Single-cell visualization of mir-9a and Senseless co-expression and larval peripheral nervous system development

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Abstract

The Drosophila melanogaster peripheral nervous system (PNS) comprises the sensory organs that allow the fly to detect environmental factors such as temperature and pressure. PNS development is a highly specified process where each sensilla originates from a single sensory organ precursor (SOP) cell. One of the major genetic orchestrators of PNS development is Senseless, which encodes a zinc finger transcription factor (Sens). Sens is both necessary and sufficient for SOP differentiation. Senseless expression and SOP number are regulated by the microRNA miR-9a. However, the reciprocal dynamics of Senseless and miR-9a are still obscure. By coupling single-molecule FISH with immunofluorescence, we are able to visualize transcription of the miR-9a locus and expression of Sens simultaneously. During embryogenesis, we show that the expression of miR-9a in SOP cells is rapidly lost as Senseless expression increases. However, this mutually exclusive expression pattern is not observed in the third instar imaginal wing disc, where some Senseless-expressing cells show active sites of mir-9a transcription. These data challenge and extend previous models of Senseless regulation and show complex co-expression dynamics between mir-9a and Senseless. The differences in this dynamic relationship between embryonic and larval PNS development suggest a possible switch in mir-9a function. Our work brings single-cell resolution to the understanding of dynamic regulation of PNS development by Senseless and miR-9a.

Keywords: microRNA; mir-9a; Senseless; peripheral nervous system; embryogenesis; wing disc

Introduction

One of the most impressive demonstrations of developmental robustness is the specification of the Drosophila melanogaster peripheral nervous system (PNS), which comprises all the organs that allow the fly to detect movement, pressure, temperature, and more. Drosophila sensilla number and position exhibit little or no variation from individual to individual, even in diverse environmental conditions. The early development step involves the selection and specification of sensory organ precursor (SOP) cells from a field of equipotent cells. During early embryogenesis (~5 h from fertilization), groups of epidermal cells start to express Achete–Scute complex genes. These proneural genes impart the potential to become neurons. As embryogenesis proceeds, these isolated sens-expressing SOPs ultimately give rise to the entire sensory organ. Sens expression becomes repressed around stage 13, when SOPs are fully specified. Since sens maintains proneural gene activation, loss-of-function sens mutant embryos exhibit a decreased number of SOPs, corresponding to a loss of sensory organs in the adult fly (Nolo et al. 2000). Gain of function mutations and ectopic expression of Sens cause an increased number of SOPs and consequently sensory organs (Jafar-Nejad et al. 2003; Li et al. 2006). Therefore, it is suggested that sens is necessary and sufficient for through subsequent changes in cell death and proliferation (Orgogozo et al. 2001; Orgogozo and Schweisguth 2004).

One of the major effects of PNS development is a gene named Senseless (sens) (Nolo et al. 2000). sens encodes a transcription factor (Sens) whose expression is initially activated and subsequently maintained by the proneural genes achete and scute (Jafar-Nejad et al. 2006). sens in turn maintains the expression of proneural genes to direct proper neuronal cell differentiation (Nolo et al. 2000; Acar et al. 2006). sens expression is first detectable during stage 10 of Drosophila embryogenesis, as isolated cells start to specify according to their SOP fate potential. As embryogenesis proceeds, these isolated sens-expressing SOPs ultimately give rise to the entire sensory organ. sens expression becomes repressed around stage 13, when SOPs are fully specified (Nolo et al. 2000). Since sens maintains proneural gene activation, loss-of-function sens mutant embryos exhibit a decreased number of SOPs, corresponding to a loss of sensory organs in the adult fly (Nolo et al. 2000). Gain of function mutations and ectopic expression of Sens cause an increased number of SOPs and consequently sensory organs (Jafar-Nejad et al. 2003; Li et al. 2006). Therefore, it is suggested that sens is necessary and sufficient for...
SOX differentiation (Nolo et al. 2000). The robustness and reproducibility of sensory organ development between individuals implicates senS as a keystone gene whose fine-scale regulation involves multiple feedback inputs.

