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Neuronal cell culture from transgenic zebrafish models of neurodegenerative disease

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ABSTRACT

We describe a protocol for culturing neurons from transgenic zebrafish embryos to investigate the subcellular distribution and protein aggregation status of neurodegenerative disease-causing proteins. The utility of the protocol was demonstrated on cell cultures from zebrafish that transgenically express disease-causing variants of human fused in sarcoma (FUS) and ataxin-3 proteins, in order to study amyotrophic lateral sclerosis (ALS) and spinocerebellar ataxia type-3 (SCA3), respectively. A mixture of neuronal subtypes, including motor neurons, exhibited differentiation and neurite outgrowth in the cultures. As reported previously, mutant human FUS was found to be mislocalized from nuclei to the cytosol, mimicking the pathology seen in human ALS and the zebrafish FUS model. In contrast, neurons cultured from zebrafish expressing human ataxin-3 with disease-associated expanded polyQ repeats did not accumulate within nuclei in a manner often reported to occur in SCA3. Despite this, the subcellular localization of the human ataxin-3 protein seen in cell cultures was similar to that found in the SCA3 zebrafish themselves. The finding of similar protein localization and aggregation status in the neuronal cultures and corresponding transgenic zebrafish models confirms that this cell culture model is a useful tool for investigating the cell biology and proteinopathy signatures of mutant proteins for the study of neurodegenerative disease.

KEY WORDS: Primary neuronal cell culture, Transgenic zebrafish, Amyotrophic lateral sclerosis (ALS), Spinocerebellar ataxia type-3, Fused in sarcoma (FUS), Ataxin-3 (ATXN3)

INTRODUCTION

The zebrafish (Danio rerio) is increasingly used to successfully model neurodegenerative diseases (Babin et al., 2014; Bai and Burton, 2011; Bandmann and Burton, 2010; Bosco et al., 2010; Kabashi et al., 2010; Laird et al., 2016; Lemmens et al., 2007; McGown et al., 2013; Miller et al., 2005; Paquet et al., 2009; Ramesh et al., 2010) and holds promise for testing potential disease treatments (McGown et al., 2013; Schiffer et al., 2007). There are straightforward methods available for modulating gene expression in zebrafish (Don et al., 2017; Hruscha et al., 2013; Suster et al., 2009) and female zebrafish spawn large numbers of embryos making it possible to perform behavioral testing and drug study screens with relatively high throughput (Zon and Peterson, 2005). Many proteins associated with neurodegenerative disease in humans are homologous in zebrafish, highlighting potentially conserved molecular-cellular functions that can be readily investigated in the zebrafish model (Howe et al., 2013).

Zebrafish cells, including neural cells, can be cultured directly from developing embryos (Liu et al., 2010; Myhre and Pilgrim, 2010; Robles et al., 2011; Sakowski et al., 2012; Fassier et al., 2010; Ciarlo and Zon, 2016; Sassen et al., 2017). The potential of this method for investigating differentiated neurons has previously been achieved with later-stage embryos (>19 h post-fertilization (hpf)) (Sakowski et al., 2012). Here we focused on exploring the potential to study neurodegenerative diseases by applying and optimizing the technique using transgenic zebrafish expressing mutated forms of the proteins fused in sarcoma (FUS) and ataxin-3 to model amyotrophic lateral sclerosis (ALS) and spinocerebellar ataxia type-3 (SCA3), respectively. ALS is a fatal neurodegenerative disease that causes progressive paralysis due to loss of motor neurons within the brain and spinal cord. ALS can be caused by either non-inherited (sporadic) and/or inherited causes, with more than 25 different genes currently identified to be linked with the disease (Nguyen et al., 2018; Ling et al., 2013; Renton et al., 2014). One gene known to cause ALS is FUS (Vance et al., 2009). FUS is a ubiquitous, predominantly nuclear, multifunctional DNA- and RNA-binding protein (reviewed in Deng et al., 2014). More than 50 different FUS mutations have been discovered to cause familial ALS (fALS) (Vance et al., 2009; Deng et al., 2014; Kwiatkowski et al., 2009). SCA3 is a somewhat similar fatal neurodegenerative disease that results in gradual loss of control and coordination of muscles due to neuronal loss. The genetic cause of SCA3 is inheritance of an expanded CAG trinucleotide repeat region in the ATXN3 gene (Costa Mdo and Paulson, 2012). Abnormal CAG nucleotide repeat expansions (>40 repeats) result in an ataxin-3 protein with a long polyglutamine (polyQ) repeat region that has multiple potential toxic effects (Costa Mdo and Paulson, 2012). We have recently reported that zebrafish expressing ataxin-3 with an expanded polyQ tract harbor disease hallmarks such as ataxin-3 positive cleavage fragments and impaired movement at 6 days post-fertilization (dpf) (Watchon et al., 2017).

