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In Vivo Visualization of the Development of the Enteric Nervous System Using a *Tg(-8.3bphox2b:Kaede)* Transgenic Zebrafish

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Abstract

The *phox2b* gene encodes a transcription factor that is expressed in the developing enteric nervous system (ENS). An enhancer element has been identified in the zebrafish *phox2b* locus that can drive tissue specific expression of reporter genes in enteric neuron precursor cells. We have generated a transgenic zebrafish line in which the Kaede fluorescent protein is under the control of this *phox2b* enhancer. This line has stable expression of the Kaede protein in enteric neuron precursor cells over 3 generations. To demonstrate the utility of this line we compared the migration and division rates of enteric neuron precursor cells in wild type and the zebrafish ENS mutant *lessen*.

Keywords

Enteric nervous system; *phox2b*; Kaede; in vivo; *med24*

The enteric nervous system (ENS) is the largest subdivision of the peripheral nervous system. Normal function of the ENS is essential to maintain proper function of the digestive system (Furness 2012). The development of a fully functional ENS requires enteric neuronal precursor cells (ENPCs), which are largely derived from the cranial neural crest, to migrate to and along the gut while proliferating appropriately. Any errors in this developmental process can lead to aganglionosis of the gut, a condition commonly seen in the human clinical condition Hirschsprung's disease (Young 2008, Obermayr et al. 2013).

While the general course of ENS development in many vertebrate species is known, better tools are needed to help understand all the intricacies of the process (Shepherd and Eisen 2011, Holmberg et al. 2004, Young 2008). Zebrafish are an ideal model system to develop these tools, given the ease with which one can image live embryos in vivo. Zebrafish embryos are externally fertilized, are transparent, and have a relatively fast generation time. Genetic tools are freely available that make it possible to generate transgenic animals in a time efficient and cost effective manner.

While green fluorescent protein is an excellent tool for visualizing developmental processes, alternative fluorescent proteins have shown great potential in unlocking new ways to observe biological phenomenon. One such protein, the Kaede protein, has the ability to shift its

emission spectra when exposed to UV light (Ando et al. 2002, Mizuno et al. 2003). Normally this protein fluoresces green, but when exposed to UV light the protein shifts to expressing red fluorescence (Ando et al. 2002, Mizuno et al. 2003). We have utilized this property of the Kaede protein, along with the live imaging benefits of the zebrafish, to develop a transgenic line to visualize development of the ENS in vivo.

To create this transgenic line we utilized the Tol2 transgenic system (Kawakami et al. 2000, Kawakami 2004, Kwan et al. 2007). In this system, plasmids are generated with two Tol2 transposition sites flanking the target sequence of DNA to be integrated. When this Tol2 construct is injected into one-cell embryos along with the TOL2 transposase mRNA, the target sequence gets efficiently integrated into the host's genome. To express the Kaede protein in the ENPCs we utilized a zebrafish ENPC specific enhancer. The transcription factor Phox2b has previously been shown to be an excellent marker for ENPCs in zebrafish and other vertebrate species (Elworthy et al. 2005, Pattyn et al. 1999). Subsequent studies have shown that specific regions of the zebrafish *phox2b* enhancer are sufficient to drive GFP expression in ENPCs (McGaughey et al. 2009, McGaughey et al. 2008). Utilizing the -8.3b*phox2b* enhancer element we generated a plasmid containing the Kaede protein under control of this enhancer flanked by Tol2 sites (Fig 1). Following injection into one-cell embryos, potential transgenics were isolated and raised to adult-hood. These adults were then outcrossed with wild type adults and their progeny were re-screened for Kaede expression. One male was identified and was further outcrossed to establish the line. These transgenic embryos show stable expression of the Kaede protein in the neural crest and the ENPCs with the ability to shift from red to green fluorescence after exposure to UV light (Fig 2).

To demonstrate the utility of this line in studies of ENS development we carried out live imaging experiments with the transgenic embryos. We had previously identified a zebrafish ENS mutant in the lab, named *lessen*, that has a significantly reduced number of enteric neurons (Pietsch et al. 2006). This phenotype is caused by a null mutation in the *med24* gene, a component of the mediator co-transcriptional activation complex (Pietsch et al. 2006). Subsequent experiments showed that *med24* translation blocking morpholino produced a similar ENS phenotype to that seen in *lessen* mutants (Pietsch et al. 2006). The ENS phenotype in the *lessen* mutant has been shown to be due to a decreased proliferation rate in the ENPCs (Pietsch et al. 2006). Utilizing the *Tg(-8.3bphox2b:Kaede)* transgenic line we were able to look more closely at the *med24* knockdown ENPC cellular phenotype in live imaged *med24* morphant embryos.

