Making a mes: A transcription factor-microRNA pair governs the size of the midbrain and the dopaminergic progenitor pool

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Canonical Wnt signaling is critical for midbrain dopaminergic progenitor specification, proliferation, and neurogenesis. Yet mechanisms that control Wnt signaling remain to be fully elucidated. Wnt1 is a key ligand in the embryonic midbrain, and directs proliferation, survival, specification and neurogenesis. In a recent study, we reveal that the transcription factor Lmx1b promotes Wnt1/Wnt signaling, and dopaminergic progenitor expansion, consistent with earlier studies. Additionally, Lmx1b drives expression of a non-coding RNA called Rmst, which harbors miR135a2 in its last intron. miR135a2 in turn targets Lmx1b as well as several Wnt pathway targets. Conditional overexpression of miR135a2 in the midbrain, particularly during an early time, results in a decreased dopaminergic progenitor pool, and less dopaminergic neurons, consistent with decreased Wnt signaling. We propose a model in which Lmx1b and miR135a2 influence levels of Wnt1 and Wnt signaling, and expansion of the dopaminergic progenitor pool. Further loss of function experiments and biochemical validation of targets will be critical to verify this model. Wnt agonists have recently been utilized for programming stem cells toward a dopaminergic fate in vitro, highlighting the importance of agents that modulate the Wnt pathway.

Keywords: midbrain, dopamine neurons, microRNA, Wnt signaling, Lmx1b

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revealed that mir135a2 is expressed in the ventral midbrain in the mDA progenitor pool. It is also expressed in the Wnt-rich isthmus and roof plate. This was confirmed by analysis of embryos harboring sensor transgenes, in which a ubiquitous eGFP reporter containing miR binding sites in the 3’UTR, was specifically downregulated in miR135a2 rich regions. Thus, in the 11.5 dpc embryo miR135a2 is enriched in Wnt-rich regions of the midbrain, suggesting a role in modulating this critical signalling pathway.

Through bioinformatic analysis and RT-PCR, we revealed that miR135a2 was embedded in and co-expressed with the long non-coding RNA, Rmst. Previously, miR135a2 was thought to be intergenic, but a separate screen for ventrally expressed microRNAs suggested that miR135a2 was actually located between 2 exons of an uncharacterized gene. Thus, we performed bioinformatic analysis to identify genes near the miR135a2 locus. We found miR135a2 to be in close proximity to the 3’ end of a non-coding RNA, Rmst, and hypothesized that Rmst might have longer variants that encompass the miR135a2 locus. Therefore, we performed RT-PCR on 11.5 dpc ventral midbrain RNA using a forward primer in a known Rmst exon and a reverse primer in the predicted exon downstream of miR135a2. Our experiment revealed multiple bands, likely indicating splice variants, of which the most prominent was sequenced. We used a BLAST search to determine that this RNA was a fragment of the Rmst transcript that contained 3 previously unknown exons and that miR135a2 was located in the final intron.

miR135a2 and Rmst are dynamically expressed in a manner initially similar to both Wnt1 and Lmx1b, a key midbrain transcription factor.15,19,20 These genes are initially (~8.0 dpc) broadly expressed, but are quickly restricted (~9.0 dpc) to the fp (traditionally defined by the expression of Foxa2, Shh), roof plate, and isthmus. Within the fp, they are mainly expressed in the mDA progenitor domain (defined by the transcription factors Lmx1a/b). At 11.5 dpc, in addition to these regions, miR135a2/Rmst transcript can also be detected in cells exiting from the ventricular zone, throughout the midbrain. Within the mDA progenitors, Wnt1 and Lmx1b are downregulated over time, and the miR135a2/Rmst transcript is maintained. By the end of the mDA neurogenic interval (~14.5 dpc) the miR135a2/Rmst transcript becomes mutually exclusive from Wnt1 and Lmx1b, although not from Lmx1a. This is consistent with a phenomenon referred to as temporal exclusion, in which microRNAs are often initially coexpressed with targets, but ultimately become exclusive.21 Collectively, these expression studies open the possibility that miR135a2/ Rmst may be involved in fine-tuning or downregulating the expression of Lmx1b and Wnt1 in the early midbrain, and later within the mDA progenitor pool. In accordance with this notion, bioinformatic analysis predicted various components of the Wnt signalling pathway (including upstream transcription factors, ligands, positive and negative modulators) and Lmx1b to be targets of miR135a2. TGFβ/BMP pathway genes are also frequently predicted, but few genes in the hedgehog pathway were predicted targets of this miR. Luciferase assays in heterologous cells revealed that miR135a2 is indeed sufficient to repress constructs containing critical components of the canonical Wnt cascade (Ccdn1, Gsk3b, Tcf7l2 and Lmx1b).15 Another key Wnt pathway gene, APC, is also a target of miR135a2. In contrast Lmx1a was not a predicted target of this miRNA, consistent with the lack of temporal exclusion.

