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ORIGINAL RESEARCH

EGFR/MAPK Signaling Regulates the Proliferation of *Drosophila* Renal and Nephric Stem Cells

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ABSTRACT

Tissue homeostasis, accomplished through the self-renewal and differentiation of resident stem cells, is critical for the maintenance of adult tissues throughout an animal's lifetime. Adult *Drosophila* Malpighian tubules (MTs or fly kidney) are maintained by renal and nephric stem cells (RNSCs) *via* self-renewing divisions, however, it is unclear how RNSC proliferation and differentiation are regulated. Here we show that EGFR/MAPK signaling is dispensable for RNSC maintenance, but required for RNSC proliferation *in vivo*. Inactivation of the EGFR/MAPK pathway blocks or greatly retards RNSC cell cycle progression; conversely, over-activation of EGFR/MAPK signaling results in RNSC over-proliferation and disrupts the normal differentiation of renablasts (RBs), the immediate daughters of RNSC divisions. Our data further suggest that EGFR/MAPK signaling functions independently of JAK/STAT signaling and that dMyc and CycE partially mediate EGFR/MAPK signaling in MTs. Together, our data suggest a principal role of EGFR/MAPK signaling in regulating RNSC proliferation, which may provide important clues for understanding mammalian kidney repair and regeneration following injury.

KEYWORDS: *Drosophila* Malpighian tubules; Renal and nephric stem cells; EGFR/MAPK signaling

INTRODUCTION

Stem cells are a group of cells with unique potential, which undergo asymmetric divisions to self-renew and produce differentiating daughters at the same time. The maintenance of adult tissue homeostasis, during which out-going differentiated cells are replenished by differentiating progeny of resident stem or progenitor cells, is essential for life. Growing evidence suggests that stem cell self-renewal and differentiation are controlled by both intrinsic factors and extrinsic signals (Morrison and Spradling, 2008). De-regulation of stem cell self-renewal *versus* differentiation could result in depletion or excessive proliferation of stem cells, which eventually leads to premature aging or cancer. Thus, understanding the molecular mechanisms governing stem cell self-renewal *versus* differentiation is crucial for the use of stem cells in regenerative medicine and cell therapy.

The function of excretory systems (such as kidney in vertebrates and Malpighian tubules (MTs) in *Drosophila*) is important for adult homeostasis by removing metabolic wastes, foreign toxins and maintaining ionic, acid/base and water balance (Dow and Davies, 2006). It is well known that the mammalian kidney has a great potential for tissue regeneration following an ischemic or toxic injury. Ischemic injury to the mammalian kidney causes acute renal failure, loss of tubular polarity, necrosis and cell death, followed by tubular

Abbreviations: EGFR, epidermal growth factor receptor; JAK/STAT, Janus kinase/signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; MTs, Malpighian tubules; RNSC, renal and nephric stem cell.

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regeneration and recovery of renal function (Anglani et al., 2008; Gupta and Rosenberg, 2008; Vaidya et al., 2008; Yokoo et al., 2008). Furthermore, many animal models provide evidence of regeneration of completely degenerated renal tissues after injury. These data strongly suggest the existence of adult kidney stem cells and that a stem cell-based system may function during the regeneration processes in vertebrates (Elger et al., 2003; Haller et al., 2005; Anglani et al., 2008). However, these hypotheses await further experimental supports.

Drosophila MTs show great similarities in development and function to the mammalian kidney (Dow and Davies, 2006; Singh et al., 2007; Singh and Hou, 2009). Even some of the pathways and molecules utilized during development are conserved in both systems (Ainsworth et al., 2000; Denholm et al., 2003; Jung et al., 2005). Adult *Drosophila* has two pairs of MTs, a longer anterior pair that runs through the hemolymph on both sides of the midgut and a shorter posterior pair that runs along the hindgut, converging through common ureters onto the alimentary canal at the midgut-hindgut junction (Sozen et al., 1997; Pugacheva and Mamon, 2003; Singh et al., 2007). Genetic mapping revealed that each tubule can be divided into four compartments with six regions including initial segment, transitional segment, main segment, lower tubules, upper ureter and lower ureter (Sozen et al., 1997). Following its formation during late embryogenesis *Drosophila* MTs are thought to be stably maintained throughout development. However, using lineage tracing and molecular marker labeling, it was shown that *Drosophila* MTs actually contain multipotent stem cells (known as renal and nephric stem cells, RNSCs), which are located at the lower tubule and ureter region and produce several differentiated cell types. Moreover, autocrine JAK/STAT signaling was shown to regulate RNSC self-renewal (Singh et al., 2007). Both RNSCs and RBs (the differentiating daughters of RNSC divisions) are small diploid cells expressing STAT92E, Armadillo (Arm, the fly β-Catenin) and DE-Cadherin (DE-Cad). However, it is likely that only RNSCs express Upd, a ligand of the JAK/STAT pathway, and undergo cell cycle progression (Singh et al., 2007). Whether RNSCs are regulated by additional signaling pathways remains elusive.

