D. DISCUSSION

D.1. Post-transcriptional regulation of miRNA expression during neural development

Post-transcriptional mechanisms, such as alternative mRNA splicing, mRNA trafficking and translational control, are believed to play an important role in the regulation of gene expression (reviewed in [160-163]). It is becoming increasingly clear that miRNAs are part of a novel regulatory pathway of similar importance. In plants, miRNAs function in a number of essential developmental pathways, including floral and leaf morphology (reviewed in [113]). In *C. elegans* and *D. melanogaster* specific miRNAs regulate genes that control critical cellular functions such as terminal differentiation [15,24,115,164] apoptosis [117,118], proliferation [117], and cellular polarity [138]. Specific functions in neural development have been shown for *lin-4* and *let-7* in the control of *hunchback* [22,23], and for *lsy-6* and *mir-273* in sensory neuron specification [59,116]. There is less direct evidence for a developmental role in mammals. Mutations in or near miRNA genes are frequently associated with human malignancies [44,51,165] In haematopoietic differentiation, miRNA profiling revealed lineage specific expression of three miRNAs. Ectopic expression of one, *mir-181*, led to expansion of the B cell lineage [55].

D.1.1. Temporal miRNA expression during neural development. Lineage specificity

Accumulating evidence from diverse experimental sources increasingly supports an important role for miRNAs in the developmental control of the vertebrate nervous system. Several groups have cloned numerous miRNAs from neural tissue and neural cell lines [28,30,33], and a recent study verified expression of 86 distinct miRNAs in rat cortical neurons [37]. Four computational studies have screened databases of 3'UTRs for miRNA targets [107-109,166], the study by Enright et al. noted a large number of potential target genes associated with neural development and connectivity. A role for miRNAs in the developmental control of the CNS was also supported by microarray expression analysis [167,168], *in situ* hybridization [169] and phenotypic characterization of mutants in miRNA biogenesis [120].

How miRNA expression is regulated is not well understood. For *lin-4* and *let-7*, the timing of miRNA induction is critical for driving temporal patterns of cell specification (reviewed in [170,171]). In *C. elegans*, *let-7* controls the developmental timing of progenitor cell maturation (reviewed in [11,170]), a finding that has been extended to additional *let-7* family members: *mir-48, mir-84, mir-241*, which have been shown to specify the timing of the L2 to L3 translation [172]. Therefore, the studies presented here began by investigating the temporal expression patterns of a panel of highly expressed neural miRNAs in the course of mouse brain development and found significant

differences among them. In the course of this work, Krichevsky et al. reported the temporal expression patterns of several of these neural miRNAs (*mir-9*, *mir-124*, *mir-125*, *mir-128*) during rat brain development [53]. The results presented here are in general agreement, although *mir-128* was scored as predominantly post-natal and embryonic induction of *mir-124* was continuous in their analysis. The analysis of miRNA expression patterns were included in two publications, one focused on lineage specificity of neural miRNA expression [48], the other on the regulation of *let-7* [49]. Analyzing the miRNA Northern blots shown in Figure C.2, significant accumulation initiated as early as E12 for *mir-26*, *let-7a* and as late as P14 for *mir-29*. The degree of induction also varied, some miRNA displayed relatively constant expression throughout development (*mir-23*, *mir-26*), others increased well over ten-fold (*mir-124*, *mir-128*). For several of the miRNAs in the sample, the expression dynamics broadly correspond to that of cortical neurogenesis, which in the mouse is continuous between E10 and E18. In particular, the neuron-specific miRNAs (*mir-124*, *mir-125*, *mir-128*) gradually accumulated in parallel to neuronal maturation. The post-natal decline in expression seen in cortical samples may reflect the increasing contribution of non-neuronal cell types to total brain RNA. Expression of these miRNAs increased in the post-natal cerebellum, which may reflect the burst of granule neuron proliferation and maturation. Similarly, the astrocyte-specific *mir-29* was expressed at low levels throughout embryonic development.

