Please note that this exemplar is from another unit and much more extensive than required for BIOL3120

Review of Zebrafish Molecular Biology Techniques to Observe Stress Granule Formation in Motor Neuron Disease

***Abstract*— Abnormal stress granule dynamics have been previously linked to cell death and implicated in cases of motor neuron disease (MND). Understanding these dynamics in a zebrafish model organism can lead to a greater understanding of human MND pathogenesis. To investigate stress granule dynamics, researchers must image the biochemical interactions occurring at a cellular level. For this reason, preceding researchers have altered gene expression of zebrafish to enable imaging of stress granule formation. Typically, this involves identifying a gene of interest involved in stress granule formation or structure and attaching it to an imaging molecule or dye (such as Green Fluorescent Protein). Previous techniques used include the transient overexpression of genes, the Tol2 transposon transgenesis, endonucleases such as zinc fingers, TALENs, and CRISPR/Cas9, viral vector mediated gene editing, and naked DNA/Gene gun bombardment. While all techniques are validated with experiments, CRISPR/Cas9 gene editing is the most suitable of the techniques for investigating stress granule formations. In understanding molecular biology techniques in zebrafish, the observation of stress granule dynamics can be better understood at the biochemical level. This will lead to a greater understanding of the role stress granules play in the pathogenesis of MND.**

I. Introduction

Stress granules are accumulations of non-membrane bound RNA-protein assemblies which have aggregated in the cytosol of cells as a response to external stimuli (1, 2). During stress granule formation, cellular stresses such as heat shock, viral infection, and oxidative stress (3) trigger the phosphorylation the mRNA translation initiation factor (eIF2α). This impairs the assembly of the eIF2/tRNAiMet/GTP complex which is essential for mRNA translation and halts non-essential protein formation in the cell (4-6) These formations are a natural and dynamic biological response to cellular stress which limit mRNA translation initiation and help the cell to survive short-term cellular stresses (7). Chronic stress granules form when the cell doesn't disperse of the stress granule formations, leading to cell death which has been implicated in the pathology of neurodegeneration and motor neuron disease (2, 8-11). Investigating stress granule dynamics is crucial to supporting the characterization of cellular pathologies in motor neuron disease. In understanding the pathology motor neuron disease at a cellular level, advancements in treatments can be made.

Zebrafish (Danio rerio) are a suitable model organism for this study as they possess highly desirable traits for the *in vivo* observation of stress granule formation. As model organisms, zebrafish are suitable for simulating human disease as they share up to 70% of their exons with humans (12). Zebrafish also have anatomy similar in structure to human organs such as nervous system and muscles (13). Comparable genetic and anatomical structures combined with transparency of the fish in their embryo and larval stages of life make *in vivo* imaging of conditions modelled in the zebrafish a possibility (14). Zebrafish are a low-cost model organism in initial purchase and maintenance (15) when compared to other higher order, model organisms such as mice or primates (16) due to their small size, shoaling behavior (showing preference to be kept with other zebrafish), (17) and their capability for daily, asynchronous reproduction (18). Zebrafish are prolific breeders, capable of producing clutch sizes of roughly 94.9 ±18.5-169 ±25.1 eggs (depending on male size) (19) every 1-1.9 days (20, 21) although optimal protocol suggests breeding once every 1-2 weeks (22). High reproduction rates are a major benefit to maintaining transgenic zebrafish lines as the likelihood for offspring fish carrying the transgenic manipulation is high. Finally, zebrafish are amendable to genetic manipulation with an established history as model organisms in the field. For these reasons, zebrafish will be the model organism of choice for genetic manipulation to observe stress granules.

There are different technologies used in the genetic manipulation of zebrafish to study and image *in vivo* and *in vitro* stress granule development. This review aims to identify the most appropriate technology for this application.

II. Transient Overexpression in Zebrafish

A common method for the study of physiological and disease processes in zebrafish is the transient overexpression of a mRNA of interest in developing zebrafish embryos. This technique involves the microinjection of mRNA into the 1-2 cell stage embryo in order, to induce short term expression of a protein of interest (23, 24). These transient expressions are measured over the course of hours-days as the genetic material is not integrated into the genome of the zebrafish model (25). The benefits for this type of expression are vastly quicker results when compared to traditional transgenic methods, lower cost of implementation, and lower skill barrier to the approach as genetic material design and microinjection are relatively simple.