Neurogenesis is extensively regulated by microRNAs (miRNAs) (Nolo et al. 2000; Hilgers et al. 2010; Caygill and Brand 2017). These small regulators of translation and mRNA stability contribute to the robustness of many biological processes. It has been shown that miR-263a/b stabilize sensory organ patterning in the retina by inhibiting sensory organ cell apoptosis (Hilgers et al. 2010), and that miR-7 stabilizes neuronal differentiation in the Drosophila larval brain by targeting the Notch pathway (Caygill and Brand 2017). In addition, Li et al. (2006) showed that miR-9a regulates Sens function through multiple target recognition sites in the sens 3′ UTR. When sens’ miR-9a binding sites are mutated, Sens levels are not only higher but more sensitive to temperature perturbations (Cassidy et al. 2013), resulting in an altered distribution of sensory organs in the wing margin (Cassidy et al. 2013; Giri et al. 2020).

Loss of function and overexpression of miR-9a produce opposite phenotypes with respect to sens in both embryos and larvae. Thus, the phenotypic consequences of miR-9a disruption mirror those of sens, suggesting that miR-9a is necessary to ensure appropriate Sens expression in the right cells and at the right level to convey robustness to SOP specification (Li et al. 2006).

The miR-9a miRNA is a member of one of the ~30–40 families that are predicted to pre-date the divergence of protostomes and deuterostomes, and therefore to be conserved in essentially all bilaterian animals (Wheeler et al. 2009; Ninova et al. 2014). In every animal where miR-9 family members have been studied functionally, they are found to regulate processes related to neurogenesis and neuronal progenitor proliferation (Sempere et al. 2004; Wheeler et al. 2009; Delaloy et al. 2010). For instance, overexpression of miR-9 in zebrafish embryo (Leucht et al. 2008), mouse embryonic cortex (Zhao et al. 2009), and chicken spinal cord (Otaegi et al. 2011) all lead to a reduction of the number of proliferating neural progenitors and impairment of PNS development. These studies demonstrate clear similarities between miR-9 expression and function in Drosophila and vertebrates. Disrupted miR-9 function has also been linked with some human pathologies, including cancer progression (Nowek et al. 2013) and neurodegenerative amyloid diseases (Packer et al. 2008). For instance, tumorigenic cells from medulloblastoma appear to have decreased expression of miR-9, while a subclass of glioblastoma tumor cells express miR-9 at a higher level (Ferretti et al. 2009; Kim et al. 2011). In addition, miR-9 has been also found to have a role as a proto-oncogene and/or as a tumor-suppressor gene during progression of cancers not directly related with the nervous system (Coolen et al. 2013).

The current model of miR-9a function in Drosophila SOP specification suggests mutually exclusive reciprocal expression of miR-9a and sens in SOPs (Li et al. 2006). This in turn suggests a role for miR-9a in ensuring that only one of the cells in the progenitor field takes on SOP identity. In this work, we use single-cell quantitative fluorescent in situ hybridization (FISH) and nascent transcript FISH to investigate the miR-9a/Sens/SOP regulatory model in hitherto unseen detail. This use of single-molecule FISH (smFISH) coupled with immunofluorescence (IF) allows us to simultaneously visualize active sites of miR-9a transcription and Sens protein in both embryos and larval wing disc at the single-cell level. We use these data to analyze the dynamics of miR-9a transcription and Sens protein abundance. We find that miR-9a and Sens are initially co-expressed but ultimately exhibit a dynamic reciprocal expression pattern. We observe that miR-9a transcription becomes rapidly repressed in high Sens-expressing SOPs during embryogenesis, presumably as Sens protein accumulates in the cell nucleus. A subtly different co-expression dynam was observed during wing disc development, where many SOPs also express miR-9a. These SOPs exhibit an inverse relationship between Sens abundance and miR-9a transcription. These new data refine and expand the previous model to provide key new insights into miR-9a/Sens regulation in PNS development (Li et al. 2006). In particular, we include for the first time a temporal element to the understanding of the dynamics of miR-9a regulation of SOP differentiation.