Both ALS and SCA3 are characterized by the mislocalization, accumulation and aggregation of the respective mutated proteins in neurons, accompanied by neuronal cell dysfunction and death (Rub et al., 2013; Saberi et al., 2015). In this study, cell cultures derived...
from transgenic zebrafish larvae allowed investigation of the subcellular localization of mutated human FUS and ataxin-3 and the presence or absence of protein inclusions in different cell types, including differentiated neurons. We confirmed that the subcellular localization of the disease-causing proteins were the same in the cell cultures as in the living transgenic zebrafish for both models of neurodegenerative disease. These neuronal cell cultures, obtained from transgenic zebrafish lines of neurodegenerative diseases, have potential for use in drug screening assays for effectors of protein aggregation and mislocalization. In combination with zebrafish behavioral and physiological analysis, this is an additional tool to investigate the functional effects of cellular pathology in neurodegeneration.

RESULTS
Optimization of zebrafish neural cell cultures
Cells harvested from transgenic zebrafish embryos expressing GFP in motor neurons under the islet1 promoter (islet1:GFP) were used to generate primary zebrafish cell cultures and optimize the growth of neurons. From this, we determined the percentage of cells that expressed GFP to give us an indication of the degree of motor neuron survival. Embryos up to 48 hpf were cultured with ease, whilst embryos up to 96 hpf required longer incubations in trypsin to achieve cell dissociation, which was detrimental to subsequent cell survival. For this reason, we predominantly worked with cultures from 24 hpf zebrafish embryos. Cell cultures derived from both 24 and 48 hpf embryos maintained motor neuron integrity, with GFP positive neurons representing 10-12% of the total cells in culture and exhibiting rapid neurite outgrowth after 1 day in vitro (div; Fig. 1A). There was no difference in the percentage of GFP expressing cells or cellular morphologies when comparing cells grown at 28°C (controlled temperature in captivity) and 37°C (standard mammalian cell culturing temperature) (Fig. 1B), suggesting that both temperatures are suitable for culturing zebrafish motor neurons. In an attempt to improve the cell dissociation step we tested the effect of de-yolking the embryos by microsuction prior to culturing (Sakowski et al., 2012). We found that absence of the yolk gave rise to motor neurons with shorter neurites and widespread cell death after 2 div (although the percentage of motor neurons after 1 div remained unchanged relative to cultures from embryos with intact yolks) (Fig. 1C). In cultures derived from larvae with intact yolk sacs, motor neurons were viable for up to 1 week. A schematic representation of the optimized workflow required to derive these cultures is summarized in Fig. 2.
Demonstration of mixed neural subtypes in culture
As well as islet1:GFP-positive motor neurons, a variety of other neuronal subtypes were also present in the cultures, demonstrated by immunolabeling with neural cell antibodies obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Anti-islet1/2 antibodies (39.4D5) labeled all GFP positive motor neurons as expected and additionally some GFP negative neurons presumably representing those expressing islet2 but not islet1 transcription factors (Fig. 3A). Some islet1 expressing cells and other cells were also labeled with by Zn12 antibodies against L2/HNK-1 carbohydrate epitope, a neural cell adhesion molecule expressed by a variety of different neural subtypes (Fig. 3B). In summary, a variety of motor neurons and other neural subtypes were evident in the mixed cultures.