We examined *Tg(-8.3bphox2b:Kaede)* expression in uninjected control embryos and embryos that had been injected with the *med24* morpholino. We compared both the migration and division rates between the two sets of embryos during the period ENPCs migrate along the gut (36–60 hours post fertilization (hpf)) (Elworthy et al. 2005, Shepherd et al. 2004). We analyzed in detail a specific time period during this migration, comparing control and *med24* morphant embryos from 50–56hpf (Fig 3). From simple observation it is apparent that already at 50hpf ENPC migration in *med24* morpholino injected embryos lags behind control embryo ENPC migration and the rate of this lag continues to increase over

this time period. *med24* morphant embryos show a 37.3% (+/- 6.7%) reduction in migration rate as compared to age-matched controls (Fig 4).

From observation of our control and *med24* morphant embryos, we also see that there appears to be a decrease in division rate in the *med24* morphants and that more division occurs at the leading edge than elsewhere in the migrating chain (Fig 4). In photoconverted ENPCs, each cellular division decreases the amount of red photoconverted Kaede that is present in the cells. As these cells are dividing they are also continuing to make new green Kaede due to the continued expression of the *phox2b* enhancer. Cells that are dividing rapidly will have higher levels of green fluorescence and lower levels of red fluorescence than cells that are dividing more slowly. To quantitate this data, we analyzed the levels of green and red fluorescence in the ENPCs over time. If the *-8.3bphox2b* enhancer is equally active in control and *med24* morphants we would expect to see ENPCs in both gain green fluorescence back at the same rate after photoconversion. However, control embryos gain green fluorescence back faster than the *med24* morphants (Fig 5a). Some of this difference in the rate of change in color of the Kaede fluorescence could be due to higher *-8.3bphox2b* activity in our control embryos than compared to morphants. Alternatively, if the level of enhancer activity is the same in morphants and control embryos and the rate of ENPC cell division is less in *med24* embryos we would expect to see the amount of red fluorescence in ENPCs to decrease at a slower rate than in control embryos. We do indeed see a significant decrease in the rate of loss of red fluorescence in our *med24* morphants (Fig 5b). This result is further validated when the number of divisions per cell is calculated, as we see a drop in this rate of ENPC cell division in our *med24* morphants (Fig 5c). This cell division count also confirms that division rates in the first two cells of the migrating chain are higher than the general rate for all ENPCs (Fig 5c). This apparent proliferative migration driving the migration along the gut is consistent with our previous results and with experiments and observations in other species, as well as mathematical modeling of this developmental process (Barlow et al. 2008, Young et al. 2004).

In summary the *Tg(-8.3bphox2b:Kaede)* transgenic line provides a powerful tool for studying ENS development. The ability to observe ENS development in vivo as well as selectively label specific cells will allow for a wide range of applications. In addition to what we have highlighted here, there are other potential uses for this transgenic line (Sato et al. 2006, Pan et al. 2012). One such use is the ability to label individual ENPCs and perform lineage analyses to determine if specific ENPCs give rise to specific ENS neuronal lineages at different time points during ENS development.

Methods

Construct Generation

The *-8.3bphox2b* sequence was PCR amplified from genomic DNA and subcloned into the MCS of pBto1_ *cfos*EGFP, a modified PBluescript vector containing the human *cfos* promoter and tol2 transposition sites, using EcoR1. The Kaede sequence was PCR amplified from a PCS2 Kaede containing plasmid and subcloned into pGW_ *cfos*EGFP using BamH1 and Cla1 sites to attach SV40 poly A (Fisher et al. 2006b). Kaede SV40 was then subcloned

into pBtol_*cfos*EGFP-8.3*bphox2b* by excising the EGFP in pBtol_*cfos*EGFP with BamHI and XbaI.