Methods

Fly stocks, embryo collection, and fixation and larval dissection

Flies were grown at 25 or 18°C. Embryos were collected after ~20 h and fixed in 1 V heptane + 1 4% formaldehyde for 30 min shaking at 220 rpm. The embryos were then washed and shaken vigorously for one minute in 100% methanol. Fixed embryos were stored in methanol at ~20°C. Larvae were dissected in 1× PBS, carcasses were fixed in 1 V 1× PBS + 1 10% formaldehyde for ~1 h, washed with methanol, and stored in methanol at ~20°C.

Genotypes used for this study are: W [1118], Bloomington stock 3605 and 2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG-Sens, VDRC stock ID 318017.

Probe design, smFISH, and IF

We adapted the inexpensive version (Tsanov et al. 2016) of the conventional smFISH protocol in Drosophila (Trecek et al. 2017). Primary probes were designed against the mature sens and sGFP mRNA and a genomic region flanking the miR-9a gene locus, all from FlyBase, using the Biosearch Technologies Stellaris probe Designer (version 4.2). To the 5′ end of each probe was added the Flap sequence CCTCCTAAGTTTGCGTGGACTACGTG. Multiple secondary probes that are complementary to the Flap sequence were tagged with fluorophores (CAL Fluor Orange 560, CAL Fluor Red 610, Quasar 670) to allow multiplexing. Antibodies used were Anti Green Fluorescent Protein rabbit IgG fraction (Invitrogen #A1122) at 1:500, anti-Sens (Nolo et al. 2000) at 1:1000, Goat anti-Guinea Pig IgG (H + L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 555 (Invitrogen #A21435) at 1:500, and Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488 (Invitrogen #A10080) at 1:500.

Imaging and quantification

Imaging was performed using a Leica SP8 Inverted Tandem Head confocal microscope with LAS X v3.5.1.18803 software (University of Manchester Bioimaging facility), using 20x, 40x, and 100x magnifications. Deconvolution was performed using Huygens Pro v16.05 software. Protein fluorescence levels were measured using Fiji for Macintosh. From each picture, five measurements of background mean intensity were taken. Each single-cell measurement was then adjusted using the formula: integrated density of nucleus – (area of nucleus × background mean).

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Supplementary material is available at G3.
Results

mir-9a is expressed in the dorsal ectoderm during embryogenesis and ubiquitously in the wing disc

In order to understand the interaction between mir-9a and its target Senseless (sens), we first systematically describe the mir-9a expression pattern in the embryo and imaginal disc at the single-cell level. Imaging mature miRNAs is difficult. Previously applied techniques require amplification and often have significant background noise problems (e.g. probes labeled with digoxigenin) (Biryukova et al. 2009). Many researchers have tried to overcome this (Lu and Tsourkas 2009), but these approaches still have very limited use for single-cell analysis and quantification.

We have used a nascent transcript approach using smFISH to detect expression in cells that are actively producing the miRNA primary transcript (pri-miRNA). To do so we have designed sets of ~48 probes (reported in supplementary material) that hybridize to a ~1-kb region in the primary miRNA transcript flanking the mir-9a locus. This allows the detection of active mir-9a transcription in the cell nuclei as previously described by Aboobaker et al. (2005). We find that expression of mir-9a initiates in early stage 5, at which point it is expressed throughout the dorsal ectoderm of the embryo (Figure 1, A and A'). The pattern displays a precise boundary between actively transcribing cells and nonexpressing cells, which correlates with the mesoderm–ectoderm boundary similar to that described by Fu et al. (2014). There are also small domains at the posterior and anterior embryonic ends lacking mir-9a expression. Later, during Drosophila embryonic stages 6 and 7, mir-9a expression is maintained in this pattern throughout the ectoderm (Figure 1, B, B’, C, and C’), clearly marking the boundary between ectodermal cells and invading mesodermal cells. At stage 8, the mesoderm is almost completely invaginated and the mir-9a expression domain covers the embryonic surface (Figure 1, D and D’), with the exclusion of the same regions at the anterior and posterior ends. At stages 11 and 14, mir-9a continues to be expressed throughout the ectoderm, except from a dorsal anterior region, and it is largely absent from the amnioserosa (Figure 1, E and F).