Culturing cells from transgenic zebrafish expressing pathogenic human motor neuron disease associated proteins
We next cultured cells from transgenic zebrafish that expressed fluorescently tagged human neurodegenerative-disease related proteins (Fig. 4). In post-mortem tissue, cytosolic mislocalization and aggregation of FUS occurs in motor neurons (Kwiatkowski et al., 2009; Mackenzie et al., 2010). In our zebrafish cell cultures, fALS mutant human FUS (FUS-R521C) fused to GFP showed greater cytosolic distribution than wild-type human FUS (Fig. 4A). This is consistent with mislocalization seen in an FUS zebrafish model described previously (Acosta et al., 2014) and with other cell model studies (Dormann et al., 2010).

Cells were also cultured from a transgenic zebrafish model of SCA3 that express EGFP-fused to human ataxin-3 containing either a pathogenic (84Q) or non-pathogenic (23Q) polyglutamine tract. In the SCA3 zebrafish cell cultures, we did not detect any mislocalization or aggregation of EGFP-ataxin-3-84Q within the nucleus of neurons, a common phenotype reported in SCA3 patient brain and spinal cord samples (Rub et al., 2013). No major qualitative differences were present in the neurons from EGFP-ataxin-3-84Q zebrafish compared to non-pathogenic EGFP-ataxin-3-23Q zebrafish after 2 div (Fig. 4B). We did note some aggregation of the mCherry protein used as a neural cell reporter in our transgenic zebrafish, within cells cultured from both EGFP-ataxin-3-23Q and EGFP-ataxin-3-84Q zebrafish (Fig. 4B,C).

To confirm that the EGFP displayed in the cultured neural cells was indicative of the expression of EGFP-fused to human ataxin-3 we performed immunolabeling with a polyglutamine (polyQ) antibody (Fig. 4C). The polyQ staining pattern was found to be mostly cytoplasmic, and similar for the EGFP-ataxin-3-23Q and -84Q samples (Fig. 4C). This subcellular localization was consistent with that found in the live transgenic zebrafish expressing either EGFP-ataxin-3-23Q or -84Q at 3 dpf (Fig. 4D).

DISCUSSION
Protocols exist for culturing cells from dissociated zebrafish embryos (Liu et al., 2010; Myhre and Pilgrim, 2010; Robles et al., 2011; Sakowski et al., 2012; Sassen et al., 2017; Andersen, 2001). A previous study demonstrated that differentiated zebrafish motor neurons can be cultured and maintained from embryos older than 19 hpf, up to 96 hpf, demonstrating the potential for this technique to be used to investigate the development and cell biology of motor neurons in vitro (Sakowski et al., 2012; Sassen et al., 2017). We adapted this method and used it to culture neurons from transgenic zebrafish models of neurodegenerative diseases. We show that zebrafish motor neurons grow neurites, differentiate and can be maintained in culture at either 28°C or 37°C. We found that de-yolking embryos prior to dissociation was detrimental to the survival of motor neurons in the cultures and led to stunted neurite outgrowth. This suggested the importance of endogenous factors and nutrients found...
in the yolk for growth and sustenance of differentiating neurons and other cell types (Carvalho and Heisenberg, 2010). Despite this, recent work in primary cell culture of zebrafish embryos has been successful in culturing a variety of neuronal cell types without the preservation of the yolk sac (Sassen et al., 2017). In previous work, selection of spinal neurons amongst a heterogeneous mixture of cells was achieved by performing larvae spinal cord dissections or fluorescent activated cell sorting (FACS) purification of neurons (Sakowski et al., 2012; Sassen et al., 2017). However, optimization of this particular protocol is required due to high mortality rates (Sassen et al., 2017). Enhanced motor neuron purity could enable investigation of cell-autonomous factors that have been shown to be important for the degenerative mechanisms of other motor neuron disease-related proteins such as superoxide dismutase-1 (SOD1) (Yamanaka et al., 2008). On the other hand, co-culturing motor neurons together with other cell types is biologically relevant and advantageous to cell survival due to available trophic factors in culture (Sassen et al., 2017). Similar to previous studies related to culturing zebrafish cells (Sakowski et al., 2012; Sassen et al., 2017), passaging of cells was not attempted.

