tissue. The mode of tissue organization for teleostean interrenal organ is distinct from that of mammalian adrenal gland.[14] The interrenal cells intermingle with, rather than encapsulate, the chromaffin cells which are functional equivalent of the adrenal medulla, and the assembled organ is embedded at the anterior portion of the head kidney. However, the molecular determinants for specifying steroidogenic lineages have proven to be highly conserved between teleosts and mammals. Two Ftz-F1 genes, *ff1b* (*nr5a1a*) and *ff1d* (*nr5a1b*), have been identified in zebrafish to be co-orthologues of mammalian SF1/Ad4BP (NR5A1).[15-17] While SF1 is obligatorily required for both adrenal and gonadal development, *ff1b* and *ff1d* appear to function predominantly in interrenal tissue and gonads, respectively. *ff1b* is the earliest molecular marker detected in the developing interrenal tissue of zebrafish embryo.[16] The knockdown of Ff1b by the antisense morpholino oligo approach led to specific ablation of interrenal cells, as revealed by the loss of either functional differentiation (Fig. 1) or interrenal-specific RNA transcripts, indicating that Ff1b is absolutely required for the initiation of interrenal primordium.

Pronephros vs. interrenal tissue: Parallel morphogenetic movements under differential endothelium-derived controls. Temporal and spatial analyses revealed that early interrenal tissues arise as bilateral clusters of non-steroidogenic cells within the pronephric

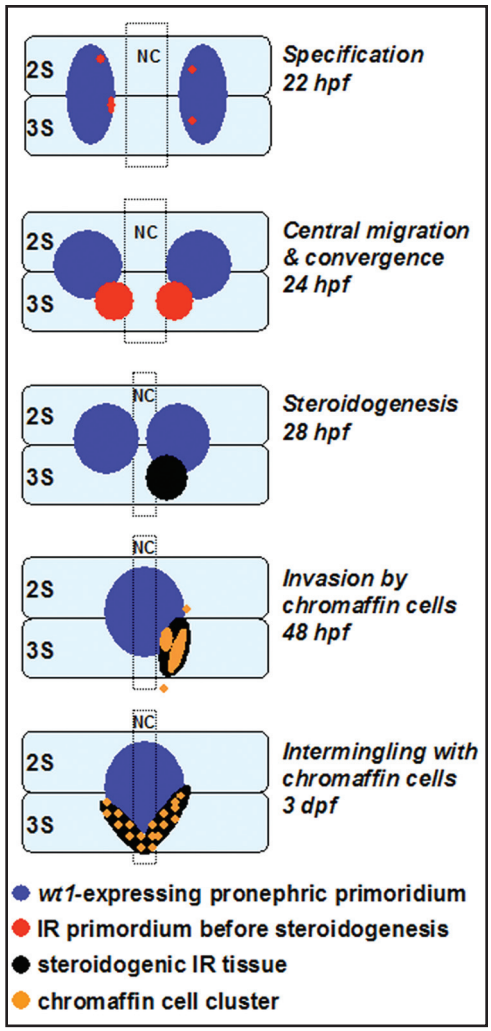

Figure 2. Early morphogenetic processes in the interrenal development of zebrafish. The parallel migrations of interrenal tissue, pronephros and chromaffin cells in this diagram are depicted based on the results of references 18, 20 and 39. The panels represent dorsal views of embryos, at the indicated stages, oriented with anterior to the top. Through the sequential stages of interrenal specification, migration, steroidogenesis and chromaffin cell invasion, an assembled interrenal organ is evident by 3 dpf. NC, notochord; 2S and 3S, the second and third somite, respectively.

primordia (Fig. 2), while both pronephros and interrenal tissue are derived from intermediate mesoderm.[4,18] The interrenal tissues then migrate out of the pronephric fields, before the initiation of steroidogenesis. After separation, the interrenal and pronephric tissues stay in close proximity to one another throughout development, and both undergo central migration followed by fusion. Temporally, the convergence of bilateral interrenal primordia well precedes the central assembly of pronephros. Interestingly, both pronephros and interrenal tissue receive endothelium-derived signals, albeit via different mechanisms, as guidance cues for central migration.[19,20]

