Chapter 4

A system to study microRNA function

As described in the Introduction (section 5.2) perturbation of miRNA expression in primary E17.5 forebrain cultures was the basis for experimental determination of miRNA function and targets in this thesis. Perturbation of miRNA expression levels was achieved through chemical transfection of miRNA mimics (for ectopic expression or overexpression of endogenous miRNAs) and inhibitors (for antisense mediated inhibition of endogenous miRNAs) in primary cultures (Methods, section 2.5). All transfection experiments in this work were conducted at one of the three developmental timepoints: **3DIV**, **4DIV** and **6DIV**. The 4DIV timepoint was selected because of its significance as a switch point in developmental gene expression program of primary E17.5 neuronal cultures, after which the ratio of neuritic and somatic transcripts resembles that in mature neurons (Chapter 3, section 3.1.3) (Valor et al., 2007; Manakov et al., 2009). The other two timepoints were selected on either side of the 4DIV timepoint. To transfect the mimics and the inhibitors, I used a protocol that was developed by a colleague in the laboratory, Dr. Erik MacLaren (Maclaren et al., 2011), for transfection of siRNAs into primary neuronal cultures. The first part of this chapter describes testing of the transfection protocol, and the second part describes adjustments to the protocol that improved detection of direct targets of miRNAs.

4.1 Efficient transfection of neurons

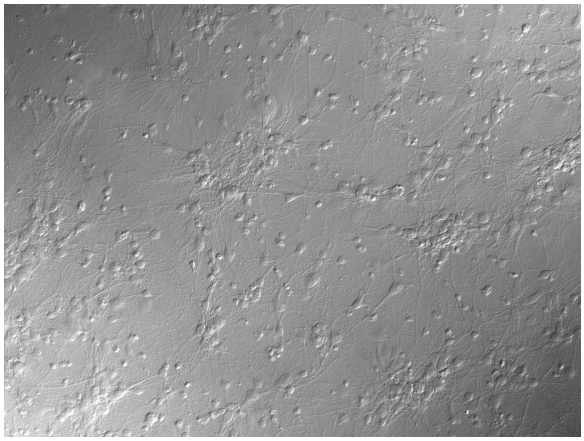
4.1.1 Microscopy confirmed efficient transfection of neurons in primary forebrain cultures

To confirm that transfection of neurons in primary E17.5 forebrain cultures was possible with the original siRNA transfection protocol, a plasmid expressing eGFP was transfected into the cultures at 6DIV. At 36 h after transfection, cultures were fixed and visualised (Methods, section 2.6). A strong fluorescence was detected in some neurons in the culture (Figure 4.1). This experiment unequivocally demonstrated that the protocol enabled transfection of neurons and it also showed that neurons were a predominant cell type among transfected cells (see images at a low magnification, Figure 4.1).

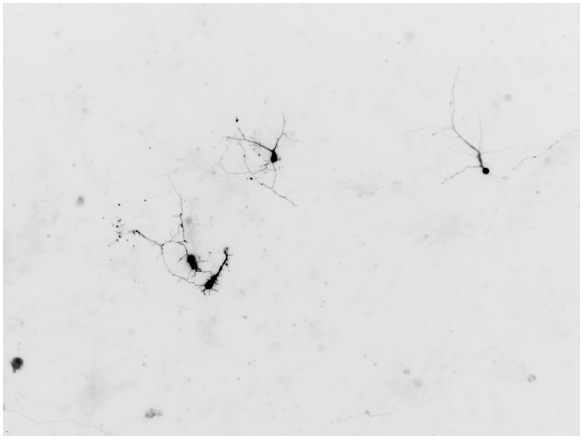
Transfection of a plasmid expressing eGFP demonstrated unambiguously that neurons were transfected, but it was not informative of the efficiencies to be expected in miRNA transfection experiments. The sizes of the plasmid and of miRNA mimics or inhibitors were different, thus, frequency of plasmid delivery was not a good estimate of the transfection efficiency for short polynucleotides. Additionally, the inefficient process of transfer to the nucleus is required for reporter gene expression (Zabner et al., 1995), while activity of miRNA mimics and inhibitors is thought to take place in the cytoplasm. Therefore, transfections of fluorescently labelled oligonucleotides were suggested by a manufacturer of miRNA mimics and inhibitors (Qiagen N.V.) to be a better estimate of the efficiency expected in transfections of miRNA mimics and inhibitors. The AlexaFlour 488 labelled oligonucleotide RNA was transfected into primary cultures at 3DIV and 6DIV (Methods, section 2.6). After 36 h of incubation, an abundant bright punctate fluorescence was detected (Figure 4.2). This was likely due to coagulation of the fluorescently labelled oligonucleotides outside the cells. At the same time, a fainter diffuse fluorescence was observed in a majority of the cells. This signal was unlikely to be due to the autofluorescence of the cells, as mock transfected cultures did not display the signal when viewed with the same settings (Figure 4.2e).

In summary, transfection of an eGFP expressing plasmid and AlexaFlour 488 labelled oligonucleotide demonstrated that the transfection protocol was capable of delivering various nucleic acids (e.g. a plasmid and a fluorescently labelled RNA oligomer) into primary neurons at different developmental timepoints. Additionally, these experiments showed that it was possible to transfect a majority of neurons with an RNA oligomer.

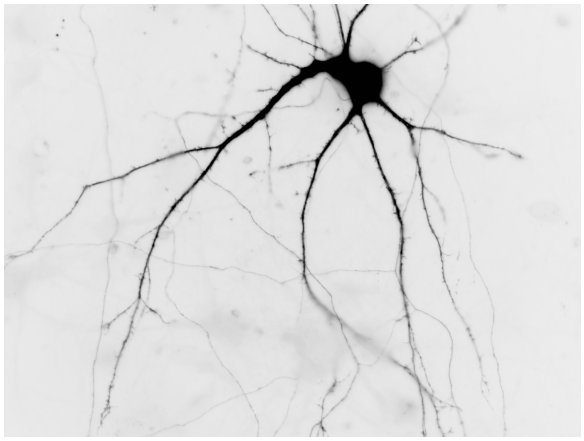
(a) transfections at 6DIV (DIC)



(b) transfections at 6DIV (eGFP)



(c) transfections at 6DIV (eGFP)



(d) transfections at 6DIV (eGFP)