It has previously been reported that mir-9a is expressed widely in the third instar wing disc but not in cells expressing sens (Li et al. 2006; Biryukova et al. 2009). Similarly, we observed that mir-9a is actively transcribed throughout the wing disc (Figure 1G). We note that chromosomal pairing in imaginal tissue results in the two nascent transcription sites (TSs) merging into a single detectable spot (Figure 1G').

Immunodetection of Sens-sfGFP fusion protein allows the study of Sens expression in Drosophila embryos at single-cell resolution

The role of mir-9a in regulating the transcription factor sens is well characterized genetically during SOP specification (Li et al. 2006; Cassidy et al. 2013). To investigate the dynamics of mir-9a regulation of sens, we developed a strategy to simultaneously observe sens transcription and protein accumulation at the single-cell level via confocal fluorescent microscopy. In principle, the efficient detection of proteins in fixed samples using IF relies on the availability of antibodies that can specifically detect the protein of interest. Alternatively, Drosophila lines that express the protein of interest fused to a reporter protein can be detected enzymatically or by fluorescence (Timmons et al. 1997; Chatterjee and Bohmann 2012). We have therefore used a transgenic Drosophila reporter line that, in addition to the endogenous sens locus, carries two additional copies of a C-terminally tagged Sens-sfGFP reporter that can be detected either directly (live imaging of GFP fluorescence) or by IF using either anti-GFP or anti-Sens antibodies (Sarov et al. 2016). We were therefore able to use two methods in order to examine Sens dynamics: direct detection of Sens using antibodies against the endogenous protein, and indirectly using anti-GFP antibodies. To validate that the reporter accurately reflects endogenous Sens protein pattern and level we performed a double staining experiment against endogenous Sens using an anti-Sens antibody (obtained from H. Bellen lab; Nolo et al. 2000), and an anti-sfGFP antibody, in both embryos and wing discs (Figure 2). Under these conditions, we expect that anti-Sens antibodies will detect protein products derived from all four sens loci (the two endogenous loci and the two sfGFP-tagged reporter loci inserted at the VK00033 landing site on the third chromosome), while anti-sfGFP antibodies will detect only proteins from the two sfGFP-tagged loci.

The co-localization of the WT and sfGFP-tagged signals during embryogenesis and wing disc development are shown in Figure 2. The data clearly show that during both embryogenesis (Figure 2, A–D) and wing disc development (Figure 2, E–H), the two signals co-localize in the same cells. In the wing disc, we also noticed a small number of cells with clear presence of Sens protein but reduced detection of sfGFP. This occurs particularly in cells near the intersection of the dorsal–ventral and anterior–posterior boundary. It is likely that detection of Sens is more robust in this dynamic region as Sens protein is produced from four loci. Given the minimal differences between Sens and sfGFP detection, we are confident that the reporter accurately reflects endogenous Sens expression patterns during embryonic and wing imaginal disc development. We further measured the relative fluorescence from each of the two antibodies in single cells to ensure that the reporter gene reflects Sens abundance. As expected, the sfGFP reporter is expressed proportionally to sens in both embryos (Figure 2D) and wing discs (Figure 2I). Taken together, these observations indicate that the fluorescence signal from antibodies against sfGFP provides reliable information on both endogenous Sens localization and relative expression levels.

mir-9a expression pattern and Senseless protein levels are inversely correlated during embryogenesis