Tg(-8.3*bphox2b*:Kaede) Construct Injections

Wild type embryos were collected and injected at the one cell embryo stage. We established the line by injecting 400 embryos. The injection solution was prepared with 25 ng/μl of *Tg(-8.3bphox2b:Kaede)* transposon construct, 35 ng/μl of transposase RNA, phenol red for visualization, and RNase-free water and kept on ice (Fisher et al. 2006a). 1 nl of solution was injected into one cell embryos and embryos were raised at 28.5° C.

Transgenic Screening and Raising

Injected *Tg(-8.3bphox2b:Kaede)* embryos were screened for integration of the targeting construct by looking for Kaede fluorescence protein expression at 48 hpf. 20 positive embryos were identified and raised to adulthood and outcrossed with wild type adults in the hope of generating germ line transmission of the transgene. 1 adult male with germ line transgenic integration was identified and isolated. This male was then outcrossed to establish the line.

Med24 Morpholino Injections

Tg(-8.3bphox2b:Kaede) transgenic adults were outcrossed with wild type adults and their embryos were injected at the one cell stage. One-cell embryos were injected with 1 ng of a *med24* morpholino at a concentration of 5ng/μl. The sequence for the translation blocking morpholino was +1/+25, CCTGTTTCAGATTCACCACCTTCAT (Pietsch et al. 2006).

Stop Motion Filming

Embryos were raised to the desired age at 28.5° C. *med24* morphant and control embryos were then embedded in 0.6% agar (ringer's solution) in 35 × 10 mm filming dishes (Falcon) and covered with embryo media and mesab. Embryos were then exposed to UV light (358nm) for ten minutes to convert the Kaede protein from fluorescing green to red. Z-stacked images were captured in SlidebookTM4.2 every fifteen minutes for 6–10 hours (Olympus IX81). Subsequent images and movies were analyzed using NIH image J software (1.46r) (Rasband 1997–2012). To calculate the migration rate 24 control embryos and 20 *med24* morphant embryos were analyzed. Migration distances were obtained using the particle tracker plug-in for Image J to follow the path of the leading edge cell of the migrating ENPC chain (Sbalzarini and Koumoutsakos 2005). To calculate fluorescence and division rates 64 starting ENPCs were analyzed. Fluorescence was measured by calculating fluorescence intensity of the individual cell over the cell area. The particle tracker plug in was again used to follow the paths of ENPCs and new appearances of cells from previous cells to calculate division rate (Sbalzarini and Koumoutsakos 2005). P-values were calculated with a t-test for division rate and within subjects ANOVA for all other cases.

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Figure 1.