The second step was to study expression patterns of the selected set of neural miRNAs in primary cell culture, in order to compare expression in neurons and astrocytes. Whereas *mir-124* and *mir-128* were restricted to neurons, *let-7* homologs and *mir-125* were preferentially expressed in neurons, and *mir-23* expression was observed exclusively in astrocytes (Figure C.4). In their study of haematopoietic differentiation, Chen et al. found that the majority of miRNAs were common to all lineages, and only three lineage specific [55]. Because the miRNAs chosen for this thesis were biased toward strong expression in adult tissue, it was surprising to find strong lineage specificity in their expression. Lagos-Quintano et al. have estimated, for example, that *mir-124* accounts for 25% to 50% of total adult brain miRNAs [28]. The samples analyzed in this work are not comprehensive but did include highly expressed and brain-specific miRNAs. The presence of disparate miRNA populations in neuronal and astrocytic cells may help the protein repertoires of the two lineages to diverge. Lai first suggested that a group of *D. melanogaster* miRNAs might regulate proneural transcription factors that act in the Notch pathway [173]. Large scale computational target site prediction supported and extended this hypothesis. The Notch pathway is a key determinant of cell fate choice after asymmetric cell division. In the case of *lin-4* and *let-7*, evidence for a role in terminal differentiation and cell-cycle exit has been presented. Members of the *let-7* family and *lin-4* cooperate in the post-transcriptional regulation of the *C. elegans hunchback*

homolog *hbl-1* [22,23,172], a key gene in the specification of neural progenitor cell fate. It is likely that differential miRNA expression in neural lineages reflects a role in the establishment of cell identity.

To confirm the observation of temporal regulation of miRNA expression during neural development, the same set of miRNAs analyzed by developmental Northern blots were investigated in two well-established models for neural differentiation, ES and EC cells. Houbaviy et al. were the first to catalog miRNA expression in ES cells using expression cloning [34], and a similar study has also been performed with human stem cells [45]. Sempere et al. have extensively characterized miRNA profiles after RA induction in the related P19 EC cell model [54]. Adult neural stem cells have also recently been reported to express characteristic repertoires of miRNAs [140]. Both cell types were treated with RA for 4 days to induce neural differentiation. The expression of each of the neural miRNAs in our collection was induced in response to RA stimulation, no signal was observed in undifferentiated ES cells (Figure C.7, Figure C.8). In the case of the *let-7* homologs and *mir-125* the level of expression in differentiated ES cells was comparable to that observed with RNA from embryonic brain (E15). *mir-23* was more readily detected in ES cells, in contrast *mir-128* was difficult to detect in differentiating ES cells. Strikingly, the more complex temporal expression pattern shown by *mir-124* in brain development appears to be reflected in the transient expression observed during ES cell differentiation. The temporal regulation of neural miRNAs during differentiation of ES cells was confirmed by neural induction of P19 EC cells (Figure C.9). The expression of all tested miRNAs was restricted to differentiated P19 EC cells. A complete description of miRNA expression during stem cell differentiation will require the application of array technology [53,174].

These results raise interesting questions regarding the mechanisms underlying temporal regulation of miRNA during brain development and stem cell differentiation and also on the impact of miRNA expression on genetic pathways active during neural differentiation. The timing of *let-7* expression triggers terminal cell differentiation in the *C. elegans* heterochronic gene networks [21], and it has been widely proposed that miRNAs may be involved in the regulation of either the timing, outcome or maintenance of cell fate decisions (reviewed in [11,170]).

The temporal expression of miRNAs during neural development and differentiation has already been shown [53;54]. In particular, Lagos-Quintana et al. were the first to demonstrate high representation of *let-7* family members in miRNA populations from mouse brain [28]. The expression of *let-7* in the developing nervous system of the zebrafish has recently been characterized by *in situ* hybridization [169], and the technique has been extended to mouse embryogenesis [175]. However, the function of *let-7* during brain development has not been adequately addressed.

D.1.2. Regulation of miRNA processing activity during neural differentiation

In ES cells and embryonic neural progenitors the transition from the pluripotent, self-renewing state to cell fate restriction is accompanied by a profound shift in the miRNA populations present in the cell. Little is known about the regulatory signals directing this shift. Transcriptional regulation is likely to play an important role in defining the marked tissue specificity characterizing miRNA expression patterns [28,169,176]. Several studies have been begun to address the transcriptional control circuitry involved [47,177-179]. In perhaps the first of these studies, mesoderm specific transcription of *mir-1* in *D. melanogaster* is sequentially regulated by the *Twist* and *Mef2* transcription factors [178]. Post-transcriptional mechanisms may also participate, as a lag was observed between the onset of *pri-mir-1* transcription at the blastoderm stage and the first detectable mature *mir-1* signal during gastrulation [178].