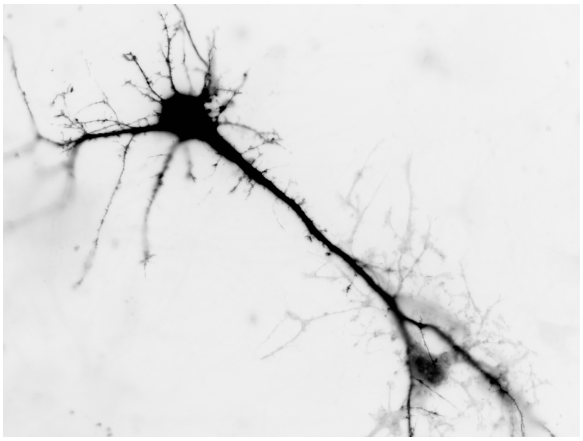
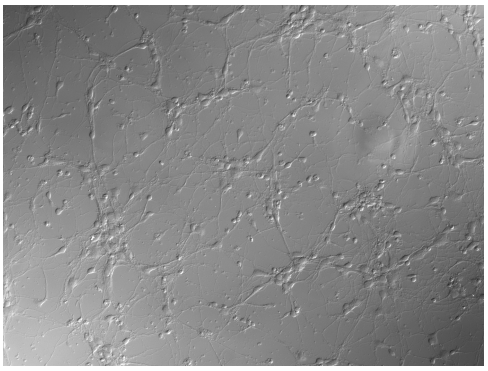
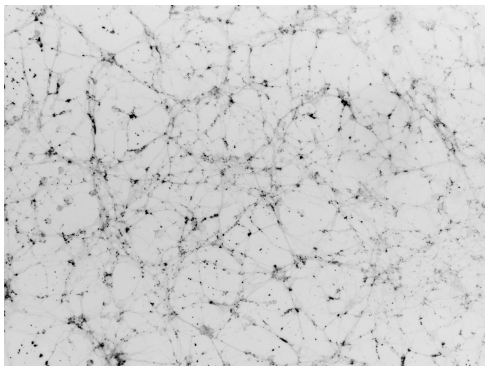


Figure 4.1: Transfection of primary cultures with eGFP-expressing plasmid.
Figures 4.1a and 4.1b show the same area of the culture viewed with differential interference contrast (DIC) or fluorescence microscopy (495 nm light for excitation of enhanced GFP (eGFP)) settings at a low magnification. Figures 4.1c and 4.1d show the fluorescence microscopy images (495 nm light for excitation of eGFP) at a high magnification. The slide preparation and the microscopy settings are described in Methods (section 2.6).

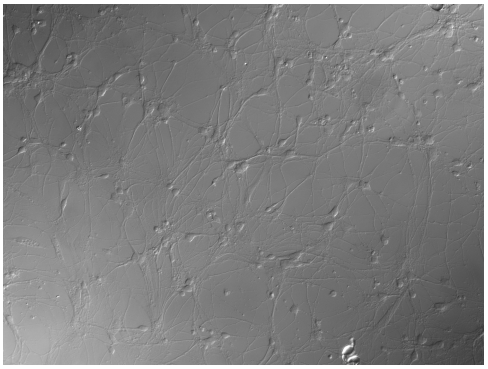
(a) transfections 3DIV (DIC)



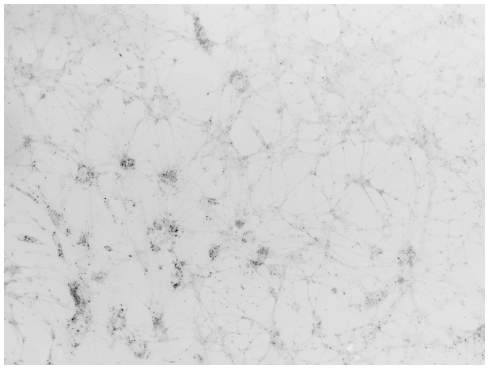
(b) transfections 3DIV (AlexaFlour 488)



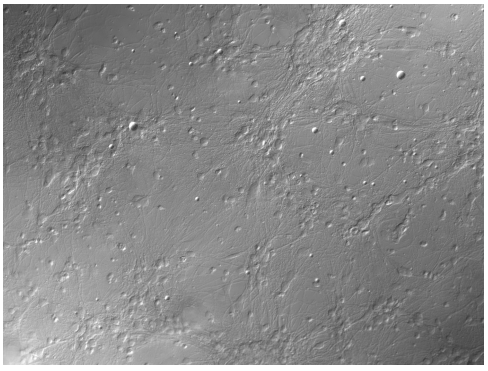
(c) transfections 6DIV (DIC)



(d) transfections 6DIV (AlexaFlour 488)



(e) mock 6DIV (DIC)



(f) mock 6DIV (AlexaFlour 488)

Figure 4.2: Transfection of primary cultures with AlexaFlour 488 labelled oligo at 3DIV and 6DIV.
Pairs of figures 4.2a and 4.2b, 4.2c and 4.2d, 4.2e and 4.2f, show the same areas of transfected cultures viewed with differential interference contrast (DIC) or fluorescence microscopy (495 nm light for excitation of AlexaFlour 488) settings at a low magnification. The exposure time for all AlexaFlour 488 images was fixed at 220ms. The slide preparation and the microscopy settings are described in Methods (section 2.6).

4.1.2 Transfection did not cause neuronal death

Four types of evidence showed that no significant neuronal loss was associated with transfections of primary cultures. These four sources of evidence came from visual examinations, measurement of differential gene expression, recordings of electrophysiological activity in transfected cultures and Trypan blue assay (Altman et al., 1993).

Visual inspections of transfected cultures did not reveal neuronal loss

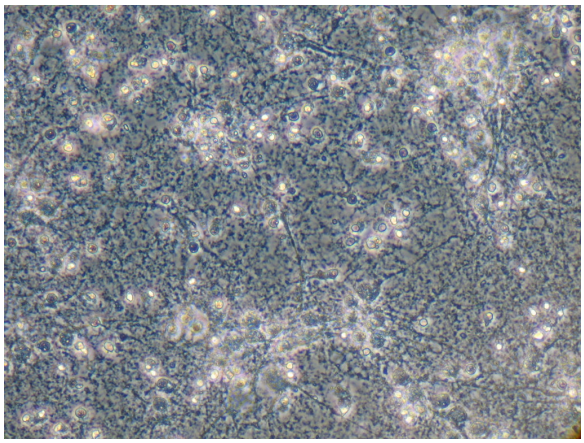
Concentration of mimics and inhibitors in transfection mixtures was either 0 nM (in case of mock transfection), 115 nM or 230 nM (Methods, section 2.5). No significant cell loss was visually detected in any of the experiments, neither in mock transfections nor in transfection of mimics and inhibitors. Moreover, addition of RNA in even higher concentration ($>$ 1,300 nM) to the mixture did not cause an observable neuronal loss (Figure 4.3). A complete degradation of nearly all neurites was associated with the death of neurons (Figure 4.3a), and this was not found to be the case in a mock transfected culture (Figure 4.3b) or cultures treated with a transfection mixture containing a mimic or an inhibitor in $>$ 1,300 nM concentration (Figure 4.3c and 4.3d).