Since microRNAs often act in synchrony with transcription factors that can drive or repress the expression of the microRNA,23 we tested the hierarchical relationship between miR135a2 and Lmx1b. Through conditional gain- and loss-of-function studies in mice, we found that Lmx1b drives Rmst/miR135a2 expression.15 Moreover, we confirmed and expanded upon previous reports to show that Lmx1b also promotes Wnt1, and Wnt signaling in the midbrain.24-27 Thus, our data suggest a novel auto-regulatory negative feedback loop, in which Lmx1b directly or indirectly via Wnt signaling, drives miR135a2/Rmst and miR135a2 inhibits Lmx1b to modulate levels of Wnt1/Wnt signaling in the midbrain (Fig. 1). It is likely that Lmx1a will also have the ability to drive miR135a2/Rmst expression, akin to its redundancy with Lmx1b in driving Wnt1 transcription.28

We next explored the role of Lmx1b in midbrain development by gain and loss of function experiments.15 Briefly, forced expression of Lmx1b throughout the midbrain (En1::Cre;Lmx1bOE), led to an overall increase in the size of the midbrain, with the ventral Foxa2+/Shh+ domain disproportionately enlarged. In controls the Foxa2 domain is normally divided into a medial Lmx1a domain, thought to generate mainly mDA neurons, and a lateral Nkx6.1 domain, thought to generate mainly Brn3a+ neurons destined for the Red Nucleus; in mutants the Lmx1a domain is expanded and partially encroaches into the Nkx6.1 domain. Consequently, we observed increased numbers of mDA neurons. Conversely, conditional loss of Lmx1b from the midbrain (En1:: Cre;Lmx1bKO), led to a reduction in midbrain size, a diminished Foxa2 domain, a compressed Lmx1a+ mDA progenitor pool and drastically depleted TH+ neuron numbers, as previously reported in Lmx1b null mutants.24,32 Oculomotor neurons, a nearby neuronal

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Example Figure 1. Model showing the potential role of Lmx1b and miR135a2 in modulating the Wnt pathway and influencing midbrain size, mDA progenitor specification and neurogenesis. The transcription factor Lmx1b, directly or indirectly, drives Wnt1/Wnt signaling and the Rmst/miR135a2 transcriptional unit. miR135a2, on the other hand, represses Lmx1b and Wnt pathway targets, among other factors. Thus, the levels of this transcription factor-miRNA pair influence the net levels of Wnt1/Wnt signaling during midbrain development, impacting overall size, patterning and expansion of the mDA progenitor pool, at least in part via modulation of downstream transcription factors like Lmx1a.

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and arguing against non-specific toxic time sensitive window for miRNA function progenitors was observed, suggesting a body in which most progenitors are aimed to determine the role of miRNAs severe.15 We developed an embryoid Lmx1bcKOs except that the account for the phenotypes observed. By several criteria examined, the transgene could be modestly overexpressed (~3 fold) upon Cre-mediated recombination. When activated with En1::Cre throughout the early midbrain (~8.0 dpc), increased miR135a2 led to reduction in the size of the Lmx1b/Wnt1 domain. The strength of Wnt signaling, measured by the domain of Axin2 (from an Axin2::d2eGFP transgene)33 was also reduced, albeit less severely than in En1::Cre;Lmx1bcKO,Axin2::d2eGFP embryos.15 Consistent with a reduction in mDA progenitor domain size, less mDA neurons were generated. Interestingly, the adjacent Nkx6.1 domain and its Bm3a descendants remained unaffected, highlighting the specificity of this phenotype. Oculomotor neuron numbers were also reduced, albeit not as drastically as in the En1::Cre;Lmx1bcKO,Axin2::d2eGFP embryos. In these mutants the isthmus was modestly affected, thus opening the possibility that isthmic defects could in part account for the phenotypes observed. When the same transgene was activated with Nev::Cre (11.0 dpc) or Shh::Cre (8.5-9.5 dpc), no effect on mDA progenitors was observed, suggesting a time sensitive window for miRNA function and arguing against non-specific toxic effects of miRNA overexpression.15 Interestingly, this temporal sensitivity has also been observed for Shh::Cre;Lmx1bcKOs.27 By several criteria examined, the miR135-a2OE mutants resembled the Lmx1bcKOs, except that the Lmx1bcKOs were more severe. In a separate series of experiments, we aimed to determine the role of miRNAs in establishment of the mDA progenitor pool in an in vitro ES cell differentiation paradigm.15 We developed an embryoid body in which most progenitors are Foxa2+ and Foxa2-t, and of these approximately half are Lmx1a/b+ whereas half are Nkx6.1+. Conditional loss of Dicer from embryoid bodies induced to express typical markers of mDA precursors, resulted in increased Lmx1a/b+ cells (mDA markers) at the expense of Nkx6.1+ cells (a marker of red nucleus progenitors). Although this experiment did not specifically assess loss of miR135a2 function, this data indicated that microRNAs could be involved in progenitor cell allocation in Foxa2+ progenitors, and that loss of microRNAs expands mDA progenitors. When considered together with the in vivo data, we postulated that miRs, including miR135a2, are likely involved in determining the size of the mDA progenitor pool.