Employing two models for neural differentiation, ES cells and EC cells, evidence for post-transcriptional control mechanisms in the induction of *let-7* family members are presented here. In Northern blots with ES and EC cells, a surprising observation was made: *let-7* precursor synthesis was constitutive and readily detected in undifferentiated cells in the absence of mature *let-7*. The mature forms of *let-7* family members, in turn, were strongly upregulated during neural differentiation (Figure C.7, Figure C.8, Figure C.9). These results were confirmed by demonstrating active transcription of primary transcripts for three *let-7* genes (*pri-let-7a-1, pri-let-7e, pri-let-7f-1* together with *mir-99* and *mir-125a*) in undifferentiated cells and also in primary astrocytes, both cell types with very low levels of the mature miRNAs (Figure C.5, Figure C.10). These observations point to the involvement of post-transcriptional mechanisms in the developmental control of mature miRNA accumulation. Moreover, using an *in vitro* miRNA processing assay, *let-7* precursor processing activity was shown to be increased in differentiated ES and EC cells in comparison to undifferentiated cells. Processing activity was also higher in primary neurons in comparison to astrocytes (Figure C.14). A similar pattern of processing activity was obtained using the unrelated *pre-mir-30* and *pre-mir-128* as substrates (Figure C.13, Figure C.14). The finding that neural differentiation leads to increased processing activity suggests that post-transcriptional control mechanisms make an important contribution to the kinetics and magnitude of miRNA induction during stem cell differentiation *in vitro* and early CNS development *in vivo*. The central nervous system expresses a particularly rich population of miRNAs, and regulation of the miRNA processing machinery may be a mechanism to coordinate their expression during development.

Two mechanisms might account for the deficiency in *let-7* processing in undifferentiated cells: either a lack of essential components of the processing machinery; alternatively, the precursor might be sequestered in an inactive complex early in differentiation. Both ES and EC cells are competent for RNA*i*, and accumulate a unique class of stem cell-specific miRNA [34], indicating that

the processing pathway is not completely lacking. Identifying the protein components of the processing pathway is the subject of intense investigation. Two proteins initially identified in the regulation of mRNA splicing, Gemin3 and Gemin4, were shown to associate with miRNAs as part of a large nucleoprotein complex [152]. However, developmental regulation or mutational phenotypes of Gemin3 and Gemin4 have not been reported. Dicer is thought to be ubiquitously expressed, although there is evidence for downregulation after terminal cell differentiation. Dicer is required for normal development in *D. melanogaster*, *C. elegans* and *Arabidopsis*; null mutations in both zebrafish and mouse display embryonic lethality (reviewed in [8,52]), most likely due to defects in the establishment of chromatin structure [180] in addition to any disruption of the miRNA pathway. Mutations in *Arabidopsis* Argonaute genes differentially affect miRNA accumulation, but it is not clear what step in the pathway is disrupted [181]. Evidence from *D. melanogaster* suggests that the Ago2 homolog is most closely associated with the RNAi pathway, and Ago1 with miRNA production and utilization [182]. Interestingly, mutations in *D. melanogaster* Ago1 cause an arrest in neural development [143]. A study of human Argonaute family members found association of each of the four proteins tested (Ago1 – Ago4) with miRNAs, with Ago2 uniquely required for miRNA-directed RNA cleavage [183].

The regulation of each of these proteins was examined by RT-PCR in the ES or in EC cell systems. No evidence was found for differential regulation of Dicer, Argonaute or Gemin proteins during stem cell differentiation. Furthermore, the expression of each of these proteins was similar in primary neurons and astrocytes (Figure C.16). Evidence from two additional lines of investigation support a model in which the processing machinery fails to engage *let-7* precursor RNAs at the earliest stages of differentiation. In the first, evidence was obtained for differential precursor RNA binding activity in primary and stem cell derived neurons. In the second, the subcellular organization of pathway components in self-renewing, pluripotent stem cells was found to differ from that of more differentiated cells.

