Gene Expression Patterns 20 (2016) 106–110

Contents lists available at ScienceDirect

Gene Expression Patterns

journal homepage: http://www.elsevier.com/locate/gep

The zebrafish fast myosin light chain *mylpfa*:*H2B-GFP* transgene is a useful tool for *in vivo* imaging of myocyte fusion in the vertebrate embryo

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ARTICLE INFO

Article history:
Received 4 December 2015
Accepted 5 February 2016
Available online 10 February 2016

Keywords:
Zebrafish myocyte fusion
mylpfa:*H2B-GFP* transgene
Genetic screen
In vivo imaging

ABSTRACT

Background: Skeletal muscle fibers are multinucleated syncytia that arise from the fusion of mononucleated precursors, the myocytes, during embryonic development, muscle hypertrophy in post-embryonic growth and muscle regeneration after injury. Even though myocyte fusion is central to skeletal muscle differentiation, our current knowledge of the molecular mechanism of myocyte fusion in the vertebrates is rather limited. Previous work, from our group and others, has shown that the zebrafish embryo is a very useful model for investigating the cell biology and genetics of vertebrate myocyte fusion *in vivo*.

Results: Here, we report the generation of a stable transgenic zebrafish strain that expresses the Histone 2B-GFP (H2B-GFP) fusion protein in the nuclei of all fast-twitch muscle fibers under the control of the fast-twitch muscle-specific *myosin light chain*, *phosphorylatable*, *fast skeletal muscle a* (*mylpfa*) gene promoter. By introducing this transgene into a mutant for *junctional adhesion molecule 3b* (*jam3b*), which encodes a cell adhesion protein previously implicated in myocyte fusion, we demonstrate the feasibility of using this transgene for the analysis of myocyte fusion during the differentiation of the trunk musculature of the zebrafish embryo.

Conclusions: Since we know so little about the molecules regulating vertebrate myocyte fusion, we propose that the *mylpfa*:*H2B-GFP* transgene will be a very useful reporter for conducting forward and reverse genetic screens to identify new components regulating vertebrate myocyte fusion.

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1. Introduction

Skeletal muscle development progresses through a series of well-choreographed events. This begins with the specification of the precursor cells, the myoblasts, their proliferation and migration to appropriate sites of muscle formation and withdrawal from the cell cycle to form post-mitotic cells called myocytes. Following this, myocytes aggregate and fuse with each other to form syncytial structures – the myotubes. Finally, the myotubes switch on genes for muscle structural proteins, and then mature into fully differentiated muscle fibers (Bentzinger et al., 2012; Buckingham and Rigby, 2014). A key and intriguing step in this elaborate myogenic program is the fusion of the myocytes with each other to give rise to the syncytial myotube. Even though myocyte fusion has been observed and investigated for several decades using mammalian myoblasts in culture, it is through systematic genetic analysis of muscle development in *Drosophila* that a dedicated molecular pathway regulating the fusion process *in vivo* was first identified (Rochlin et al., 2010; Abmayr and Pavlath, 2012; Kim et al., 2015). In flies, myocyte fusion is initiated by a group of immunoglobulin (Ig) domain-containing type I transmembrane cell adhesion proteins, which serve to promote adhesiveness among the myocytes and between myocytes and developing myotubes. Downstream of these cell adhesion molecules, an elaborate set of actin cytoskeletal regulators have been implicated. Current view posits that these proteins function to modulate the structure of the cell surface actin network to bring the membranes of the fusing cells in close apposition (Haralalka and Abmayr, 2010).

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http://dx.doi.org/10.1016/j.gep.2016.02.001
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Evidence that the *Drosophila* fusion pathway has some bearing for vertebrate muscle development has come from studies of myocyte fusion in the zebrafish embryo. Here, two distinct kinds of muscle fibers differentiate within the myotome – the slow-twitch muscle precursors are fusion-incompetent, and differentiate as mononucleate fibers, while the fast-twitch muscles are derived from the fusion of many fast-twitch myocytes with each other (Roy et al., 2001). Zebrafish embryos deficient in Kirrel3l, a protein homologous to the Ig domain-containing proteins regulating *Drosophila* myocyte fusion, showed a significant reduction in fast myocyte fusion (Srinivas et al., 2007). Moreover, Dock1 and Dock5 – the homologs of Myoblast city, as well as the actin regulator Rac, all involved in *Drosophila* myocyte fusion, were also shown to be required for fast myocyte fusion in the zebrafish embryo (Moore et al., 2007; Srinivas et al., 2007). While all of these data construe a degree of evolutionary conservation in the molecular regulation of myocyte fusion, a number of recent studies, however, have begun to provide evidence for the involvement of unique molecules in vertebrate myocyte fusion that do not function in flies. Notable among these are a distinct family of Ig domain-containing proteins called the Junctional adhesion molecules (Jam) in the zebrafish (Powell and Wright, 2011), and Tmem8c or Myomaker, a multipass transmembrane protein (Millay et al., 2013; Landemaine et al., 2014; Luo et al., 2015).