In order to study the reciprocal dynamics of mir-9a and sens during embryogenesis and wing disc development, we simultaneously tracked active sites of mir-9a transcription using smFISH and Sens abundance via IF. We investigated these patterns at three different stages of embryonic development: stages 10–12 (Figure 3, A–C) after early sens expression and the initial stages of SOP specification. Interestingly, we found that mir-9a transcription levels were inversely correlated with Sens protein levels, and that mir-9a transcription is repressed rapidly after the initiation of Sens expression. Intriguingly, we noticed that a very small number of Sens-expressing cells also displayed active mir-9a transcription (Figure 3, A–C'). Moreover, when we look at the fluorescence levels of sfGFP and the size of mir-9a TSs in the subset of cells that express both, it is evident that both miRNA active transcription and Sens level are lower in comparison to the rest of the cells. We believe that sens has just started to be translated in these cells and mir-9a transcription is stopped. We also noticed that while cells transcribing mir-9a are mostly located on the most superficial cellular layers, Sens-expressing cells are located inwards, as reported in the orthogonal projections and fluorescence intensity measurements in Figure 3, D–F and G–I, respectively.
The interpretation of these results is that the accumulation of Sens in the nucleus is associated with repression of mir-9a transcription, either by direct repression, or through an intermediary negative regulator.

To further investigate the dynamic relationship between expression of mir-9a and Sens, we developed a multichannel experiment to simultaneously study the expression pattern of mir-9a TSs and Sens-sfGFP with sens and sfGFP mRNAs (probes reported in supplementary material) (Figure 4, for panels A and C split channels are reported in Supplementary Figure S1 for detail). We observe that cells transcribing both mir-9a and sens have not yet accumulated significant quantities of Sens protein and are usually located in...
superficial layers, while cells with high Sens levels are usually not transcribing \textit{mir-9a} and are located inwards (Figure 4, B and C). At this stage, Sens-expressing cells occupy several embryonic cellular layers ranging from the superficial to deeper layers. Using an orthogonal projection to clearly distinguish these embryonic cell layers, we can see that the cells containing Sens protein are located inwards, whereas cells that are transcribing both \textit{mir-9a} and \textit{sens} mRNA, but that have not accumulated Sens protein, are usually localized on the embryonic surface (Figure 4D). For a clearer visualization, we also plotted embryonic depth against average fluorescence intensity of the four channels (Figure 4E). We believe that the rapid dynamic changes in \textit{mir-9a} expression are correlated with SOP differentiation, during which SOPs progressively migrate inwards as Sens protein accumulates and \textit{mir-9a} is turned off.

\textbf{mir-9a is actively transcribed in cells containing Sens during early SO specification in the third instar imaginal wing disc}

Since \textit{sens} regulates SOP differentiation during PNS development in the \textit{Drosophila} larvae (Singhania and Grueber 2014), we applied the approach outlined above to study \textit{mir-9a} expression pattern and Sens abundance in third instar imaginal wing discs at the single-cell level. The adult \textit{Drosophila} wing possesses a spatially organized series of bristles (a class of SO) located at the wing margin (Hartenstein and Posakony 1989). Flies in which Sens is ectopically expressed in the wing disc exhibit an increased bristle number in that wing region (Jafar-Nejad et al. 2003). During larval development, \textit{sens} starts to be expressed at around 15 h after...
third instar ecdysis in single SOPs in the wing notum. At around 30 h, sens is expressed in an increased number of isolated SOPs in the wing and notum area plus in two distinct stripes of cells in the wing disc pouch (Mirth et al. 2009). Cells belonging to these two rows of Sens-expressing cells will give rise to the adult wing margin bristles (Jafar-Nejad et al. 2006).