The central migration of pronephric primordia, starting at around 30 hours post-fertilization (hpf), is temporally correlated with the angiogenesis of paired glomeruli.[4,18,20] In the meantime, the developing interrenal cells are tightly associated with endothelium (Fig. 3A–F). This central fusion of glomeruli is disrupted upon the perturbations of blood flow, either genetically or pharmaceutically, implying a role of hemodynamic force for glomerular morphogenesis.[19] Indeed, the endothelial

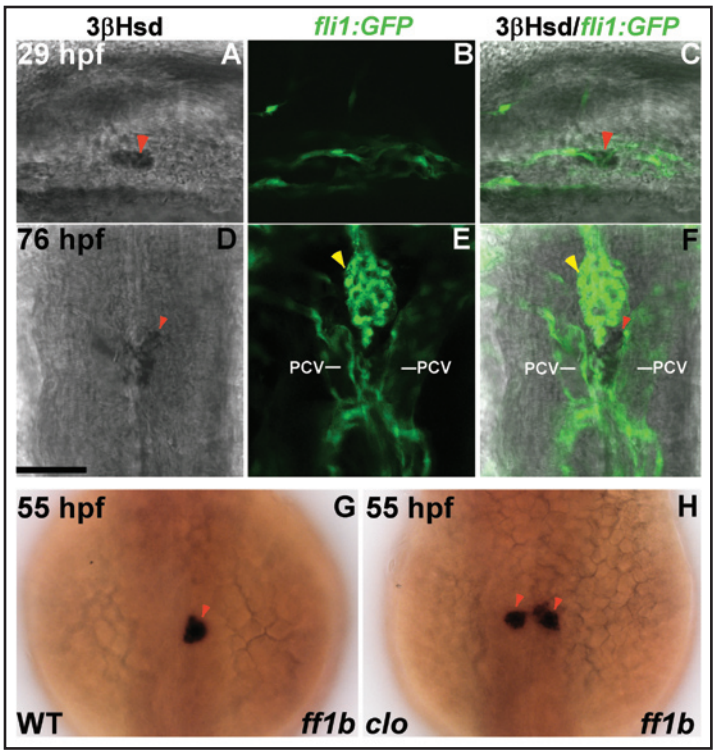

Figure 3. (A–F) Interaction of interrenal and endothelial cells as revealed in *Tg(fli1:EGFP)*^{Y1} transgenic zebrafish. Single confocal sections showing the interrenal tissues as stained by 3β-Hsd activity assay (left panel: A and D), and the neighboring ECs as labeled by the green fluorescence (middle panel: B and E), of the *Tg(fli1:EGFP)*^{Y1} embryos while staged at 29 hpf (A–C) and 76 hpf (D–F) respectively. The merged images of 3β-Hsd activity and GFP are shown in the right panel (C and F). (A–C) are lateral views with anterior oriented to the right. (D–F) are dorsal views with anterior oriented to the top. The interrenal cells are in close contact with ECs that are engaged in axial vessel assembly. PCV, posterior cardinal vein. Red arrowheads indicate interrenal tissues, while yellow arrowheads indicate kidney glomeruli. Bar, 50 μM. (G and H) *ff1b* expression in the *clo*^{m39} mutant and its wild type sibling. Embryos of *clo*^{m39} mutant (H) and its wild-type sibling (WT; G) were labeled by *in situ* hybridization for *ff1b* (dark blue) at 55 hpf. Dorsal views of embryos are shown, with anterior oriented to the top. The central migration of interrenal tissue is arrested in *clo*^{m39}, resulting in persistent distribution of a pair of cell clusters on either side of midline. Reproduced with permission.[20]

cells (ECs) invading pronephros, by sprouting of dorsal aorta, are regulated by blood circulation to express matrix-metalloproteinase-2, which in turn mediates the assembly of bilateral glomeruli at midline. However, hemodynamic force does not contribute to the central fusion of bilateral interrenal primordia, despite the close association of pronephros and interrenal tissue in the early embryo.[20] In fact, the interrenal migration and fusion is normal even as axial vessel assembly or angiogenesis is severely compromised. However, interestingly, the interrenal central convergence is disrupted in the endothelium-free mutant *cloche* (*clo*; Fig. 3G and H), and can be rescued while endothelium near interrenal area is partially restored by the forced expression of the Scl transcription factor.[20] This argues for a role of endothelial signaling in guiding interrenal migration, and provides one of the few pieces of evidence that beyond the vascular functions in supporting cell growth, tissue maintenance and endocrine secretion, endothelium also plays a morphogenetic role in endocrine development.