Trends in differential gene expression were not compatible with a consistent significant neuronal loss

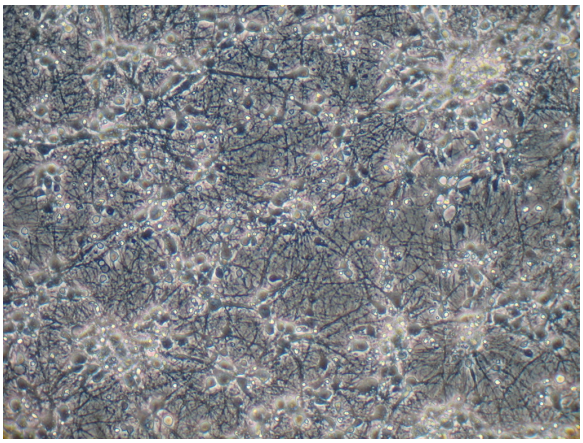
A significant loss of neurons would skew the results of gene expression analysis, because removal of neurons (i.e. neuronal mRNA) from transfected cultures would make neuron specific genes appear as downregulated. To test if this might have been the case, a list of putatively neuron specific genes was compiled (Methods, section 2.9) and their expression was compared to the rest of the genes.

Analysis of changes in expression of these neuron specific genes revealed that no consistent or significant neuronal loss was associated with transfection experiments. Although neuron-specific genes were found to be downregulated in some experiments (consistent with neuronal cell loss), they were upregulated in other experiments (inconsistent with significant neuronal loss). For example, overexpression of cel-miR-67 at 6DIV lead to significant downregulation of the neuron specific genes, while overexpression of miR-124, performed with the same protocol and at the same developmental timepoint, lead to upregulation of the neuron specific genes (Figure 4.4).

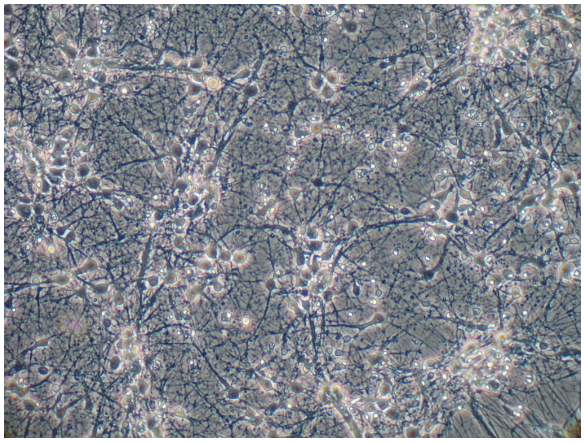
(a) killed by sodium azide



(b) mock transfection



(c) transfection with miR-103 mimic



(d) transfection with miR-103 inhibitor

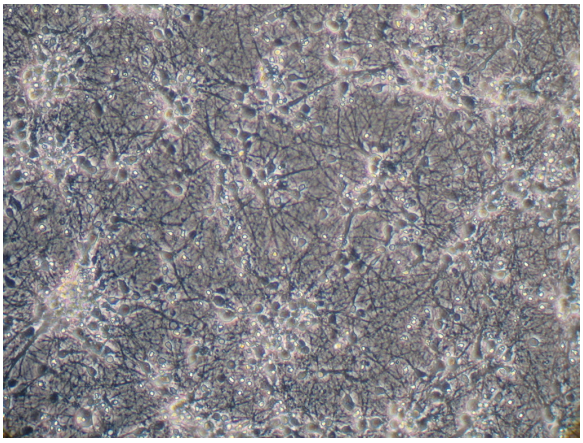


Figure 4.3: Visual inspection of transfected cultures.
The cultures were transfected at 6DIV, and the images were taken after 48h of incubation. 4.3a - killed with sodium azide at 8DIV (0.03%, 24h incubation); 4.3b - mock transfection; 4.3c - transfection with the mimic of miR-103 ($> 1,300$nM); 4.3d - transfection with the inhibitor of miR-103 ($> 1,300$nM).

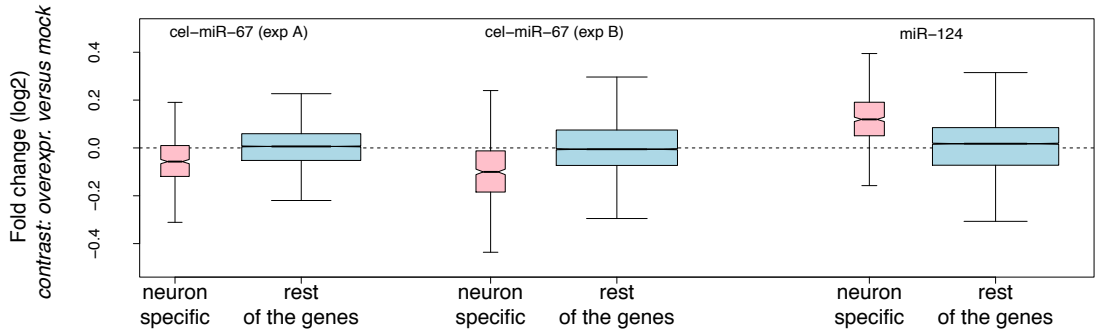


Figure 4.4: Differential regulation of neuron specific genes.
The y-axis shows the fold change (log_2) in gene expression of the *neuron specific* and the *rest of the genes* upon transfections of the cel-miR-67 and miR-124 mimics at 6DIV (the names of the over-expressed miRNAs are shown in the plot area, the types of the genelists are shown on the x-axis). The transfection of cel-miR-67 mimic at 6DIV was independently repeated twice (shown as *exp A* and *exp B*). The boxes correspond to the distribution of the fold changes (log_2) of the genes in the contrast of cultures transfected with the mimics to the matched mock transfected cultures. The width of the boxes corresponds to the number of genes within the two lists (the neuron specific and the rest of the genes) present among the genes detected in each of the experiments (using the standard Illumina detection call $P < 0.01$, see Methods, section 2.7). The notches of the boxes correspond to the median value of the distribution of the fold changes (log_2) of the genes in the genelists, the bottom and the top sides of the boxes correspond to the first and the third quartiles, the whiskers extend to no more than 1.5 times the interquartile range (IQR), or to the most extreme data-point, if it is closer to the median than 1.5 IQR. In all three experiments, the Wilcoxon test P-values for the differences between the medians of the neuron specific genes and the rest of the genes was beyond the precision limit of the test as implemented using the standard R libraries (RTeam, 2008) ($P < 1e - 320$).