Thus, our study provides an example of a transcription factor-microRNA regulatory loop that plays a broad role in midbrain development. Since each microRNA may regulate hundreds of mRNAs, it is likely that through modest changes in a large number of target genes, the balance of miR135a2 and Lmx1b significantly impacts the net output of the powerful Wnt signaling pathway. Our data are consistent with the notion that coordinated microRNA and transcriptional regulation enhances the robustness of gene regulation. Along these lines, a recent theoretical analysis of gene expression at the midbrain-hindbrain boundary proposed that a combination of transcription factor and microRNA regulation aids in sharpening the expression of Wnt1 at the isthmus. We expect more examples of this paradigm will emerge in other aspects of CNS development as well. However, the findings described in our recent study are only an initial characterization of the function of the Lmx1b-Rmst/miR135a2 pair. A number of our results lead to natural follow up questions, and here we will discuss some of the most critical for future experiments.

As mentioned above, our data revealed that miR135a2 is embedded within the long non-coding RNA, Rmst, but additional experiments are needed to determine how these 2 RNA species are regulated and if they are functionally linked. We showed that the 2 are coexpressed in both wild-type and mutant scenarios, indicating that, like more than 50% of microRNAs, miR135a2 could be processed from the Rmst transcript. In this way, it is conceivable that the 2 RNA species share similar functions or that the long non-coding RNA serves merely as a vehicle for miR135a2 expression. Alternatively, consistent with the findings of a recent study in which the levels of Rmst had no effect on the levels of miR135a2 in human stem cells, the 2 could be regulated through distinct mechanisms. In this case, a separate internal promoter might exist for miR135a2. Once it has been determined whether a single or multiple promoters exist, it will be important to elucidate which factors directly regulate the transcript(s). Our data indicate that Lmx1b is upstream of miR135a2/Rmst, as well as Wnt1/Wnt signaling. Thus, miR135a2/Rmst expression could be regulated directly by Lmx1b, through Wnt signaling, or a combination of the 2. Moreover, the closely related Lmx1a, which is expressed in mDA progenitors and known to be both up and downstream of Wnt1, could also play a role in regulating miR135a2/Rmst expression.

Our interpretation of our data set, points to a model in which Lmx1b activates Wnt1 and miR135a2/Rmst, and then miR135a2 represses among others, Lmx1b and several genes in the Wnt cascade (Fig. 1). To add to this data set, in vivo loss of function studies of miR135a2 are crucial. These studies are currently underway. However, since single microRNA knockouts often have subtle or no phenotypes, compound knockouts of miR135a2 and the closely related miR135a1 or miR135b, or even other unrelated miRNAs also expressed in the midbrain and proposed to target Wnt1, such as miR705 and miR709, will likely be required to reveal how microRNAs influence Wnt1/Wnt signaling and midbrain development. These loss of function tools will also facilitate an in depth analysis of miR135a2 direct targets, which is crucial for understanding how a miR targeting both positive and negative elements of the Wnt pathway, may elicit its effect. Determining the role of Rmst and miR135a2 in other contexts is also important. For instance, our whole mount in situ data showed that in addition to being expressed in the midbrain, miR135a2/ Rmst extends into the dorsal telencephalon.
and the hindbrain. Thus, miR135a2/Rmst expression and function should be further characterized in these regions. Moreover, in addition to demonstrating the early role of miR135a2 in fine-tuning Lmx1b/Wnt1/Wnt signaling for proper mDA progenitor domain allocation, our study pointed toward a possible later role in ultimately downregulating Lmx1b/Wnt1 within mDA progenitors. The timing of Lmx1b/Wnt1 downregulation correlates with the end of the mDA neurogenetic interval, which budding studies have revealed to be between ~10.5–14.5 dpc. During this window, a number of factors, including Wnts, could influence whether progenitors continue to proliferate, or exit the cell cycle to become post-mitotic neurons. Thus, via regulation of Wnt1/Wnt signaling, miR135a2 and possibly even the host non-coding RNA, Rmst, may influence the timing of neurogenesis. Interestingly, a separate study in mice [9] has pointed toward a possible later role in ultimately downregulating Wnt agonists during a critical window in the differentiation protocol, human embryonic or induced pluripotent stem cells can be efficiently programmed to produce large numbers of bona fide mDAs that survive grafting. Thus, understanding the agents that modulate Wnt1/Wnt signaling in vivo will aid in the refinement of such rationally designed protocols to produce the most authentic neurons for treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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