The first evidence for differential precursor binding activity was obtained using the *in vitro* processing assay (Figure C.15). Subsequent studies by our group revealed that a prominent precursor binding complex found in both stem cell-derived as well as embryonic neurons contained the core processing proteins Ago1, as well as FMRP [49]. In addition to their essential function in the miRISC effector complex, Argonaute proteins participate in a core ternary complex with Dicer and TRBP in precursor processing [80-82,184]. Several groups have shown that *D. melanogaster* and mouse FMRP homologs associate with miRNAs, polyribosomes and RISC activity (reviewed in [52,141]). Loss-of-function mutants in FMRP, as well as the Drosophila homolog dFXR, lead primarily to defects in the nervous system, including neuronal morphology, connectivity and synaptic transmission (reviewed in [185,186]). FMRP is not required for precursor processing in vitro [80,151,187,188],

but, as an important regulator of neuronal mRNA translation, it is tempting to speculate that FMRP may serve as a bridge between the miRNA processing pathway and the translational control machinery. Indeed, a recent analysis identified potential miRNA binding sites in many FMRP-associated mRNAs, including several predicted *let-7* target genes [189]. The finding that FMRP expression is induced in ES cells in response to RA, and is expressed at a higher level in primary neurons compared to astrocytes (RT-PCR and Western blot analysis, Figure C.16, Figure C.17), are consistent with a role for FMRP as a developmentally regulated processing gene. Moreover, an anti-FMRP antibody interfered with the formation of a miRNA precursor-binding complex that was strongly enhanced in neurons (Figure C.15 and [49]), supporting the hypothesis that FMRP is involved in the biogenesis of neural miRNA as well as their utilization. It will be interesting to learn if miRNA accumulation is disrupted in loss-of-function FMRP mutants.

In this work it was shown that in undifferentiated cells, the *let-7* precursor is not processed, and a *let-7* sensor is not silenced. Current models for miRNA processing involve initial precursor binding by a miRNA loading complex and subsequent transfer or rearrangement to an activated effector complex (reviewed in [190,191]). Localization of the effector complex to cytoplasmic P-bodies is thought to be essential for mRNA silencing [96,105,192]. In support of the identification of FMRP in a neuronal pre-*let-7* binding complex, it was found that a portion of ectopically expressed FMRP co-localizes with Argonaute proteins to cytoplasmic foci in HEK293 cells (Figure C.20). Moreover, extensive co-localization of Ago1, Ago2, MOV10 and FMRP in dendrites of cultured neurons was observed (Figure C.24). These results are consistent with the well-established role for FMRP in the regulation of synaptic translation (reviewed in [141,193]), the recruitment of FMRP to stress granules [194], and the identification of FMRP as a miRISC associated protein (reviewed in [141]).

In clear contrast, neither the Argonaute proteins Ago1 and Ago2, nor FMRP were organized in definable cytoplasmic foci in undifferentiated ES or EC cells (Figure C.21). This observation is related to the inefficient processing of *let-7* in these cells. At present, it is not clear where pre-miRNA processing occurs. Processing is coupled to miRISC assembly, at least *in vitro* [80,81]. Recruitment of the miRISC to P-bodies by GW182, in turn, is required for efficient mRNA silencing via cleavage or translational suppression [96,105,192]. Although it is an attractive scenario, it has not been established that P-bodies are the site of precursor processing. It is possible that additional factors, such as the stem cell specific pre-miRNA binding and processing complex described by our group [49], act as escorts and regulate accessibility to the processing machinery. This model could account for the block in processing of *let-7* precursors compared to stem cell specific miRNA such as *mir-294* that are processed in these cells. In this model, sequestration of Argonautes may be overridden by ectopic expression of the Argonaute-interacting proteins MOV10 and TNRC6B (Figure C.22). Partial co-localization of FMRP and Argonaute was consistently observed

in HEK293 cells (Figure C.20), and TNRC6B affected FMRP localization in undifferentiated EC cells (Figure C.23). These results strengthen the evidence linking FMRP and the miRNA pathway, in particular regarding the interesting pattern of localization observed in neurons.

The results presented in this work and discussed above reveal novel aspects of developmental regulation of miRNA pathway during embryonic stem cell differentiation. P-bodies, as sites of mRNA metabolism are dynamic structures that are modified in the cellular response to stress, and undergo remodeling during the cell cycle [195]. It may be relevant to note that alteration of cell cycle control is a hallmark of stem cell differentiation (reviewed in [196]). Since many miRNAs, including *let-7*, are thought to target regulators of the cell cycle and proliferation and to promote terminal differentiation, a mechanism to delay miRNA accumulation early in development may be necessary. This scenario is also consistent with the phenotype of mutations in the miRNA biogenesis pathway, which tend to affect development after completion of axis and pattern formation [120,143,197].