Molecular interplays within interrenal primordium. The expression of *ff1b* is spatially closely associated with those of transcription factor genes *wilm's tumor* (*wt) 1* and *dax1* (*nr0b1*).[18,21] *ff1b*-expressing interrenal primordia originate within the *wt1*-expressing pronephric field, while *wt1* expression is essential for the development of both pronephric and interrenal tissues.[18] Well after the specification stage, *dax1* gene is transiently expressed at interrenal tissue, and is required for the expression of steroidogenic genes *cyp11a* and *star*.[21] While *SF1* is known to interact with *WT1* and *DAX1* in the mammalian systems,[22-24] it is tempting to hypothesize that similar molecular interplays might occur in the early zebrafish embryo. Alternative splicing of *WT1* results in -KTS and +KTS isoforms which differ in protein properties as well as developmental roles.[25-27] In mammals, only the -KTS form of WT1 is able to bind to and transactivate *Sf1* promoter,[28] or to interact physically with SF1 to promote *MIS* expression.[22] Splice variants with and without the KTS tripeptide-encoding sequence have been found for both *wt1a* and *wt1b*, the two *WT1* homologues identified in zebrafish.[29] However, the biochemical properties of these *wt1* variants, in terms of their interaction with Ff1b or regulation upon *ff1b* promoter, have remained unclear. DAX1 is a corepressor for several nuclear receptors involved in the steroidogenic axis, notably SF1.[30] DAX1 negatively regulates the transcriptional activities of SF1,[23,24] as well as antagonizing the synergistic action between SF1 and heterodimeric partners such as WT1.[22] While LXXLL-related motifs are necessary for the corepressing activities of DAX1,[31] the zebrafish and tilapia *dax1* sequences lack the first and second of four LXXLL motifs seen in mammals.[21] It remains unclear whether these teleostean Dax1 proteins could exert similar corepressing activities as their mammalian counterparts.

The homeodomain protein Prox1 has however been confirmed as a novel Ff1b corepressor, and expressed at the developing zebrafish interrenal tissue.[32] Both *prox1* and *dax1* appear to function downstream of *ff1b* in interrenal development, and to be implied in steroidogenesis.[21,32] While *dax1* is only transiently expressed at around 32 hpf, *prox1* expression at interrenal tissue starts from 28 hpf and persists up to three days post-fertilization (dpf). Both *prox1* and *dax1* morphants display reduced numbers of steroidogenic cells. Although no similar coregulation of SF-1 by mammalian Prox1 has been reported, Prox1 was interestingly found to corepress another Ftz-F1 protein Liver Receptor Homolog-1 (LRH-1; NR5A2), and thus suppress LRH-1-mediated activation of cholesterol 7-α-hydroxylase gene.[33,34] Both mammalian and zebrafish Prox1 proteins are interacting physically with Ftz-F1s via LXXLL-related motifs. These studies indicate that the corepressor function of Prox1 upon Ftz-F1 appears to be conserved across the vertebrate species, yet diversified in terms of the profile of target genes regulated.

