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Differential contribution of direct-developing and stem cell-derived melanocytes to the zebrafish larval pigment pattern

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ABSTRACT

The extent of adult stem cell involvement in embryonic growth is often unclear, as reliable markers or assays for whether a cell is derived from an adult stem cell, such as the melanocyte stem cell (MSC), are typically not available. We have previously shown that two lineages of melanocytes can contribute to the larval zebrafish pigment pattern. The embryo first develops an ontogenetic pattern that is largely composed of ErbB-independent, direct-developing melanocytes. This population can be replaced during regeneration by an ErbB-dependent MSC-derived population following melanocyte ablation. In this study, we developed a melanocyte differentiation assay used together with drugs that ablate the MSC to investigate whether MSC-derived melanocytes contribute to the ontogenetic pattern. We found that essentially all melanocytes that develop before 3 dpf arise from the ErbB-independent, direct-developing population. Similarly, late-developing (after 3 dpf) melanocytes of the head are also ErbB independent. In contrast, the melanocytes that develop after 3 days postfertilization in the lateral and dorsal stripe are sensitive to ErbB inhibitor, indicating that they are derived from the MSC. We show that melanocyte regeneration mutants *kit*^{j1e99} and *skiv2l2*^{j24e1} that are grossly normal for the overall ontogenetic pattern also lack the MSC-derived contribution to the lateral stripe. This result suggests that the underlying regeneration defect of these mutations is a defect in MSC regulation. We suggest that the regulative functions of the MSC may serve quality control roles during larval development, in addition to its established roles in larval regeneration and growth and homeostasis in the adult.

Introduction

The life cycle of animals is marked by multiple developmental transitions. Such changes may be morphological, such as the wholesale and coordinated changes in form during insect or amphibian metamorphosis (Truman and Riddiford, 1999; Yaoita and Brown, 1990) or the onset of adult pigment pattern at the larva-to-adult transition in zebrafish (Johnson et al., 1995). Developmental transitions may also be physiological in nature, such has the developmental change in growth response of fins to nutritional insufficiency (Goldsmith et al., 2006). Yet additional developmental transitions may involve the origins of cells that contribute to tissues of the animal. For instance, during embryonic or fetal development of fish, amphibians or mammals, a direct developing (without a renewing stem cell precursor) blood lineage, referred to as primitive hematopoiesis, is replaced by definitive hematopoiesis, through the self-renewing hematopoietic stem cell (Bertrand et al., 2007; Davidson and Zon, 2004). Likewise, the stem cell-derived adult pigment pattern of zebrafish replaces the embryonic and larval pattern of melanocytes that is thought to develop largely without a self-renewing stem cell precursor (Budi et al., 2008; Hultman et al., 2009).

The zebrafish embryonic and larval pigment pattern is an attractive model for studying the relative roles of direct developing and stem cell-derived melanocytes (O'Reilly-Pol and Johnson, 2009). During early embryonic development, melanocytes or their precursors begin to migrate from the neural crest beginning at approximately 19 h (Kelsh et al., 2000), and are largely differentiated and in place by 72 h (Rawls and Johnson, 2003). When embryonic melanocytes are ablated with lasers (Yang et al., 2004) or chemicals (Yang and Johnson, 2006), they rapidly regenerate from undifferentiated precursors, referred to as melanocyte stem cells (MSCs). Although these MSCs have not yet been directly identified, their properties can be inferred from a variety of experiments. Thus, when embryos are mutant for the EGF-like receptor *erbb3b* or treated during early embryonic stages with the ErbB inhibitor AG1478, the ensuing embryonic pigment pattern is grossly normal (Budi et al., 2008; Hultman et al., 2009). However, when these ontogenetic melanocytes are subsequently chemically ablated, no melanocyte regeneration occurs (Hultman et al., 2009), suggesting that the ErbB inhibitor has ablated the stem cell that supports larval melanocyte regeneration. Similarly, new melanocytes fail to develop in these fish during pigment pattern metamorphosis at 14 dpf (Budi et al., 2008), strongly suggesting that larval regeneration melanocytes and metamorphosis melanocytes share a common, AG1478-sensitive, MSC. These results led to a model of two distinct types of lineages that can contribute to the larval melanocyte