The epidermal growth factor receptor (EGFR) pathway is widely utilized during animal development and is involved in cell fate specification (Shilo, 2003). In *Drosophila*, the signaling cascade is initiated upon binding of ligand (Spitz, Gurken, Keren and Vein in *Drosophila*) to the receptor and acts through the canonical RAS/RAF/MEK/mitogen-activated protein kinase (MAPK) pathway and the ETS transcriptional activator, Pointed (Pnt), to regulate gene expression (Shilo, 2003). Mis-regulation of this pathway is often associated with developmental defects including a variety of cancer (Holbro and Hynes, 2004). Previous data indicate that this pathway is also required for the normal development of stem cells under physiological conditions (Aguirre et al., 2010). In *Drosophila* midgut, EGFR/MAPK signaling acts to maintain midgut homeostasis under physiological conditions and mediate regenerative response under stress conditions (Buchon et al., 2010; Jiang et al., 2010; Biteau and Jasper, 2011; Xu et al., 2011). Whether EGFR/MAKP signaling is required for RNSC proliferation is unknown.

Here, we show that EGFR/MAPK signaling functions in *Drosophila* MTs to maintain tissue homeostasis. Our data suggest that the EGFR/MAPK pathway is likely not essential for RNSC maintenance, but is critical for RNSC proliferation and RB differentiation. Our data indicate that its role in RNSCs is independent to that of JAK/STAT signaling and identify dMyc and CycE as two downstream mediators of EGFR/MAPK signaling. Given that the regulation of EGFR/MAPK signaling is evolutionarily conserved between flies and mammals, our study may shed light on the mechanisms underlining renal regeneration after ischemic injury in mammals.

RESULTS

EGFR/MAPK signaling is activated in RNSCs and RBs

We first addressed whether EGFR/MAPK pathway components are expressed in adult *Drosophila* MTs. In wild type (WT), RNSCs are small diploid cells located in lower tubules and ureters and undergo self-renewing divisions to generate a new-born RNSC daughter and an RB daughter, which differentiates directly into a renalcyte (RC) in the region of the lower tubules and ureters, or a type I or II cell in the region of upper tubules. Using an anti-EGFR antibody in immunofluorescence analysis, we showed that EGFR was expressed in MTs and interestingly only in the small cells located in low tubules and ureters, a region with high stem cell activity (Fig. 1A). To investigate whether these EGFR-expressing cells are RNSCs and/or RBs, we co-stained MTs with both anti-EGFR and anti-STAT92E antibodies. Our results showed that all EGFR-expressing cells were also STAT92E-positive, indicating an RNSC/RB fate (Fig. 1A). In *Drosophila*, the EGFR pathway can signal through the canonical RAS/RAF/MEK/MAPK pathway to regulate target gene expression *via* the Pointed (Pnt) transcriptional activator (Shilo, 2005). *Pnt-lacZ*, an enhancer trap line for Pnt transcription activation, is specifically expressed in the small cells located in the region of lower tubules and ureters which also express STAT92E (Fig. 1B). To further address whether the EGFR/MAPK pathway is activated in MTs, we used an antibody specific for the active, double phosphorylated form of ERK (pERK), representing signal activation *in vivo* in immunofluorescence analysis (Gabay et al., 1997). Indeed, the pERK positive cells were small cells situated in the region of lower tubules and ureters and also STAT92E-positive (Fig. 1C). In adult *Drosophila* midgut, the Notch pathway is activated and Delta (Dl), one of the Notch ligands in the fly, serves as an intestinal stem cell (ISC)-specific marker (Ohlstein and Spradling, 2007). Our recent data show that the Notch pathway is activated in adult MTs and Dl serves as an RNSC-specific marker (Li et al., 2014). Interestingly, pERK signal was highly detected in these Dl-positive cells (data not shown). Together, these data show that components of the EGFR/MAPK