Interrenal steroidogenesis. The interrenal tissue is the major site of steroidogenesis in most teleosts, as is the adrenal cortex in mammals.[14,35] In zebrafish, many steroidogenic genes have been cloned and identified, including side chain cleavage enzyme *cyp11a1* (*p540scc*)[36,37] and *steroidogenic acute regulatory protein* (*star*),[38] both are known to mediate the rate-limiting steps of steroidogenesis. Cyp11a1 catalyzes the first step of adrenal steroidogenesis, while StAR regulates cholesterol shuttling across the mitochondrial membrane and the acute steroid production. Both *cyp11a1* and *star* are expressed at embryonic interrenal tissue, starting from approximately 24 hpf, shortly after the specification and central convergence of interrenal primordia.[39] However, the functional differentiation of interrenal tissue, marked by the chromogenic detection of

3-beta-Hydroxysteroid dehydrogenase (3β-Hsd) enzymatic activity, is not initiated until about 28 hpf. The expressions of steroidogenic genes are abolished upon the knockdown of Ff1b by antisense morpholino injection, implying their direct transcriptional regulation by Ff1b protein.[16,18] In addition, the transcripts of *cyp11a* and *star* are down-regulated at the interrenal tissue of *dax1* morphant, while the enzymatic activity of 3β-Hsd is reduced in *prox1* morphant.[21,32] Collectively, the interrenal steoridogenesis in zebrafish embryo might be mediated by a combinatorial action of transcriptional regulators.

The gene expressions associated with pituitary control of interrenal steroidogenesis, including those of *proopiomelanocortin* (*pomc*)[40,41] and *acth receptor* (*mc2r*),[39,42] also commence early at 18 and 32 hpf respectively. In mammals, ACTH is the major pituitary regulator derived from POMC to regulate adrenal glucocorticoid synthesis and secretion. However, in contrast to the early onset of *pomc* and *mc2r* gene expressions, the hormonal control of interrenal steroidogenesis does not occur until larval stage.[39] In either *mc2r* morphant or mutants lacking pituitary corticotrophs, no perturbation of interrenal size or steroidogenesis is evident until 5 dpf, when the developing fish reaches larval stage. This indicates a developmental transition, from autonomous pituitary-independent phase, to pituitary-dependant phase of steroidogenesis and interrenal growth. These results in zebrafish are consistent with adrenal development in *Pomc*-null mice, where adrenal glands are normal in morphology at birth and develop adrenal hypoplasia only postnatally.[43,44]

Integration of interrenal with chromaffin cells: Mechanisms unclear. In zebrafish, the parallel development of interrenal steroidogenic and chromaffin cells resembles organ formation of adrenal gland in mammals. Chromaffin cells, detected by the expression of Dopamine β-hydroxylase (Dβh) which converts dopamine to noradrenaline, are dispersed as several clusters in the zebrafish interrenal region at 2 dpf, which then converge to midline by 3 dpf and stay in intimate contact with steroidogenic cells throughout the subsequent stages (Fig. 2).[16,39] Similar to the mammalian adrenal medulla, the chromaffin component of the interrenal organ is originated from the neural crest through sequential steps of differentiation.[45] In the zebrafish embryo, the trunk neural crest gives rise to dorsal root ganglion sensory neurons and autonomic sympathetic neurons. Sympathetic neurons from cervical ganglion subsequently express adrenergic differentiation markers *dβh* and tyrosine hydroxylase, and some migrate to the interrenal region to become endocrine chromaffin cells. In vertebrates, the specification and diversification of neural crest-derived lineages require a highly regulated network of signaling molecules and transcription factors, with the factor(s) determining the final differentiation of chromaffin cells remaining unclear.[46,47] While reverse genetics to this end could not solve the question concerning how endocrine vs. neural sympathoadrenal cells develop, forward genetic approach using zebrafish might help to identify the unknown signal(s) that instruct migration and maturation of chromaffin cells.

CONCLUDING REMARKS

Since the identification of the *ff1b* gene, subsequent studies in various groups combined to demonstrate that interrenal development in the zebrafish shares many conserved molecular and developmental mechanisms with higher vertebrates. Moreover, various morphants and genetic mutants can be successfully used to manipulate molecular, cellular and hormonal signalings, for dissection of the regulatory network that directs interrenal organogenesis. The availability of specific markers for steroidogenic cells will allow chemical mutagenesis screens for the discovery of novel genes associated with interrenal disorders, such as interrenal hypoplasia. Therefore the zebrafish interrenal model is promising to provide insights on the interplay among gene, development and disease of mammalian adrenal gland, and even a platform for pharmaceutical screenings targeting adrenal functions.

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