Number of active synapses was not affected by transfections

While establishing the original protocol for siRNA transfection, it was shown that neither the mock transfection nor transfections with siRNAs (designed to target genes shown in Figure 4.5) reduced the number of neurons, or a number of active synapses in primary cultures (Maclaren et al., 2011). In order to demonstrate that, primary neuronal cultures were plated on microelectrode arrays (MEAs), where firing patterns (spikes) could be recorded for a number of days after transfections. Toxicity of the transfection reagent could be uncovered through these measurements, because it was previously shown that the total number of spikes correlated with the number of active synapses adjacent to the electrodes of MEAs (Wagenaar et al., 2006) and with synaptic density in the whole culture (Brewer et al., 2009). No significant differences ($P < 0.05$) were detected at any timepoint between mock transfected, untransfected and siRNA transfected cultures (Maclaren et al., 2011) (Figure 4.5).

Cell viability was not affected by transfections

Finally, I confirmed viability of transfected cells with Trypan assay (Altman et al., 1993). Cultures at 6DIV were transfected with the mimic of cel-miR-67 (220 nM concentration, see Methods, section 2.5) and stained with Trypan blue and DAPI at 48 h post transfection (Figures 4.6a and 4.6b, see Methods, section 2.6). Majority of both transfected and matched untransfected cells (Figures 4.6c and 4.6d) were viable (i.e. not stained with Trypan blue (Table 4.1), see for a comparison cells treated with sodium azide in Figures 4.6e and 4.6f). Additionally, the difference in percent of viable cells was not significant between transfected and untransfected cultures (t-test $P > 0.7$, based on three non-overlapping images per treatment).

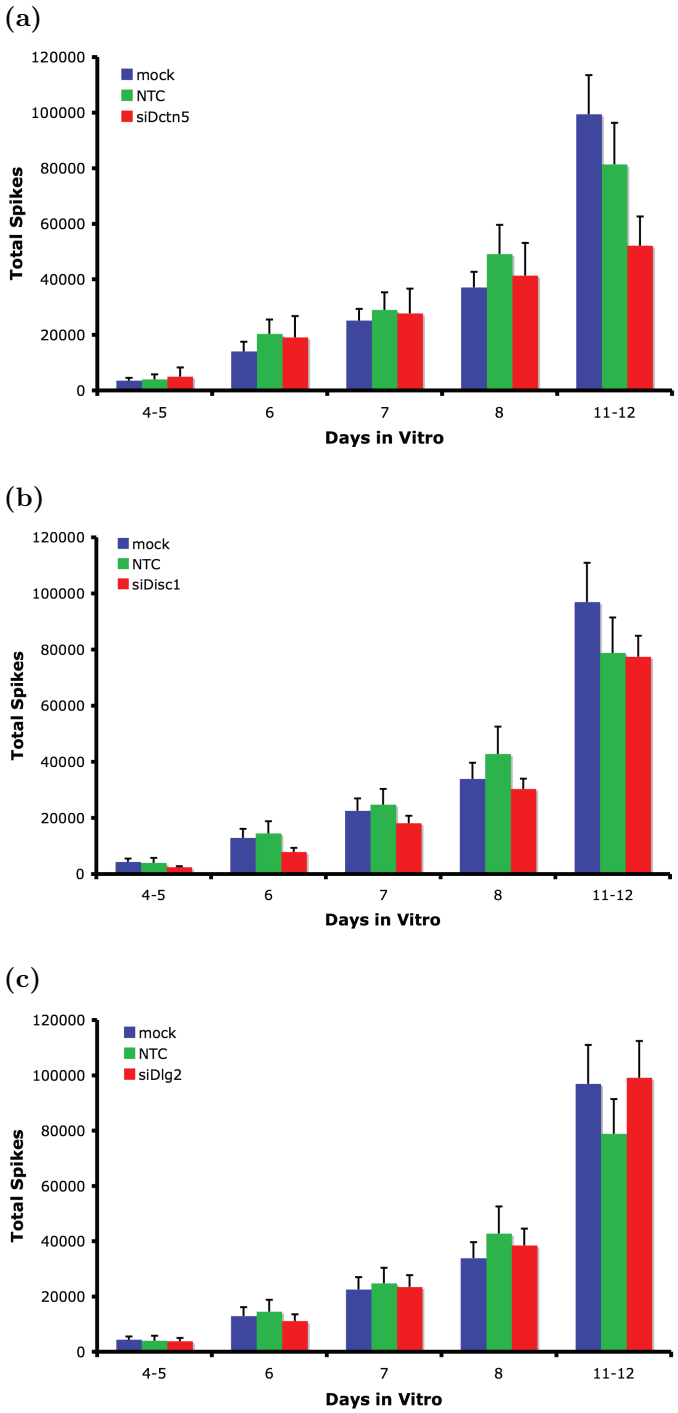
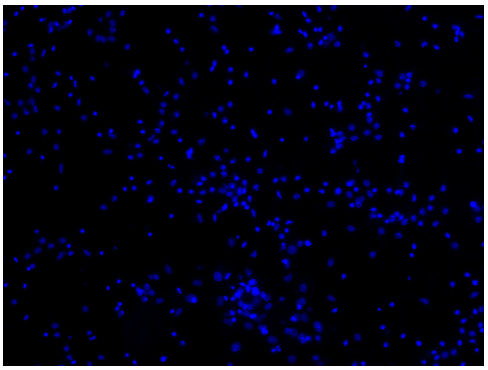
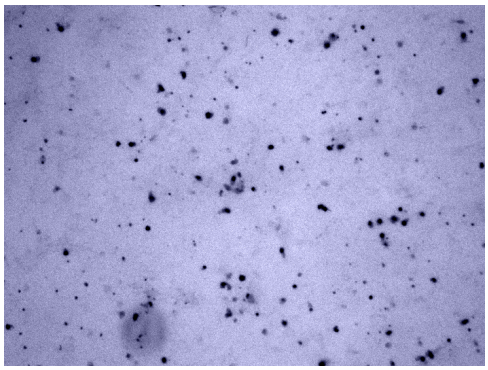


Figure 4.5: The total number of spikes recorded in cultures after transfections. The figure is reproduced from the manuscript by MacLaren *et al.*, with permission of Erik MacLaren. "*mock*" – mock transfected cultures; "*NTC*" – untransfected cultures; "*siDctn5*", "*siDisc1*" and "*siDlg2*" – cultures transfected with siRNAs designed to target Dctn5, Disc1 and Dlg2 transcripts (Maclaren et al., 2011).