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Transient overexpression has been utilized to simulate mutant TAR DNA-binding protein 43 (TDP-43) axonopathy (26). The overexpression of TDP-43 was investigated through microinjection of wild type and mutant (A315T) TDP-43 in zebrafish (26). In this experiment, Liard et al showed the overexpression of the mutant A315T TDP-43 variant produced a decrease in axonal length in zebrafish models when compared against the wild type positive control (26). The use of transient overexpression in this experiment has the benefit of being a highly controlled and fast assay to assess the impact of problematic genes of interest in the zebrafish model. Whilst transient overexpression as a technique is of interest when investigating short-term gene expression, it has drawbacks when investigating stress granule formation. Often overexpression of stress granule marker proteins (such as G3BP1) can artificially promote the stress granule response as they are stress granule nucleation sites (27). It is important when investigating or characterizing a component of a disease to not introduce artificial influences as they will impact the integrity of the model.

III. Tol2 Transgenesis in zebrafish

Historically there has been a myriad of molecular biology techniques which can be used to create long-term overexpression in zebrafish. Viral vectors, naked DNA injections and Tol2 transgenesis are such examples that have been employed in zebrafish (28-32). While not currently employed in stress granule observation experiments, these techniques expand the molecular biologist's toolkit in providing insight to gene editing. It is important to keep these historically used techniques in mind as they may provide solutions for future challenges, this review section will focus on the commonly used Tol2 transgenesis system. The Tol2 transposon is a versatile and well documented gene transfer vector used to create stably expressing transgenic zebrafish. Originally identified in the medaka fish (*Oeyzuas latipes*) (33), the Transposase enzyme allows a *Tol2* flanked DNA construct to be inserted into the target genome. This technique's ease of use has established its place as a part of the zebrafish gene editing tool kit in contemporary literature with it being first used in zebrafish in 2000 (34, 35). A transposase-donor DNA plasmid is designed with a promoter, the gene of interest (and typically a reporter such as Green Fluorescent Protein [GFP] genes) and co-injected with a synthetic transposase mRNA into early fertilized zebrafish eggs (36, 37). The injected genetic material is integrated at the Tol2 locations in the genome and the construct becomes part of the zebrafish genome. Injected fish typically display mosaic integration of the transgene and as such are bred with a wild-type genome zebrafish to produce offspring which express the desired transgene in the targeted cell types (36, 37). It is important to note that genetic uptake frequency varies depending on the plasmid and experimental design.

A major feature of the *Tol2* transposon mediated genetic editing of zebrafish is the lack of specific integration sites meaning the inserted DNA can be theoretically translocated anywhere along a chromosome – this high variability is a major consideration to be had when implementing this technique as depending on the experiment, it may represent a major drawback of the technique. A benefit of this technique is the potential for large DNA inserts which can be up to 11 kilobases in size (36). The downsides to the *Tol2* system are the high costs associated with this method of transgenesis and the uncertainty in genetic uptake of the vector. Despite high variability, Tol2 transposon mediated mutagenesis popular in zebrafish transgenesis experiments.

The reliability and coverage of literature surrounding Tol2 transposon mediated transgenesis has established the system as a standard for the creation of stably expressing transgenic zebrafish. This is evident in the use of this technique to create a real time, *in vivo* cell death reporter which could be used to live image and characterize apoptotic cells in zebrafish (38, 39). This fish line was creating a Tol2 flanked construct which included a secreted ANNEXIN5 protein genes (secA5) bound to Yellow Fluorescent Protein (YFP) genes. Using Tol2 based transgenesis, this transgene was integrated into the genome of the zebrafish and live imaging was performed to observe cell death, in real time (38). This use of the Tol2 system as a tool for imaging cellular function was also seen in studying microglial apoptosis and engulfment (39). While employing different means of cellular analysis, both studies show the value of Tol2 transposon mediated zebrafish transgenesis in creating cellular event reporter zebrafish for use in contemporary studies. This experiment in of interest to investigating stress granule reporters as cell death and stress granules can be comparable as cellular events.