The discovery of novel players like the Jams and Myomaker strongly suggests that many more components of the vertebrate myocyte fusion pathway remain to be identified. One can envisage several ways by which such components can be isolated. First, a search for proteins that interact with the known players such as Kirrel3l, Jams and Myomaker can lead us to additional components. Secondly, a candidate approach, for instance, by analyzing the functions of genes with restricted expression in skeletal muscle precursors, can also uncover new players in fusion, a strategy that was successfully used to identify Myomaker (Millay et al., 2013). Perhaps the most powerful approach, though, is forward genetic screen, as demonstrated by the success of this method in piecing together the myocyte fusion pathway in *Drosophila*. Among the vertebrates, the zebrafish embryo is particularly suitable for large-scale forward genetic screens. Although a large number of novel genes involved in a variety of developmental and physiological processes have been discovered using forward genetic screens in this organism, none of the screens have targeted myocyte fusion. Since skeletal muscle fibers are syncytial, to effectively screen for genes regulating myocyte fusion, it will be ideal to have a reporter that will label the nuclei of the fast-twitch fibers so that mutants affected in fusion can be readily scored and selected.

2. Results and discussion

A previous study had established that a 1.934 kb of promoter sequence of the zebrafish fast-twitch muscle-specific gene, *mylpfa*, is sufficient to specifically drive expression of the GFP reporter in the fast-twitch muscles of the developing zebrafish embryo (Ju et al., 2003). We reasoned that a transgene using this promoter to drive expression of a nuclear-localized reporter will be an ideal reagent for the study of myocyte fusion during muscle development. To this end, we constructed a transgene that contains the *mylpfa* promoter element and the cDNA encoding the H2B-GFP fusion protein (Fig. 1A). We injected this construct together with the *I-SceI* meganuclease into fertilized zebrafish eggs, and found nuclear-localized expression of the H2B-GFP reporter in the differentiating trunk muscles in approximately 90% of the F0 embryos that developed post-injection. We raised these embryos to adulthood, and then screened them for transmission of the transgene to the F1 generation. We obtained 3 F0 fish that transmitted the transgene to their progeny.

We fixed GFP-positive embryos from one of the lines that we established from the F1 progeny, and examined the expression of the H2B-GFP reporter at different stages of embryonic development by immunofluorescence (Fig. 1B–F). At all stages examined, we observed expression of the reporter in nuclei of multinucleated muscle fibers signifying that these are of the fast-twitch type (Fig. 1B and C). In older embryos, expression was also observed in the cranial muscles and muscles of the pectoral fins (Fig. 1D and E). To confirm that the transgene is indeed active exclusively in the fast muscle cells, we stained the transgenic embryos with antibodies that specifically label the slow- and fast-twitch muscles. Slow-twitch muscle cells are labeled with the antibody F59, which recognizes a slow-twitch muscle-specific myosin heavy chain, and there is little specific H2B-GFP expression in these F59-labeled muscle cells (Fig. 1F). This confirmed that expression of the *mylpfa*:*H2B-GFP* transgene is excluded from the slow-twitch muscles. On the other hand, labeling with the antibody F310, which recognizes a fast-twitch muscle-specific myosin light chain, showed a complete congruence in the labeling of myosin light chain in the cytoplasm of the syncytial fast-twitch muscle cells and H2B-GFP expression in the multiple nuclei (Fig. 1F). Thus, the *mylpfa*:*H2B-GFP* transgene is a faithful reporter of the fast-twitch muscle nuclei in the zebrafish embryo.

We next examined the efficacy of the transgene in allowing easy identification of zebrafish embryos compromised in fast myocyte fusion. For this, we introduced the *mylpfa*:*H2B-GFP* transgene into fish heterozygous for a mutation in *jam3b* (previously called *jamc*). It has been previously shown that in embryos homozygous for this mutation in *jam3b*, fast myocyte fusion is severely compromised (Powell and Wright, 2011). Instead of differentiating multinucleated fast-twitch muscle fibers, the mutant embryos make mononucleated fast-twitch muscles – the regular spaced-out pattern of multiple nuclei that is apparent in wild-type multinucleated fibers is replaced by a single nucleus positioned at the center of each fiber. By observing a clutch of live embryos derived from the mating of two heterozygous *jam3b* fish that also carried the *mylpfa*:*H2B-GFP* transgene under a fluorescence stereomicroscope, we were able to readily sort out the homozygous mutants from their non-mutant siblings based solely on nuclear positioning. In contrast to the linearly arranged patterns of multiple fast muscle nuclei visible in each somite of the sibling embryos, the mutants exhibited a characteristic chevron-shaped single file of nuclei at the center of each somite (Fig. 2A). We further confirmed the identity of the mutant embryos by high resolution confocal imaging on fixed specimens (Fig. 2B).

Taken together, all of these findings provide substantial evidence that the *mylpfa*:*H2B-GFP* transgene is a useful non-invasive reporter for the analysis of myocyte fusion in the zebrafish embryo *in vivo* and in real time. Perhaps the greatest utility of this reagent will be in forward genetic screens to isolate new genes regulating myocyte fusion. Since the field of vertebrate myocyte fusion has been so marginally explored, we believe that this strategy will enable the discovery of many novel players that will help to significantly extend our understanding of this important myogenic event. We envision that the strain will also be useful for reverse genetics-based interrogation of the genome for fusion molecules, for studying myocyte fusion during muscle repair and regeneration in response to injury, as well as for understanding the biology behind the stereotypical spacing pattern of nuclei within the syncytial fast-twitch fibers.

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