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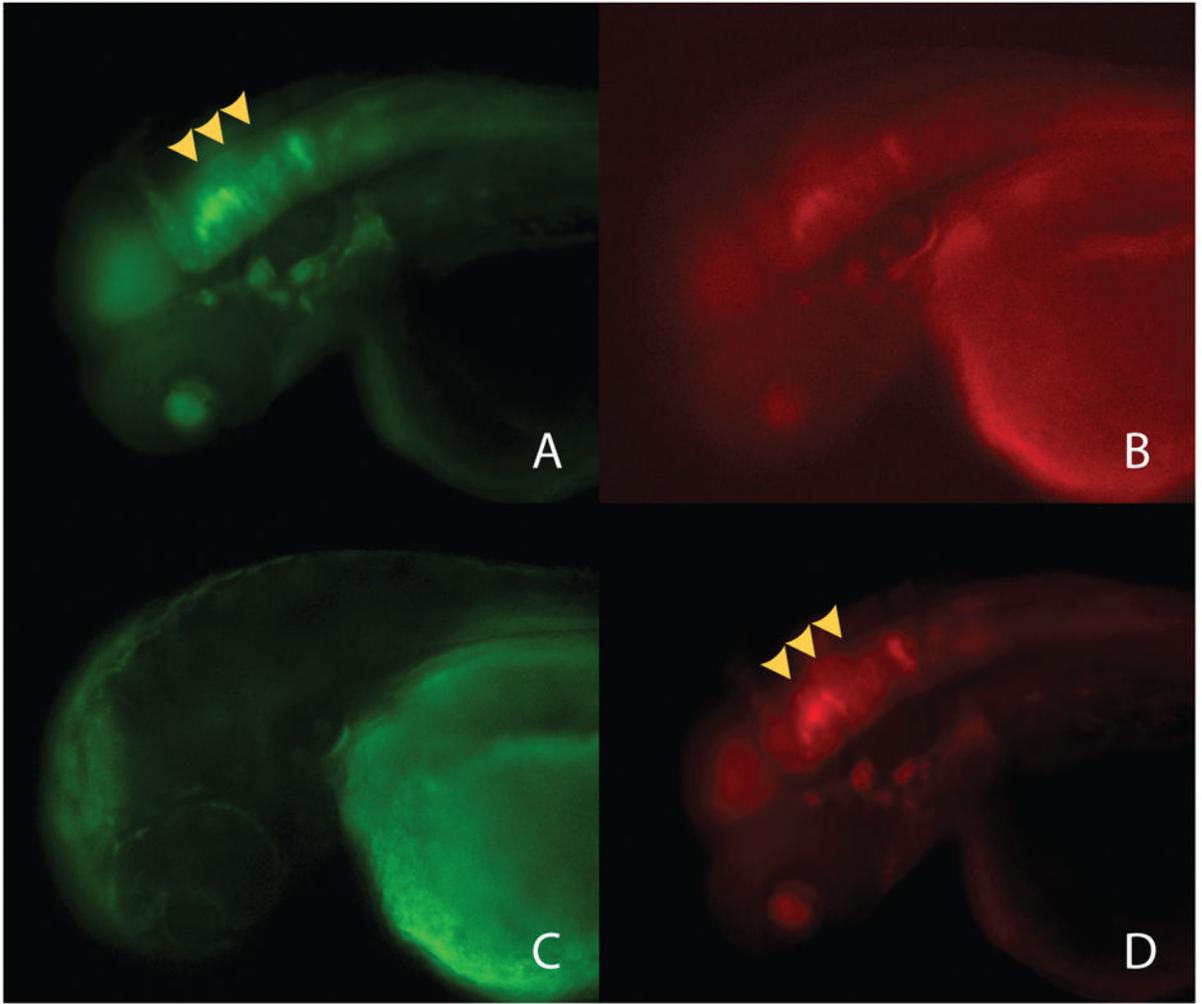


Figure 2.

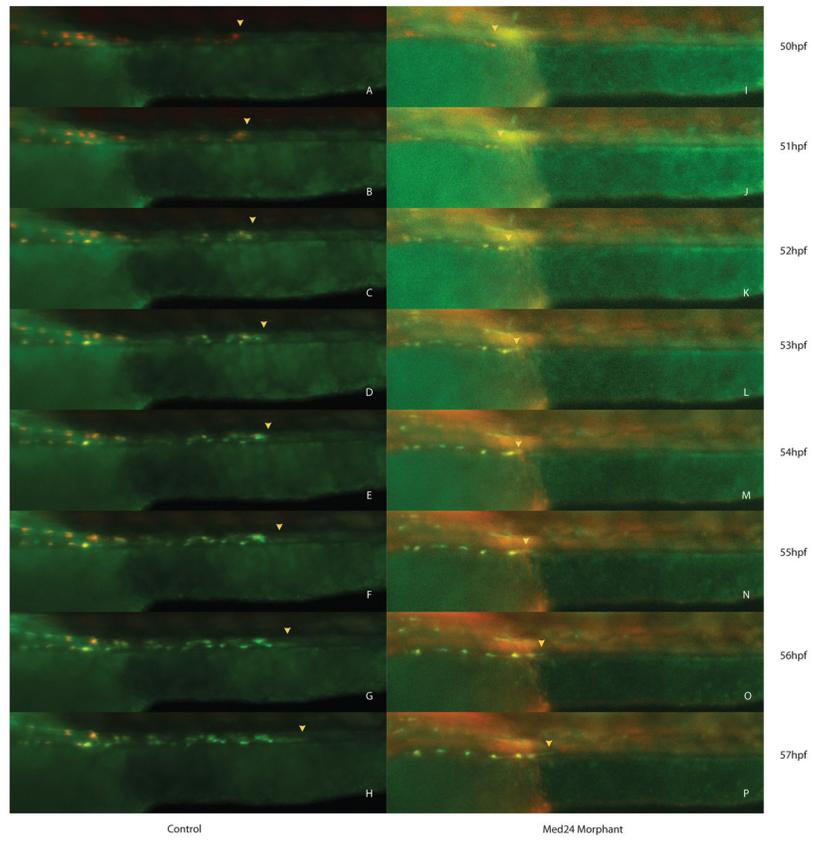


Figure 3.