Finally, Tol2 transposons have also been used to induce and investigate the formation of stress granules in zebrafish models under mutated FUS genes. When investigating FUS genes implicated in stress granule formation, the FUS-R521C gene (previously associated with MND) was inserted into a Tol2 transposon driven under the β-actin promoter (8). GFP was used to visualize the accumulation of FUS-R521C gene derived proteins in the cytosol of transgenic zebrafish (as stress granules) as well as in visualizing a wild-type derivative of FUS gene related proteins in control fish (8). Zebrafish FUS proteins were imaged by staining with polyclonal anti-FUS antibodies (8). This staining demonstrated the co-localization of the FUS accumulations and the stress granule markers indicating that the FUS accumulations were involved in stress granule dynamics in these fish. The Tol2 transposon system was used as a reliable and non-problematic means of mutagenesis in these zebrafish to observe stress granule formation. A major weakness of the Tol2 transposon system is that stress granule dynamics cannot be imaged *in vivo.*

IV. EndoNucleases

Endonucleases such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 are artificial, chimeric restriction enzyme systems. These enzymes have been employed as a DNA editing technique in a variety of different organisms. (40). In general, ZFNs, TALENs and CRISPR/Cas9 work to edit the genome by inducing a double-stranded DNA break in a region of interest (41-43). As the organism's natural DNA repair mechanisms work to repair the double-stranded DNA break, errors can occur resulting in a targeted altered genome sequence when investigating gene knockouts (44). Alternatively, gene knockins can be achieved by exposing the recently cleaved DNA of the host to DNA donors of interest, inserting it at the site (45). Both zinc finger and TALEN techniques generally employ specific binding sites to target where the double-stranded DNA break should occur and the Fok1 domain to induce the breakage as opposed to CRISPR/Cas9 which employs the Cas9 enzyme to induce breakage. All three systems have been employed for genome editing in zebrafish (46).

A. Zinc Finger Nucleases

Zinc Finger Nucleases (ZFNs) were the first DNA specific, genome editing technique to be used in zebrafish in 2008 (47, 48). They are composed of two main structures, the DNA binding domain and the Fok1 cleavage domain. The DNA binding domain is composed of repeated zinc fingers. The term 'zinc fingers' refers to multiple protein structures of different classes which while structurally differ, function in a similar way (49). Generally, a zinc finger is characterized by being a multidomain protein structure stabilized by a zinc ion in a metal binding domain (49). A chain of zinc fingers of variable length is what binds the ZFN to specified DNA sites. Once two ZFNs are bound to the DNA from opposite ends, the Fok1 domains can cleave at the designated site with a 5-7bp space (50).

ZFN constructs have been employed induce double-stranded DNA breaks in zebrafish (41). In 2008, the zebrafish *kdr* locus was targeted for editing by using a chimeric fusion between a Cys2His2 zinc finger protein and *Fok1* cleavage domain (47). In this experiment, two 9-base pair zinc finger DNA recognition domains flanked a 6-base pair space within the *kdr* exon (47). From this experiment, almost 33% of screened founder zebrafish carried lesions at the *kdr* exon site. Whilst this was an exciting first step in genome editing in zebrafish, ZFNs result in many off-target site effects with unintended double-strand DNA breaks elsewhere in the genome (47). Despite the early and successful implementation in zebrafish, ZFNs are rarely used in studying stress granules in zebrafish due to their highly variable nature and labor-intensive design process (51). While not being used in contemporary zebrafish studies, ZFNs were instrumental to the development and popularization of other endonuclease techniques such as TALENs and their uses in zebrafish.

B. TALEN

TALENs are similar to ZFNs in structure and function. Both techniques utilize the Fok1 cleavage domain as lead by DNA binding domains (52, 53), however despite these similarities, they maintain distinction in their application. The DNA binding domain for TALEN is the TAL (transcription activator-like) effectors (54, 55). In nature, these TAL effectors are linked to *Xanthomonas spp.* pathogenesis in plants (55, 56) however have been adapted for use in molecular biology. Similarly, to ZFNs, TALENs leave a spacing between their cleavage sites of 12-21bp. Generally ZFNs able to induce both insertions and deletions where as TALENs are better suited to deletions at the cleavage sites rather than insertions (57). When compared with ZFNs on the same target site (the CCR5 gene), TALEN mediated DNA breaks produced less off-target DNA breaks (58). For this reason, TALEN mediated double-strand DNA breaks are favorable over ZFN mediated double-strand breaks. TALEN is often seen as more reliable than ZFN in inducing mutations (46). However, it should be noted that there still exists potential for off-target effects in highly similar regions of DNA due to the nature of how the TALEN and ZFN techniques operate.