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pattern: (1) direct-developing melanocytes that do not go through an AG1478-sensitive MSC intermediate, that are responsible for the ontogenetic pattern and (2) MSC-derived melanocytes that contribute melanocytes during regeneration (Hultman et al., 2009). In this study, we explore the notion that MSC-derived melanocytes also contribute to the ontogenetic pattern.

Although the embryonic pattern is relatively stable from 3 dpf to the onset of metamorphosis at 14 dpf, approximately 20 new melanocytes are known to appear after 3 dpf in the lateral stripe (Milos and Dingle, 1978a). This second wave of lateral stripe melanocyte differentiation contributes approximately 50% of the melanocytes in the lateral stripe at 8 dpf. Further analysis revealed that this second wave regulates and fills in the gaps either introduced by manipulation (Milos and Dingle, 1978b) or left by first wave melanocytes (Milos et al., 1983). This regulative behavior is similar to that observed during melanocyte regeneration where the additional melanocytes will reform the normal larval pattern (Yang et al., 2004).

The assays that revealed that AG1478 ablates MSCs responsible for larval melanocyte regeneration also led to the identification of melanocyte regeneration mutants. Recessive lethal mutants for *skiv2l2*^{j24e1} that encodes an RNA helicase and *gfpt1*^{j23e1} that encodes the enzyme for the first step in the hexosamine biosynthetic pathway develop grossly normal numbers of ontogenetic melanocytes but fail to regenerate them after chemical ablation (Yang et al., 2007). Similarly, mutants for the temperature-sensitive allele of the receptor tyrosine kinase *kit* (*kit*^{j1e99}) also develop grossly normal numbers of melanocytes when reared at the permissive temperature (Rawls and Johnson, 2003) but fail to regenerate them following ablation (Yang and Johnson, 2006; Yang et al., 2004). One model for these mutants is that they are specifically defective for regeneration mechanisms. Alternatively, these mutations may identify defects in regulation of the MSC lineage. These models might be distinguished from each other if we could identify contributions of the MSC lineage to ontogenetic melanocyte pattern.

In this study, we develop a melanocyte differentiation assay to investigate the relative contributions of melanocytes developing at different stages to different regions of the larval pigment pattern. We also examine the effects of ablating the AG1478-sensitive MSC on temporal and regional populations of larval melanocytes. We show that development of early differentiating melanocytes or melanocytes that develop prior to 3 dpf is not affected by MSC ablation. Similarly, melanocytes that develop after 3 dpf, which we hereafter refer to as late developing melanocytes (LDMs) in the head, are not affected by AG1478-mediated MSC ablation. In contrast, we show that LDMs in the lateral stripe fail to develop following MSC ablation with AG1478, indicating that LDMs of the lateral stripe are derived from the MSC. Examination of larval melanocyte regeneration mutants shows that *kit*^{j1e99} and *skiv2l2*^{j24e1} are also defective for lateral stripe LDMs, suggesting that their defects are in MSC regulation, rather than specific defects in regeneration.

Materials and methods

Animal care and stocks

Adult fish were maintained at 25–27 °C at 14L:10D light-to-dark light cycle using standard fish protocols (Westerfield, http://zfin.org/zf_info/zfbook/zfbk.html). Embryos and larval fish were reared at 28 °C in 2 ppt marine salt (Coral Life, Carson, CA) in carbon-filtered tap water except for *kit*^{j1e99} mutants, which were reared at 25 °C.