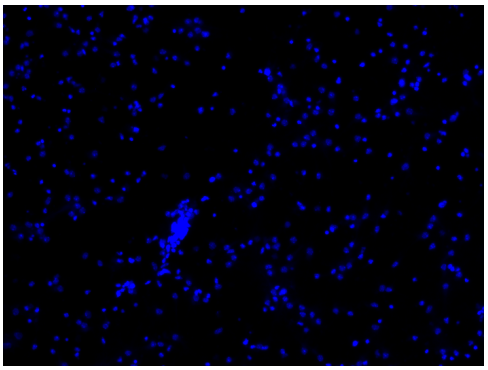
(a) transfected (DAPI)



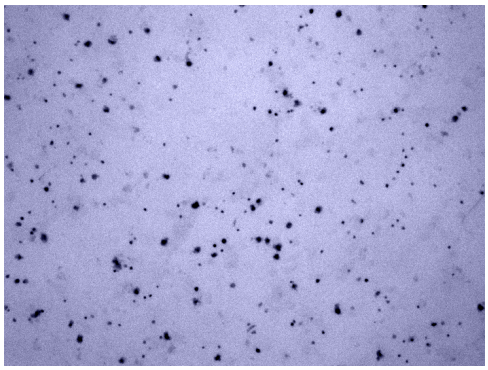
(b) transfected (Trypan)



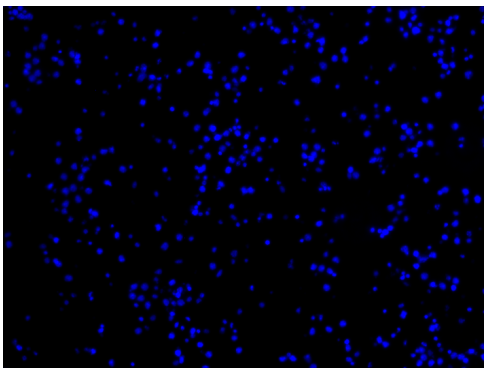
(c) untransfected (DAPI)



(d) untransfected (Trypan)



(e) killed by sodium azide (DAPI)



(f) killed by sodium azide (Trypan)

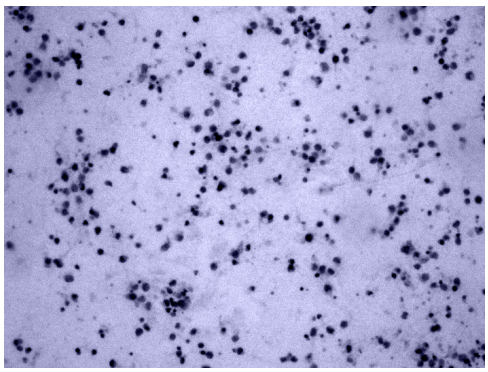


Figure 4.6: Viability of cells in transfected cultures.
Pairs of figures show the same areas of cultures stained with DAPI or Trypan blue (see titles of the subfigures). Figures 4.6a and 4.6b show a culture transfected at 6DIV with the mimic of cel-miR-67 (visualised at 8DIV); Figures 4.6c and 4.6d show a matched untransfected culture (visualised at 8DIV); Figures 4.6d and 4.6d) show a culture treated at 8DIV with sodium azide (0.03%, 24 h incubation). The assay is describe in Methods (section 2.6).

Days *in vitro*	**Viability**	**Standard deviation**
transfected	68.1%	± 2.85%
untransfected	66.4%	± 8.05%

Table 4.1: Viability of cells in transfected cultures.
Viability was estimated based on three non-overlapping 10× objective images (∼ 500 cells per image, see for example Figure 4.6). Total numbers of cells were estimated by counting DAPI stained nuclei, numbers of dead cells – counting Trypan stained cells (Methods, section 2.6).

4.1.3 Transfection of miRNA mimics consistently induced miRNA mediated changes in gene expression

Although transfections of a plasmid expressing eGFP and of a fluorescently labelled oligonucleotide (section 4.1.1) showed that neurons were efficiently transfected, it was not known if introduced miRNAs were active inside cells. It was also not known how well the changes in gene expression induced by transfections were reproducible between different transfection experiments.

Activity of introduced miRNAs and their widespread direct effect on gene expression in the cultures was made evident through analysis of the distribution of seed matching sites complementary to the transfected miRNAs. It was previously demonstrated that a widespread direct effect of an overexpressed miRNA on gene expression manifested itself as a significant enrichment of the sites complementary to the seed region of that miRNA in the 3′UTRs of downregulated genes (Lim et al., 2005; Giraldez et al., 2006). Such an effect was consistently observed upon transfection of various miRNA mimics into primary E17.5 forebrain neuronal cultures (for example see Chapter 5, Figure 5.1). Importantly, by using Sylamer (van Dongen et al., 2008), which simultaneously assess biases in occurrence of all nucleotide words (Methods, section 2.8), it was possible to show that such enrichment in many cases was exclusively specific to the seed matching sites of only the transfected miRNAs (for example see Chapter 5, Figure 5.2).

The concern that the variable nature of the primary culture system would dramatically compromise reproducibility of gene expression measurements was resolved by doing replicate transfections of the same miRNA mimic. By conducting replicate experiments of cel-miR-67 at 4DIV and 6DIV it was possible to show significant correlation in gene expression changes between the experiments (Figure 4.7). Additionally, correlation between genes that contained sites complementary to the seed region of cel-miR-67 was higher than for all genes. The latter observation was consistent with the changes induced directly by the miRNA to be among primary changes in transfected cells.