Cultures from human mutant FUS transgenic zebrafish demonstrate that mutant, but not wild-type, human FUS is ubiquitously mislocalized in zebrafish cells, consistent with previous results in whole mount zebrafish larvae and cell cultures (Bosco et al., 2010; Acosta et al., 2014) and in mammalian cell lines (Bosco et al., 2010; Gal et al., 2011). This model offers another tool for gathering insight into the degenerative mechanisms of other motor neuron disease-related proteins such as superoxide dismutase-1 (SOD1) (Yamanaka et al., 2008). On the other hand, co-culturing motor neurons together with other cell types is biologically relevant and advantageous to cell survival due to available trophic factors in culture (Sassen et al., 2017). Similar to previous studies related to culturing zebrafish cells (Sakowski et al., 2012; Sassen et al., 2017), passaging of cells was not attempted.

MATERIALS AND METHODS

Transgenic zebrafish

All experiments were carried out with approval from the University of Sydney Animal Ethics Committee (K00/3-2012/2/5709, K03/10-2010/3/5435 and K00/12-2010/3/5463) and Macquarie University (2016/04, 2015/034 and 2017/19). Transgenic zebrafish were bred on Tübingen/AB background and both male and female zebrafish were utilized. Transgenic zebrafish with GFP-expressing motor neurons driven by the islet1 promoter Tg(islet1:GFP;actb:GFP)x0Tg were described in (Higashijima et al., 2000). Transgenic zebrafish Tg(actb2:Gal4-FUS-GFP) and Tg(actb2:Gal4-VP16; mCherry) were used for co-expression experiments. The Tg(islet1:GFP) line and nearby islet1:GFP negative cells, are stained positively (red) for the neuronal marker Zn12, indicating the inclusion of other types of neurons in addition to motor neurons. Scale bar: 10 µm.

Fig. 3. Images of cultured 24 hpf Islet1:GFP zebrafish embryos stained with zebrafish-specific neuronal markers to confirm that the cell cultures contain various types of neurons. (A) An Islet1:GFP motor neuron within the cultures is positively stained (red) for the neuronal marker 39.4D5 (islet1 and islet2 homeobox). (B) Another Islet1:GFP motor neuron, and nearby islet1:GFP negative cells, are stained positively (red) for the neuronal cell surface marker Zn12, indicating the inclusion of other types of neurons in addition to motor neurons. Scale bar: 10 µm.
Fig. 4. Cultured cells derived from transgenic zebrafish larvae expressing neurodegenerative disease associated proteins FUS or ataxin-3. (A) In cells cultured from mutant human FUS-GFP (FUS-R521C) zebrafish the FUS-GFP protein was mislocalized to the cytosol, whereas it remained predominantly nuclear in cells cultured from wild-type FUS-GFP zebrafish. (B) Cells cultured from double transgenic zebrafish expressing mCherry (red) and EGFP-ataxin-3-23Q/84Q (green) showed no obvious difference in fluorescent protein distribution in cells expressing non-pathogenic EGFP-ataxin-3-23Q and pathogenic EGFP-ataxin-3-84Q. Aggregates of mCherry-positive protein (arrows) were present in some neurons (Jakobs et al., 2000). (C) Immunolabeling cell cultures with anti-polyQ (pale blue) demonstrated cytosolic distribution of the ataxin-3 protein in cells expressing either EGFP-ataxin-3-23Q or pathogenic EGFP-ataxin-3-84Q. Scale bars: 10 µm. (D) Cross-sections of the spinal cord of 3 dpf transgenic SCA3 zebrafish revealed a similar expression pattern of EGFP-ataxin-3 and mCherry to that seen in the cell cultures. Scale bars: 5 µm.
Primarily, fixation was achieved using 4% PFA in PBS and subjected to immunofluorescence with half of the media being replaced daily. For the experiments comparing the subcellular localization of the disease causing proteins FUS Is ubiquitously mislocalized and generates persistent stress granules in neurodegenerative diseases. Neurobiol. Dis. 40, 58-65.


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