D.2. Functional analysis of miRNAs

D.2.1. miRNA functional activity. Validation of new target genes

So-called "Sensor" assays have become a widely used tool to confirm the activity of individual miRNAs *in vivo*, and also to validate predictions of miRNA binding sites in target genes. 3'UTRs from predicted targets are fused to luciferase or GFP reporter constructs and the regulatory capacity of the UTR-containing miRNA complementary elements are assessed in animal cells [107,166]. In this work, the GFP reporter was used in the construction of sensor plasmids. The *eGFP* mRNA encoded by the peGFP-N1 vector was fused with the 3'UTR region from C. *elegans lin-41* or *lin-14* mRNAs to visualize the functions of *let-7* and *mir-125* respectively. Direct interaction of the *let-7* miRNA with at least two binding sites on the *lin-41* sequence contained in the sensor construct is well established [25]. As controls, these miRNA binding sites were either cloned in the antisense orientation or were destroyed by the introduction of point mutations. The sensor constructs were used in transfection assays in primary neurons and astrocytes to confirm neuron-specific activity of *let-7* and *mir-125*. Similarly, sensor constructs were strongly downregulated in the course of neural differentiation of EC cells. In both cases, the magnitude of the observed suppression suggests that the action of a single miRNA on its target gene is sufficient to effectively silence the target even in the absence of other regulatory control mechanisms. One potential problem with the sensor assay is that it can be difficult to predict and confirm the functional interactions of individual miRNAs with the target. For example, one binding site algorithm (RNA22, http://cbcsrv.watson.ibm.com/rna22.html, [198]) predicts 141 miRNA interactions with the 3'UTR of the mouse *lin-41* ortholog. The majority of these potential interactions, however, are not

conserved between the mouse, rat, and human mRNAs (data not shown). Potential binding sites for *let-7* and *mir-125*, however, are conserved from *C. elegans* to humans, and the corresponding region of the mouse mRNA was chosen in the construction of the sensor. Nevertheless, even the defined region on the sensor contains additional potential binding sites for *mir-26*, *mir-30* and *mir-218*. This can explain the observation that the control construct with mutations in the *let-7* binding sites is still slightly downregulated after neural induction of EC cells in comparison to the peGFP-N1 control vector (Figure C.45).

A second goal in the analysis of miRNA/target gene interactions is the assessment of their *in vivo* relevance. Even when a predicted site is experimentally validated in the sensor assay, that site may not normally function *in vivo* if the miRNA and mRNA are not normally expressed in the same cell. It is therefore important to correlate the spatial and temporal expression profiles of miRNAs with that of their potential target genes. In the experiments presented here, the activity of endogenously expressed miRNAs was assayed as a first step in verifying *in vivo* relevance. Furthermore, the expression profiles of both *let-7* and *mlin-41* in the course of EC cell differentiation and in the developing brain were analyzed (Figure C.47). The reciprocal expression of *let-7* and *mlin-41* in the brain and in embryonic stem cells supports data from the sensor assays identifying *mlin-41* as a target gene for *let-7* in the mouse.

The validation of mouse *lin-41* as a *let-7* target gene in the mouse serves as a proof of principle demonstrating the utility of primary cell transfection and EC cell differentiation assays in characterizing *bona fide* miRNA/mRNA interactions. Using these results as starting point, further investigations will include a screen and experimental validation of predicted miRNA target genes involved in neuronal specification and migration, axonal guidance and synaptic function. Characterization of miRNA target genes is one of the greatest challenges facing the field, the cell-based assays described here represent versatile and well-controlled tools. In addition to visualization of endogenous miRNAs, ectopically expressed miRNAs will be used to verify the predicted target genes in order to speed up and simplify the experimental procedure.