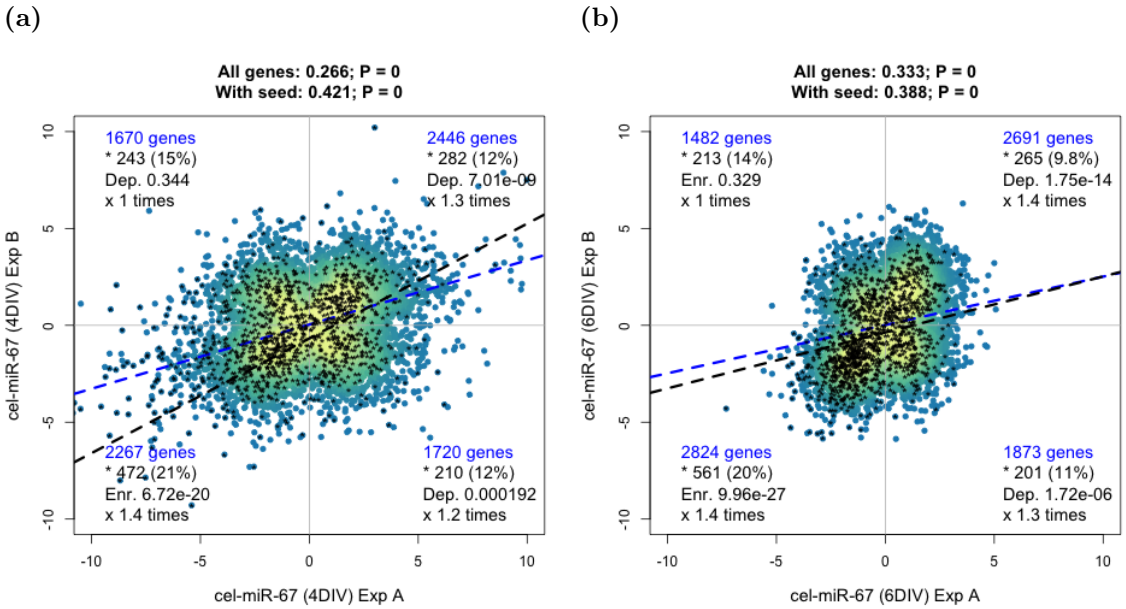


Figure 4.7: Correlation of cel-miR-67 experiments at 4DIV and 6DIV.
The points correspond to 8,103 and 8,870 genes detected in the replicate cel-miR-67 mimic transfection experiments at 4DIV (4.7a) and 6DIV (4.7b). The axes correspond to the moderated t-statistic for differential expression between the cultures transfected with the mimic of cel-miR-67 and the matched mock transfected cultures in each of the experiments. The differential expression analysis is described in Methods (section 2.7). The colors of the points depend on the density of the points in a given region of the plot (yellow – highest, blue – lowest). Black asterisks mark genes that encode transcripts with 3′UTRs harbouring one or more seed matching sites (7(2) or 7(1A)-types) for cel-miR-67. The text gives the following information: 1) The total number of genes in each of the quadrants of the plots; 2) The number (and percentage) of genes [encoding transcripts] with seed matching sites for cel-miR-67 in their 3′UTRs; 3) Hypergeometric P-value for the enrichment (*Enr.*) or depletion (*Dep.*) of genes with the seed matching sites in each of the quadrants; 4) Fold enrichment or depletion of genes with the seed matching sites for cel-miR-67 "$\times$ *times*" the number of genes that is expected by chance alone. Mapping of microarray probes to mRNA transcripts, and transcripts to genes is described in Methods (section 2.7). Pearson correlation between differential expression of all genes (*All genes* – all points) and genes with the seed sites (*With seed* – the points with asterisks), is given at the top of the plots, together with P-value of correlation (in each case it was beyond the precision of the correlation test as implemented via the standard R libraries (RTeam, 2008), which is equivalent to $P < 1e-320$). The blue dashed line is a linear model fitted through the all points (*All genes*), and the black dashed line - through points with asterisks (*With seed*).

Summary of section 4.1

Imaging cultures transfected with an eGFP expressing plasmid proved that the transfection protocol was efficient for delivery of nucleic acids into primary neurons. Transfection of the cultures with a fluorophore labelled oligonucleotide indicated that a majority of neurons was transfected in experiments at 3DIV and 6DIV. Visual inspection and directions of change in expression of neuron specific genes showed that transfections of primary cultures were unlikely to be associated with significant death of neurons. Additionally, measurement of electrophysiological parameters confirmed that transfections did not reduce the total number of active synapses in cultures (Maclaren et al., 2011), i.e. transfections had low toxicity to neurons. Significant miRNA mediated changes in gene expression were detected in transfection of cultures with mimics of several different miRNAs. A significant correlation was observed in differential gene expression that was triggered by transfections of cel-miR-67 mimics in replicate experiments. Therefore, I concluded that transfection protocol was capable of transfecting primary neurons, that transfections of miRNA mimics elicited miRNA mediated effects on gene expression in the cultures and that experimental results were reproducible.

4.2 Improving detection of miRNA targets

4.2.1 The use of miRNA inhibition instead of mock transfection improved detection of putative direct targets

The key modification of the design of the original siRNA transfection strategy (Maclaren et al., 2011), which improved detection of miRNA targets, was the use of cultures transfected with miRNA inhibitors instead of mock transfected cultures. Matched mock transfected cultures were previously used to contrast gene expression changes in transfection experiments (Lim et al., 2005; Selbach et al., 2008; Hendrickson et al., 2009). The contrast of transfected cultures with mock transfected cultures can be justified, because it compensates for the changes induced by technical manipulation and the transfection reagent itself. However, the use of cultures transfected with miRNA inhibitors has an important advantage for miRNA target identification, as it favours detection of direct miRNA targets, because the direct targets are expected to have an inverse response to miRNA overexpression and inhibition. For example, it was demonstrated that upon overexpression of miR-124, its targets were downregulated, and upon miR-124 inhibition – upregulated (Conaco et al., 2006). In the remainder of this thesis, transfection experiments where differential expression was identified through the contrast of cultures transfected with miRNA mimics to mock transfected cultures are referred to as **unidirectional** overexpression experiments. Experiments where cultures transfected with miRNA mimics were contrasted to the cultures transfected with miRNA inhibitors are called **bidirectional** perturbation experiments.

The first miRNA investigated with the bidirectional perturbation strategy was miR-124, as the inverse response of its targets was shown to take place upon overexpression of miR-124 versus its inhibition (Conaco et al., 2006). These bidirectional perturbation experiments were performed at two developmental timepoints (3DIV and 6DIV). The original transfection protocol was employed for this experiment (Methods, section 2.5), and both the mock transfection and the transfection with the miR-124 inhibitor were conducted for comparative purposes.

Transition from the contrast with mock transfected cultures to the contrast with the inhibition increased enrichment of transcripts with miR-124 seed matching sites among the downregulated transcripts (Figure 4.8). In transfections at 3DIV, the P-value of enrichment changed from $1.42e-14$ to $5.82e-29$, and at 6DIV from $6.67e-39$ to $3.11e-43$. Importantly, Sylamer analysis of the distribution of miRNA seed matching

sites also identified the increase of the enrichment (Figure 4.9). In 6DIV experiments, the peak of Sylamer enrichment increased by over 10 orders of magnitude in transition from the use of mock transfection to the miR-124 inhibitor (Figures 4.9c and 4.9d). I concluded that transition from unidirectional to bidirectional experimental strategy improved detection of putative miR-124 targets. This conclusion was based on previously reported observations, where, in miRNA overexpression experiments, downregulated transcripts with seed matching sites for the overexpressed miRNAs were enriched in validated direct targets of these miRNAs (Lim et al., 2005; Giraldez et al., 2006).