Spectrophotometric determination of melanin synthesis

Relative melanin content was determined with a modification of the method of Ozeki et al. (1995). For each treatment, 25 embryos were placed in 0.5 ml Soluene-350 (PerkinElmer) and heated at 95 °C for 1 h. After cooling and centrifugation, melanin content in supernatant was assayed by absorption at 500 nM on a Nanodrop spectrophotometer (Thermo Fisher Scientific Inc.).

PTU melanocyte differentiation assay

In order to determine when and where melanocytes develop, we took advantage of the property of phenylthiourea (PTU), also known as phenylthiocarbamide (Sigma, St. Louis) to inhibit the function of the melanin synthesizing enzyme tyrosinase (Rawls and Johnson, 2000). Melanin synthesis is inhibited within a few hours of addition of PTU (Fig. 1). Thus, melanin-positive melanocytes after the addition of PTU (0.1–0.2 mM) were differentiated prior to drug treatment. To reveal melanocytes that differentiated after PTU treatment, without visible melanin, we took advantage of a transgenic line *Tg(Tyrp1:GFP)*^{j900} expressing GFP under the control of the *Fugu rubripes* tyrosinase-related protein 1 promoter. This is a transgenic insertion generated in our laboratory from a transposon previously described by Zou et al. and is similar to the pt102 insertion allele described therein (Zou et al., 2006). In embryonic and larval stages, this line expresses GFP exclusively in differentiated (melanized melanocytes) or in differentiating melanocytes immediately prior to overt melanization. A small fraction of GFP+ cells are melanin- during melanocyte development (6/175 or 3.3% of GFP+ cells, $n=5$ embryos at 20 hpf). A smaller fraction of melanin+ cells are observed to be GFP- (1/181 melanin+ cells, or 0.5% of melanin+ cells, $n=5$ embryos at 20 hpf), indicating that GFP is typically expressed only slightly before the first signs of melanin synthesis in melanocytes. Thus, melanin+ melanocytes observed after the addition of PTU (0.1–0.2 mM) differentiated prior to PTU treatment. For our differentiation assay, we incubated larvae in PTU beginning at 3, 5 or 7 dpf and examined them for GFP+, melanin- melanocytes at 5, 7 and 10 dpf, respectively, using a Nikon SMZ1500 stereo-fluorescence microscope.

Photographic melanocyte differentiation assay

To determine late stage melanocyte development in animals not carrying *Tg(Tyrp1:GFP)*^{j900}, we photographed the right and left lateral stripe of individually segregated larvae at 3 dpf and at 6 dpf. The number of melanocytes that developed from 3 to 6 dpf was calculated

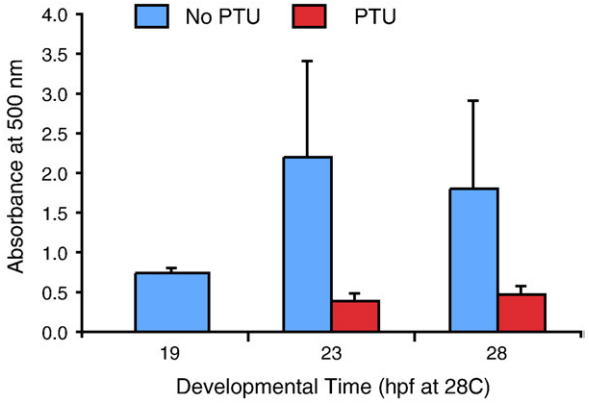

Fig. 1. PTU exposure blocks melanin synthesis within 4 h of treatment. Wild-type embryos were allowed to develop until first signs of melanization (19 hpf). At this time, 3 samples of 25 embryos each were anesthetized and solubilized in Soluene-350 for spectophotometric melanin determination (see Materials and methods). 0.2 mM PTU was then added to one-half the remaining embryos, and additional samples were prepared from PTU-treated and untreated controls at 23 and 28 hpf. Note that, while melanin absorption increased in untreated animals, it stayed the same or decreased in PTU-treated animals, suggesting that PTU blocks melanin synthesis within 4 h of application to embryos.