D.2.2. Competitive interference strategy for study miRNA functions

Another challenge facing the miRNA field is the lack of genetic tools for the investigation of miRNA function. Many miRNA genes are present in multiple copies in the genome. In the most extreme case, there are 12 *let-7* genes scattered over 8 chromosomes. This obviously constrains traditional mutational analysis. Several groups have shown that chemically modified antisense RNA oligonucleotides can interfere with miRNAs under some assay conditions [199,200]. Up to the time of the thesis preparation, the efficacy of this approach for knockdown of miRNA-mediated translational regulation has not been demonstrated. Two inherent disadvantages of this strategy are

the high cost of the 2'-O-methyl or LNA-modified oligonucleotides, and the reliance on transient transfection to introduce the oligonucleotides into cells.

To probe the function of the *let-7* regulatory circuit, an alternative strategy was explored based on competitive interference. This strategy is analogous to the squelching strategy originally devised for the study of transcriptional control elements. It was postulated that overexpression of *let-7* binding sites contained on sensor mRNAs could disrupt the balance between *let-7* and its endogenous target genes at a critical period in stem cell determination. Using this assay to knockdown *let-7* function in precursor cells led to increased glial cell maturation. This result indicates that *let-7* may regulate genes involved in cell fate determination and proliferation in the CNS.

Verified targets for *let-7* have been identified in *C. elegans*, and it is likely that these genetic pathways have mammalian counterparts. Mutations in *lin-41* affect the number of cell divisions undertaken by progenitor cells in the hypodermis and the timing of their terminal differentiation [24]. One of the *lin-41* orthologs in *D. melanogaster*, *Brat*, participates in the translational regulation of *hunchback* [201,202], which is itself a target for regulation by *let-7* in the *C. elegans* nervous system [22,23]. Significantly, mutations in *Brat* result in overproliferation of neuronal precursors and a block in terminal differentiation [203]. The *lin-41* homolog *Trim2* is expressed in the neural tube early in development [204] and has been identified as a potential target for *mir-9* [107]. In a recent advance, Johnson et al. have demonstrated widespread and evolutionarily conserved regulation of the RAS family of growth control proteins by the *let-7* family of miRNAs [134]. Human RAS is a critical oncogene and is overexpressed in lung cancer tissue, whereas *let-7* is downregulated. The relevance of the RAS pathway for the expansion of the astrocyte compartment in the competitive interference assay was not further examined.

Computational prediction of mammalian *let-7* targets identified a diverse group of genes [9,189,205,206], several of which have been implicated in early development or neurogenesis. As shown above, the closest mouse *lin-41* relative, *mlin-41*, has been identified as a novel *let-7* target gene. Similarly, during the preparation of this thesis Kloosterman et al. determined that a Xenopus *lin-41* ortholog is regulated by *let-7* [156]. It was shown that *mlin-41* is expressed in a reciprocal manner with *let-7* in ES and EC cells and in the developing CNS (Figure C.47). Moreover, the *mlin-41* sensor construct is downregulated in the course of neural differentiation of EC cells and in primary neurons. Therefore, *mlin-41* is an intriguing candidate for disregulation in the competitive interference experiments. Continued efforts should clarify the contribution of the *let-7* regulatory network, including *mlin-41*, to neural development and its disturbances. The strategy for the investigation of mLIN-41 function in early differentiation events will be the targeted destruction of the *mlin-41* mRNA using siRNA. EC cells will be transfected with siRNA against *mlin-41* in order

to knock down mLIN-41 expression. The effects of mLIN-41 absence during differentiation of EC cells will be analyzed by RT-PCR and Western blot with a panel of stem cell-, neuron-, and cardiac-specific markers.

In addition to *let-7*, the effects of *mir-125* knockdown on the neural differentiation of EC cells were analyzed. Competitive interference with *mir-125* during RA-mediated differentiation of EC cells also led to an increase in the number of glial cells generated. One candidate target gene for *mir-125* with the potential to influence astrocyte differentiation is the cytokine leukemia inhibitory factor (LIF) [107]. Further experiments are necessary to answer the question if LIF is really a target gene for *mir-125* and to demonstrate that LIF is missregulated during squelching experiments.

In conclusion, it was demonstrated that *let-7* and *mir-125* affect lineage specification during EC cell differentiation and can alter the balance between neurogenesis and gliogenesis. With the availability of computational methods for predicting miRNA target genes, and the stem cell differentiation assay described here for verifying those targets in a developmental context, in the future it should be possible to characterize the developmental programs regulated by any given miRNA.