By nascent transcript smFISH, we observed that mir-9a is expressed in nearly all cells in the wing disc. When we correlated...
mir-9a expression with that of Sens in third instar discs we identified a small population of Sens-expressing cells with no mir-9a expression. These cells always had high levels of Sens protein, similar to our observations in the embryo. We also observed that many cells that have low or intermediate Sens protein levels are actively transcribing mir-9a (Figure 5, A and B). It is interesting to note that mir-9a TSs size in Sens-expressing cells do not differ from the size of TSs belonging to cells that are not expressing Sens protein. This indicates that these cells may not shut down mir-9a transcription, which might be kept active or modulated via transcriptional bursting. Nonetheless, at this stage only a minority of cells that contain Sens protein are not transcribing mir-9a.
We therefore measured the intensity coming from sfGFP antibody (a proxy for Sens protein levels) at the single-cell level to see if there was a difference in Sens levels between mir-9a-expressing and nonexpressing cells. The data clearly show that Sens is more abundant in cells that are not transcribing mir-9a (Figure 5C). However, our finding of concurrent expression of Sens and mir-9a contradicts aspects of the previously established model of triple row bristle specification (Li et al. 2006), which suggested a binary co-expression pattern.

Discussion

The stable and reproducible development of the Drosophila PNS is an extraordinary model of how stereotyped stability of cellular differentiation is achieved (Jan and Jan 1994; Barad et al. 2011). In this study, we focused on the role of Drosophila mir-9a in regulating the function of Senseless (sens), a crucial transcription factor that orchestrates SOP differentiation and PNS development in embryos and larvae. Dysregulated mir-9a expression results in disrupted sens function leading to altered SOP differentiation and loss of stable stereotyped neural development (Li et al. 2006; Cassidy et al. 2013). It has been hypothesized that Notch signaling plays a key role in regulating mir-9a transcription in epithelial cells adjacent to potential SOPs thus preventing accumulation of Sens and unintended differentiation of additional SOPs (Li et al. 2006). Despite extensive study of sens expression (Nolo et al. 2000; Mirth et al. 2009), there is little information regarding the developmental profile of sens and mir-9a co-expression.

During embryonic development, we show that mir-9a is initially expressed throughout the neurogenic ectoderm, and a mutually exclusive expression pattern with sens is established. Our single-cell analysis shows that cells just initiating sens expression, who therefore have not accumulated measurable Sens protein, actively transcribe mir-9a. However once Sens protein levels increase, mir-9a transcription is lost. The data suggest that mir-9a expression is turned off when the level of Sens protein reaches a specific threshold. The intriguing possibility that the regulation may be direct cannot yet be confirmed. We do not observe any evidence that mir-9a expression is reinitiated during SOP cell lineage differentiation in the daughter cell and subsequently repressed as Sens levels rise. The co-expression dynamics are only observed in the selection of the initial SOP cell and, without subsequent repression by mir-9a, Sens protein accumulates rapidly (Figure 6, A and B). sfGFP staining also revealed the presence of Sens-expressing cells in different cell cycle stages (Figure 3C), as previously reported for the differentiating R8 photoreceptor cells in the eye imaginal disc (Firth and Baker 2005; Ruggiero et al. 2012). We therefore suggest that mir-9a repression of Sens protein accumulation initially plays a regulatory role to buffer SOP specification and ultimately to stabilize the geometrical pattern of differentiating R8 cells. This negative feedback loop involving Sens and mir-9a may be key in the regulative establishment of the SOP pattern. It is currently unclear if sens directly switches off mir-9a transcription or if it is an indirect effect of a multilevel genetic cascade.

Li et al. presented evidence that in the third instar wing imaginal disc cells that express Sens do not express mir-9a, which is otherwise widely expressed throughout the disc (Li et al. 2006). We find that mir-9a and Sens are often though not always co-expressed in the wing disc. More specifically, we find that among the Sens-expressing cells, those that are also transcribing mir-9a present a lower level of nuclear Sens, similar to that seen fleetingly prior to the establishment of the terminal and mutually

Figure 5 in the wing disc, Sens-expressing cells are generally actively transcribing mir-9a. (A, B) Third instar larval transgenic wing disc stained with probes against pri-mir-9a and antibody against sfGFP. Many cells that are actively transcribing mir-9a are also expressing Sens. (C) Adjusted sfGFP intensity coming from Sens-expressing cells that are and are not actively transcribing mir-9a. Different colors represent data coming from different disks (n = 4). Data from each replicate were normalized using the maximum adjusted fluorescence value from the group mir-9a off from that replicate. Scalebars: (A) 100 µm and (B) 10 µm.
exclusive pattern of SOP specification in the early embryo. The main difference here is that this subset of cells in the wing disc does not seem to be stopping mir-9a expression as it was happening in the embryo. This suggests that mir-9a and sens have an intricate reciprocal dynamic expression during embryogenesis and larval development.