TALENs were utilized in editing the zebrafish genome in 2014 for targeted mutagenesis (59). In this experiment, the zebrafish *eln* gene was targeted by a TALEN flanking exon x and inducing double stranded breaks (59). This targeted knock-out of portions of the *eln* gene enabled researchers to identify glucocorticoid response elements (GREs) in the zebrafish genome (59). The benefits of using TALEN to identify GREs in zebrafish are the relatively low cost of the technique and its greater specificity when compared with ZFNs.

C. CRISPR/Cas9

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a highly specific technique to induce double-stranded DNA breakages in the genome. Derived from the adaptive immune system of *Streptococcus pyogenes*, the technique employs a guide RNA bound to the Cas9 enzyme (CRISPR associated protein 9) and the target sequence of DNA (60). Once bound to the target sequence as defined by the guide RNA, the Cas9 enzyme induces the double-stranded DNA break in the genome (61). In the years following it's conception, CRISPR has been optimized as an effective and reliable molecular biology technique in zebrafish (62, 63). CRISPR/Cas9 has been shown to have the capability to efficiently induce biallelic mutations in zebrafish (64). This is a significant development to the *in vivo* study as it reduces the number of generations of breeding fish required to achieve homozygosity as seen with previous molecular biological techniques in generating monoallelic mutations.

CRISPR/Cas9 has been used to create an *in vivo* zebrafish stress granule reporter. This was accomplished by using CRISPR to tag endogenous Ras GTPase-activating protein-

binding protein 1 (G3BP1) genes with GFP genes (65). G3BP1 was selected for tagging as it has been found to be a key component of stress granule dynamics (66). GFP was tagged such that it was possible to visualize and image the formation of stress granules in the embryonic fish. G3BP1 proteins are diffusely expressed in the cytosol of zebrafish cells, as such there was a level of background fluorescence in the embryonic fish. As G3BP1 itself can initiate and promote stress granules (66) the GFP-tagged G3BP1 complex was controlled by the *g3bp1* gene promotor (66). A major weakness of this study resides in the possibility that previously expressed G3BP1 protein may influence the stress granule dynamics in the cells. This research was significant because using CRISPR/Cas9, Wang et. al were able to reportedly create the first *in vivo* stress granule reporter in an embryonic zebrafish model (67). As a novel technique and technology, the reproducibility of CRISPR/Cas9 experiments are fairly low however this technique boasts lower initial and ongoing costs with higher with generally higher DNA cutting efficiency when compared to ZFNs and TALEN (68). CRISPR/Cas9 mediated gene editing is responsible for the first instance of an *in vivo* stress granule reporter in zebrafish. As such, the technique holds much promise for future investigations.

V. Conclusion

This concise literature review has covered the most prominent *in vivo* molecular biology tools to investigate stress granule formation in zebrafish. As molecular biology techniques and genetic engineering practices become more advanced in zebrafish models, so too will the investigation into stress granules and their role in MND pathogenesis. In assessing the time of experiment, total cost, success rates, and benefits/drawbacks of the techniques presented, it is clear that CRISPR/Cas9 mediated zebrafish knock-in models are to be strongly considered for investigating stress granule formation. While CRISPR/Cas9 knock in models in zebrafish may represent an initially high barrier for optimization in zebrafish, the accuracy of the technique and potential knowledge creation from utilizing it in a zebrafish model is of high importance to stress granule investigations. As one of the newer members of the molecular biologist's toolkit, CRISPR/Cas9 hold promise in zebrafish modeling. None of the techniques presented in this report are mutually exclusive. Each of them may be considered for use in zebrafish models based on the experiment, time limits for research, and skills of the investigating staff.

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