During preparation of this thesis Krichevsky et el. have demonstrated similar effects on neural differentiation of ES cells by two brain specific miRNAs, *mir-9* and *mir-124* using gain-of function and loss-of-function approaches [127]. They have shown that overexpression of *mir-9* and *mir-124* led to a reduction of glial-like cells differentiated in culture, whereas inhibition of the *mir-9* caused a reduction of neurons and a slight increase of glial-like cells. In addition, the NRSE dsRNA regulates cell fate specification of adult neural stem cells by interacting with the NRSE transcription factor [140]. It seems that neural fate determination can be influenced by different classes of small non-coding RNAs, which are involved in both transcriptional and post-transcriptional regulation.

D.2.3. Influence of miRNA on the target mRNA stability

The first models for miRNA action in animal cells stressed imperfect complementarity between a miRNA and its target as a signal that marks the target mRNA for translational repression without target mRNA degradation (reviewed in [8]). Initially, these models were based on the observation that in *C. elegans* the level of the *lin-14* mRNA remained constant as LIN-14 protein levels dropped in response to regulation by *lin-4* [20]. Furthermore, *lin-4*-mediated inhibition of protein expression did not change the association of the *lin-14* mRNA with polyribosomes [18]. Several *in vitro* studies with reporter systems in mammalian cells have supported this model [66,103,104]. Unexpectedly, in contrast to published reports, a reduction of target mRNA levels was observed in our experimental system in spite of the imperfect complementarity of the tested binding sites (Figure C.40,Figure C.41). Similarly, Bagga et al. have revisited the regulation of *let-7* and

lin-4 target mRNAs during *C. elegans* development and demonstrated that miRNA/mRNA interactions led to a reduction in mRNA stability [159]. However, there is no explanation for the discrepancy between these results and the previous studies of *lin-4*-directed regulation. In support of the work by Bagga et al., Lim and colleagues have shown that ectopic expression of individual miRNAs in Hella cells led to decreased levels of scores of potential target mRNAs [157]. These results argue against a simple model that states that perfectly complementary binding sites trigger mRNA degradation and imperfect sites translational control. The mechanism by which miRNAs target mRNAs for degradation remains unclear. One possibility is the activation of endonuclease cleavage of the target RNA, as in the RNAi pathway. However, this seems to be unlikely, because destabilization of the mRNA has been observed in miRNA/mRNA pairs containing mismatches at the tenth and eleventh positions of the duplex, usual sites of cleavage by siRNAs [159]. Alternatively, translational inhibition may precede destabilization of the mRNA. miRNAs may recognize the cognate targets during translation, this interaction could result in translational inhibition followed by target degradation. In the case of *lin-41*, Bagga and colleagues observed accumulation of a possibly decapped, ~500 nt degradation product upon depletion of cellular 5'-3' exonuclease activity. These observations suggest that miRNAs either target mRNAs to an unknown decay pathway, or might promote mRNA decapping and 5'-to-3' degradation. The colocalization of 5'-3' exonucleases, decapping enzymes, together with Argonaute proteins and mRNA targets of miRNAs to P-bodies in mammalian cells (observations of this work and also [96,97,105]) strongly support the prediction that miRNAs target mRNAs to P-bodies, increasing their association with the decapping machinery and thereby potentially reducing their levels by decapping and 5'-to-3' degradation. Most likely relocalization of the repressed mRNA to P-bodies is a consequence rather than a cause of the repression. Recently, a third mechanism of regulation of mRNA expression was proposed, namely deadenylation and decay of mRNA [207]. It was shown that *mir-125b* and *let-7* can mediate the trimming of the poly(A) tail. Poly-A trimming is expected to facilitate subsequent decay of target mRNA by exonucleolytic degradation. Moreover, absence of 3'-poly(A) tail does not abolish the translational repression, suggesting that deadenylation and decay of mRNA is an additional independent mechanism to translational repression pathway.

In conclusion, in this thesis it was shown that both *let-7* and *mir-125* can destabilize their target mRNAs in the course of mouse development. This observation is of interest for further studies of the mechanism of mRNA regulation by miRNAs. It also has important practical consequences, as it can no longer be assumed that miRNA regulation does not result in loss of transcripts, making the discrimination between transcriptional control in development and post-transcriptional regulation by miRNAs more difficult to discriminate.