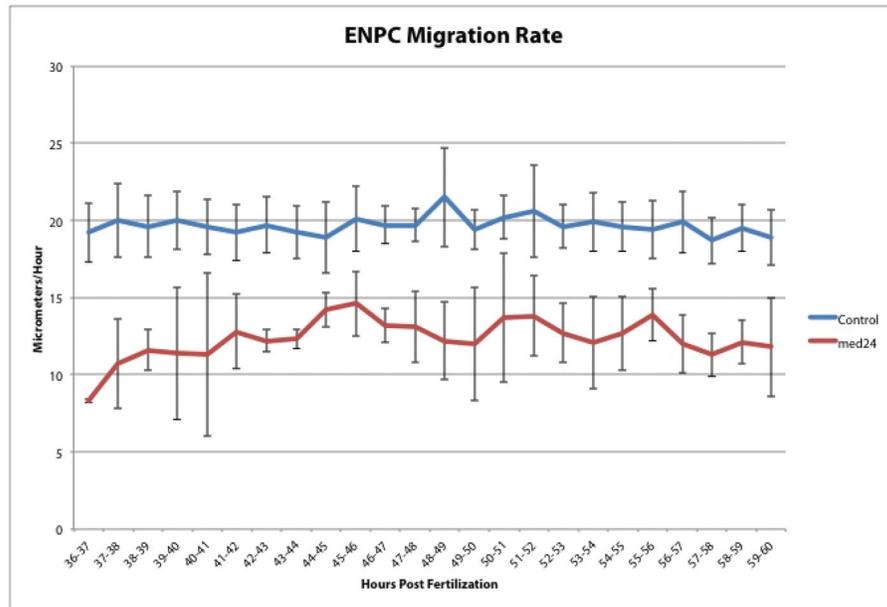


Figure 4.

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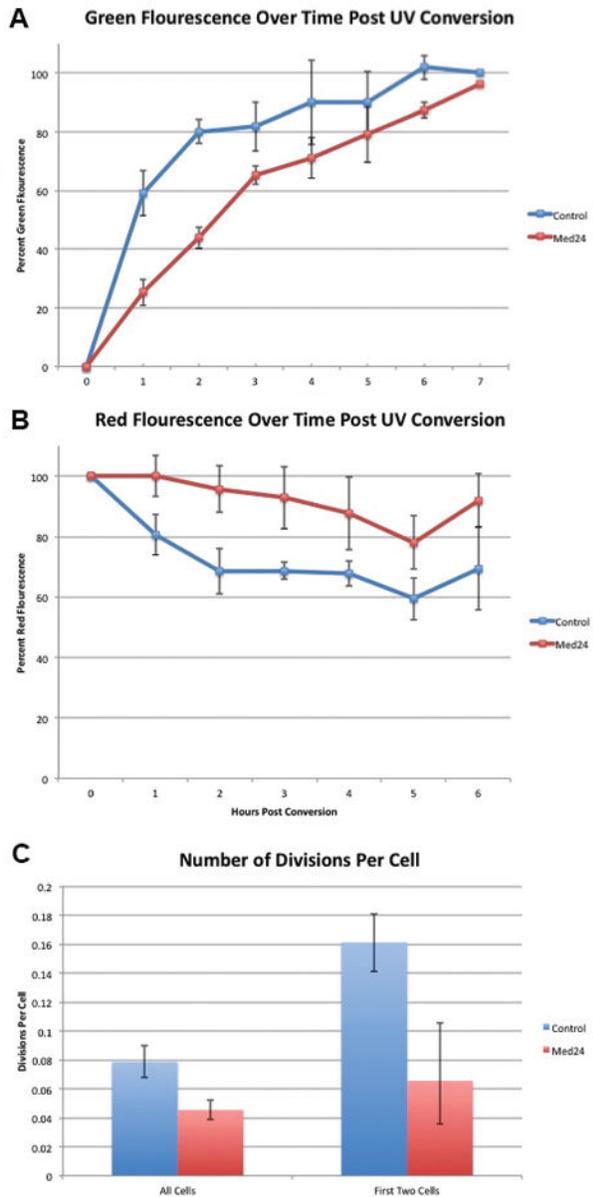


Figure 5.