Our findings complement the model (Figure 6, D and E) presented by Li et al. (2006) and show that mir-9a and sens exhibit dynamic co-expression rather than a binary one. Our findings are also in concordance with the suggestion by Jafar-Nejad (2006) that the genes that orchestrate PNS development in embryos and larvae might be different, even though the process follows a similar pattern. For instance, during embryogenesis achete and scute are necessary for sens activation, while during larval development this function is accomplished by Wingless (Jafar-Nejad et al. 2006). miR-9a function might therefore differ between embryonic and larval SOP development via the presence or absence of other miR-9a targets. We propose that mir-9a repression during embryogenesis allows sens to reach a specific threshold in order to establish the correct number and pattern of embryonic SOPs. During larval development, sens might be expressed at different levels depending on the subclass of SO and this in part involves regulatory feedback by miR-9a. The fly wing margin possesses two different kinds of SO, the mechanoreceptors and chemoreceptors, and these have a very precise pattern (Hartenstein and Posakony 1989; Raad et al. 2016). mir-9a expression may be involved in a regulatory loop with sens to set different Sens levels and thereby control the kind of SO that will develop from sens-expressing cells that are not transcribing mir-9a and will adopt chemoreceptor SOP fate: chemoreceptors are lower in number than mechanoreceptors and their localization and alternation resembles the pattern of cells with high Sens expression level. Therefore, we believe that miR-9a serves to keep Sens expression low in mechanoreceptor precursor cells to ensure they adopt the correct SOP cell fate. Temporally, our model suggests that mir-9a is initially expressed in all sens-expressing cells, delaying the adoption of a specific SOP fate (Figure 6, F and G). As SOPs adopt their specific cell fate, mir-9a is switched off as a consequence of the transition from a multipotent precursor to a determined cell. Recent work on the evolution of sensory organ identity suggests that the gene network underlying specification is labile and complex (Klann et al. 2020). Future investigation to understand the possible role of mir-9a and Sens levels as upstream regulators of the expression of sensory organ identity genes may provide insight into this hypothesis.

Our work suggests that mir-9a has a dynamic role in the specification of SOP differentiation, tuning sens expression to ensure that the correct number of cells adopt the appropriate SOP fate. The molecular mechanism that dictates mir-9a transcription during PNS development remains unknown; its elucidation is

Figure 6 Model of mir-9a and sens interaction during embryogenesis and wing disc development. (A) In the embryo, sens is expressed in clusters of cells. (B) The orthogonal view of one of these clusters shows that mir-9a is transcribed in cells at the top, some of which are turning sens transcription on. As sens mRNA gets translated, these cells stop transcribing mir-9a and move inwards. (C) In the Drosophila wing disc, sens is expressed in two rows of cells (plus additional isolated cells, not shown). (D, E) In a static model, mir-9a and sens are not co-expressed. (F, G) Dynamic model in which all the cells that contain Sens are transcribing mir-9a, which is then turned off in a specific class of SOPs.
important for complete understanding of this dynamic process. A fundamental question that needs to be answered is the mechanism by which the mutual regulation of mir-9a and sens act to establish the observed co-expression dynamic, switching from mutually exclusive to co-expressed depending on the fly developmental stage. This study demonstrates the importance of examining miRNA regulation and miRNA-target gene expression dynamics at a single-cell level in the developing organism. We suggest that these dynamic co-regulatory processes are a general feature of miRNA function during development.

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