Data obtained from bidirectional miR-124 experiments was used to compile the list of miR-124 targets, which will be described in Chapter 5 (section 5.2.1). It was decided to use the bidirectional strategy for identification of targets of other mouse miRNAs, because it worked to improve detection of targets of miR-124. However, it was expected that of all selected miRNAs, transfection of miR-124 would likely have the strongest effect on gene expression in primary neuronal cultures (the selection is described in Chapter 3, section 3.3). Unlike other selected miRNAs, miR-124 was well known for its importance for neuronal biology (Chapter 3, Table 3.3), its expression was shown to neuron specific (Christodoulou et al., 2010; Clark et al., 2010) and it was shown to be one of the most abundant miRNAs in brain (Landgraf et al., 2007). Therefore, to maximise the chances of detection of targets of other miRNAs in bidirectional experiments, it was decided to use miR-124 transfections to optimise the original siRNA transfection protocol for miRNA target detection. The next section describes the use of miR-124 transfections for selection of an optimal post-transfection incubation time and cell plating density.

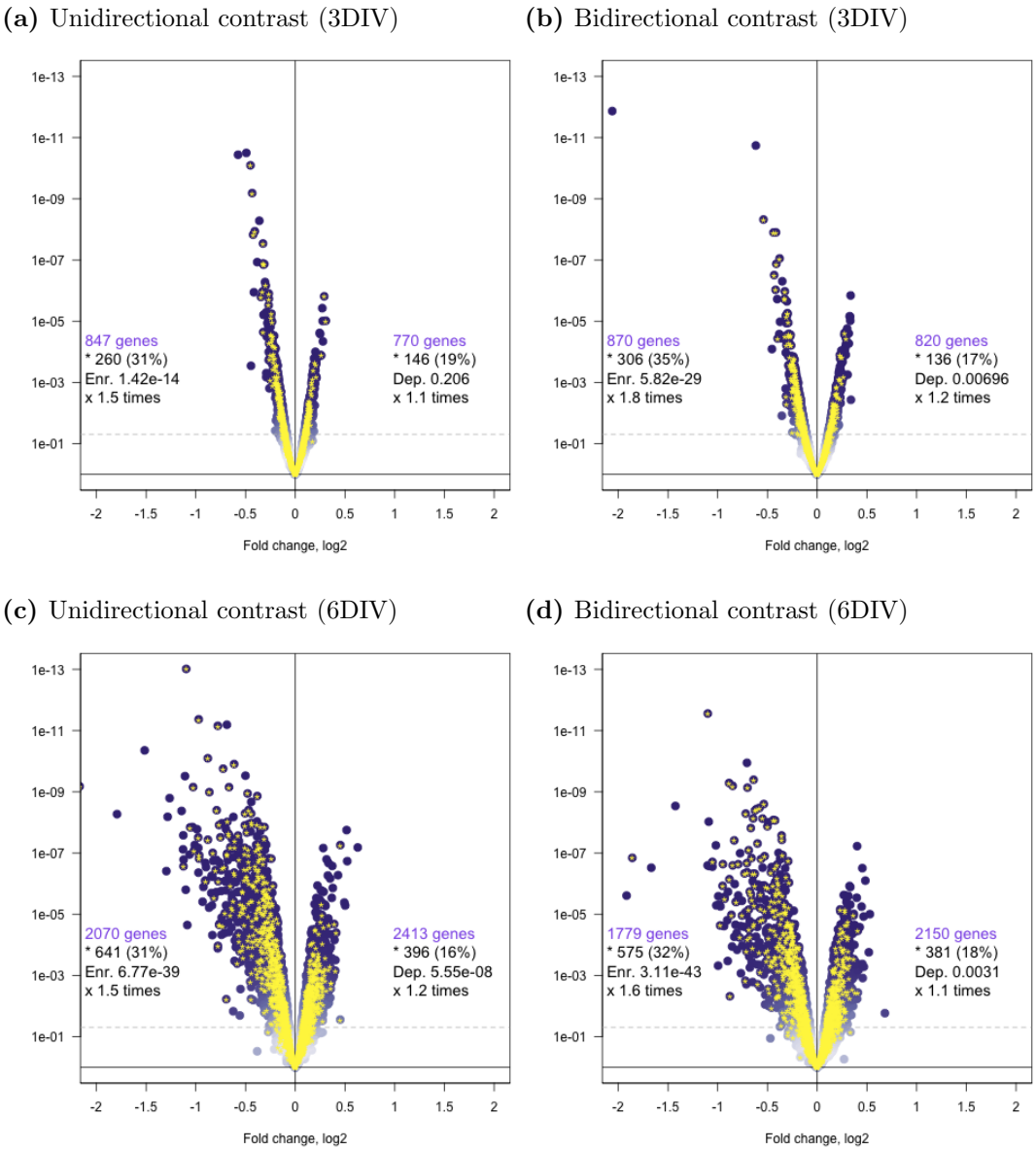


Figure 4.8: Differential gene expression and seed matching site enrichment in miR-124 transfection experiments at 3DIV and 6DIV.
Genes detected by microarrays (using the standard Illumina detection call $P < 0.01$) are shown as the purple dots (the analysis of microarray data is described in Methods, section 2.7). The x-axes represent log_2 of gene expression fold change between samples transfected: 4.8a – with the mimic of miR-124 in comparison to the matched mock transfection at 3DIV; 4.8b – with the mimic of miR-124 in comparison to the transfection with the inhibitor of miR-124 at 3DIV; 4.8c – with the mimic of miR-124 in comparison to the matched mock transfection at 6DIV; 4.8d – with the mimic of miR-124 in comparison to transfection with the inhibitor of miR-124 at 6DIV. The y-axes represent P-value of differential expression (log_{10} scale), and the horizontal dashed grey lines show P-value cutoff of 0.05. The yellow asterisks mark genes [encoding transcripts] with 3′UTRs harbouring one or more seed matching sites (7(2) or 7(1A)-types) for miR-124. The text in the two halves of the plot area provides the following information: 1) The total number of genes with differential expression P-value more significant than the cutoff (0.05); 2) The total number (and percentage) of genes with seed matching sites for miR-124; 3) The hypergeometric P-value of enrichment (*Enr.*) or depletion (*Dep.*); 4) Fold enrichment or depletion of genes with the seed matching sites "× *times*" the number that is expected by chance alone. The mapping of microarray probes to mRNA transcripts, and transcripts to genes, is described in Methods (section 2.7). The identification of the seed matching sites and the hypergeometric enrichment test is in Methods (section 2.8).

