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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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Wnts are a family of highly conserved secreted molecules that act in a dose-dependent manner to initiate signaling pathways in target cells.[1] Previous studies have revealed extensive roles for Wnts during the development of various neural systems, including the midbrain.[2,3] They contribute to overall expansion and patterning of this region, as well as specification and neurogenesis of the ventrally located midbrain dopaminergic neurons (mDAs). Recent work from our lab and others has highlighted the importance of the dosage of canonical Wnt/β-catenin signaling in the ventral midbrain[4-8]; too much or too little is detrimental for mDA production, and thus, it must be tightly regulated. Thus, better understanding the modulators of this powerful signaling pathway is of critical importance.

Of all the Wnt ligands in the midbrain, Wnt1 has been best defined and perhaps has the most significant role in instructing the canonical Wnt signaling pathway, although other Wnts may supplement this function.[9-14] The expression of *Wnt1* is dynamic, occurring in 2 phases. In an early phase at ~8.0 dpc, *Wnt1* is expressed in a prominent transverse band in the presumptive midbrain region.[15-17] *Wnt1::Cre* fate maps confirm that this transverse band is the source of the entire midbrain. Subsequently, by ~9.5 dpc, pan-midbrain expression is downregulated and expression is restricted to the isthmus, roof plate, and floor plate (fp).[15,17,18] Within the fp, *Wnt1* expression is robust, but between 11.5 and 14.5 dpc, *Wnt1* expression is downregulated.[15] In a recent study, we used gain and loss of function experiments to determine mechanisms that regulate *Wnt1*/Wnt signaling in the early embryonic midbrain. Our data point to a model in which the transcription factor Lmx1b promotes *Wnt1*/Wnt signaling, whereas *miR135a2* may serve to limit this pathway (**Fig. 1**).[15]

Our study uncovered *mir135a2* during a screen for robustly expressed microRNAs in the Wnt-rich midbrain. Locked nucleic acid *in situ* hybridization analysis

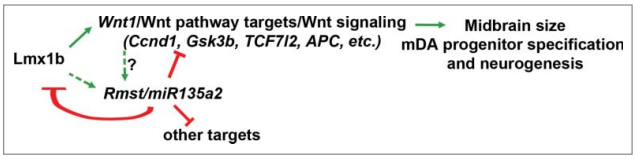

Figure 1. Model showing the potential role of Lmx1b and *miR135a2* in modulating the Wnt pathway and influencing midbrain size, mDA progenitor specification and neurogenesis. The transcription factor Lmx1b, directly or indirectly, drives *Wnt1*/Wnt signaling and the *Rmst/miR135a2* transcriptional unit. *miR135a2,* on the other hand, represses *Lmx1b* and Wnt pathway targets, among other factors. Thus, the levels of this transcription factor-microRNA 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.

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 signaling 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 neurogenetic 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 signaling pathway (including upstream transcription factors, ligands, positive and negative modulators) and *Lmx1b* to be targets of *miR135a2*. TGFb/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 (*Ccnd1, Gsk3b, Tcf7l2*) and *Lmx1b*.[15] Another key Wnt pathway gene, *APC*, is also a target of *miR135a2*.[22] In contrast *Lmx1a* was not a predicted targeted 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*;[29,30]) 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;Lmx1bcKO*;[29,31]), 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

population, were also reduced as previously reported.[15,24] Isthmic integrity and FGF expression were drastically affected in this mutant and are likely to contribute significantly to the overall phenotype. Altogether, these data indicate that *Lmx1b*, likely in part via Wnt signaling, is a critical determinant of midbrain size, fp size, mDA progenitor domain size, and mDA neuron numbers.[15]

To begin to elucidate the role of *miR135a2* in midbrain development, we next generated transgenic mice, in which *miR135a2* 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 Brn3a 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* 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 *Nes::Cre* ([34,35]~11.0 dpc) or *Shh::Cre* ([36,37]; ~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 *miR135a2OE* 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 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.[38] 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.[39] 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 co-expressed in both wild-type and mutant scenarios, indicating that, like more than 50% of microRNAs,[40] *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,[41] 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**).[15] 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,[42] 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*,[39] 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 birthdating studies have revealed to be between ~10.5–14.5 dpc.[43,44] 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 human neural stem cells has found that *Rmst* interacts with Sox2, and this binding is necessary for the coregulation of a large set of downstream neurogenic transcription factors.[41] It is conceivable that the 2 regulatory RNAs, *miR135a2* and *Rmst*, generated from the same locus could have multiple functions in regulating neurogenesis, and further studies to uncover the underlying mechanisms will be important.

Our data indicate that *miR135a2/Rmst* may be present, albeit at lower levels, in postmitotic mDA neurons.[15,19] In the case of *Rmst*, Uhde et al. suggest that it is expressed in a subset of mDA neurons in the developing VTA. Further work will be required to define their expression in postnatal midbrain, particularly in the context of our recent classification of mDA subtypes.[45] It is likely that microRNAs will also be differentially expressed by mDA neurons as has been shown for other microRNAs including miR133b.[46,47] Lmx1b is also expressed in post-mitotic mDAs, and it is conceivable that the precise balance of this transcription factor-microRNA pair also governs aspects of mDA neuron maintenance and function. In this regard, a recent study revealed a role for the miR135 family in modulating key serotonin neuron functions.[48]

Finally, given the function of these molecules in regulating the size of the dopaminergic progenitor pool, this study could be of importance in developing cell replacement therapies for Parkinson's disease. It has recently been reported that by using 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.[49-51] 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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