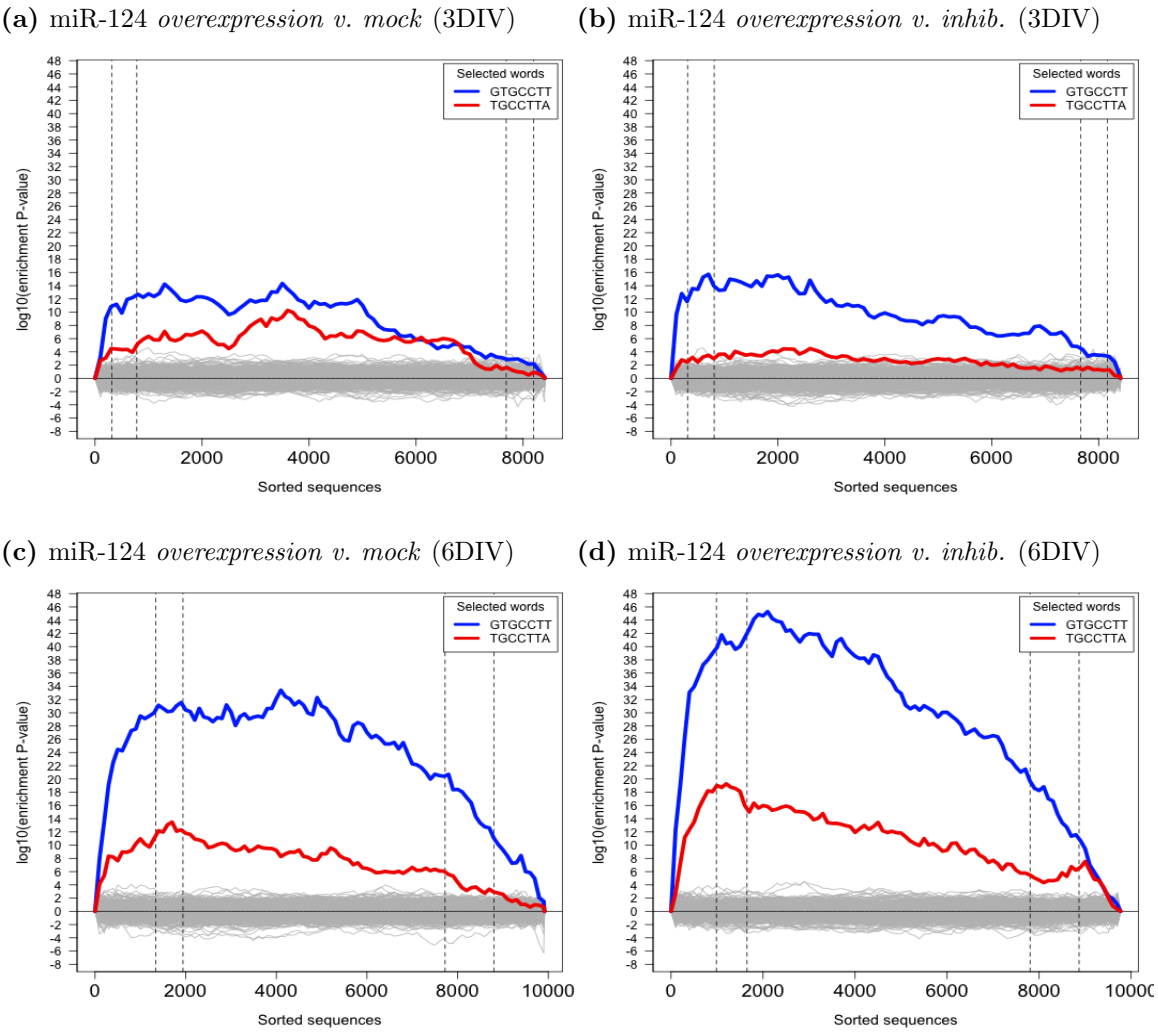


Figure 4.9: Sylamer analysis of biases in distributions of seed matching sites in miR-124 transfection experiments at 3DIV and 6DIV

The x-axes represent sorted 3′UTRs corresponding to detectably expressed genes (detected with the standard Illumina detection call $P < 0.01$, see Methods, section 2.7). These genes are ordered **from the most downregulated to the most upregulated** by fold change t-statistic for differential expression in the following transfections: 4.9a – with the mimic of miR-124 in comparison to a mock transfection at 3DIV; 4.9b – with the mimic of miR-124 in comparison to transfection with the inhibitor of miR-124 at 3DIV; 4.9c – with the mimic of miR-124 in comparison to a mock transfection at 6DIV; 4.9d – with the mimic of miR-124 in comparison to transfection with the inhibitor of miR-124 at 6DIV. The y-axes represent the hypergeometric P-values for occurrence biases of 876 nucleotide words complementary to the seed regions (7(2) and 7(1A)-types) of the complete set of 581 distinct mouse miRNAs, according to miRBase Release 14 (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). Positive values on the y-axes correspond to an enrichment ($+|log_{10}$(P-value)|) and negative values to a depletion ($-|log_{10}$(P-value)|). The vertical dashed lines mark the P-value cutoffs (0.01 and 0.05) on both sides of the ranked gene lists. The blue and the red lines show the enrichment profiles of 7(2) and 7(1A)-type seed matching sites for miR-124. The grey lines show the enrichment for the rest of the distinct seed matching sites. The mapping of microarray probes to mRNA transcripts, and transcripts to genes, is described in Methods (section 2.7). The identification of the seed regions and parameters of Sylamer (van Dongen et al., 2008) is in Methods (section 2.8). The full description of the Sylamer method is in the Introduction (section 2.8).

4.2.2 Selection of an optimal incubation time and cell plating density

A long post-transfection incubation time was used in transfection experiments described up to now (36h or 48 h), because similarly long post-transfection times were shown to be efficient in siRNA experiments (Maclaren et al., 2011). However in mutant ES cell lines with deficient miRNA biogenesis, ectopic expression of miRNAs was shown to have the strongest effect on their targets at a short incubation time of $\approx$ 10 h (Matthew Davis, personal communication) and between 12 h to 16 h (Hanina et al., 2010)). Therefore, test experiments were carried out in order to assess the effect of miR-124 transfections on gene expression upon relatively short post-transfection incubation times (i.e. 24 h or less).

Based on miR-124 experiments described in section 4.2.1, two genes were selected as indicators of the impact of miR-124 transfection on gene expression. One of these was Lass2, a previously identified direct target of miR-124 (Conaco et al., 2006). As expected for a direct miRNA target, the changes in the expression level of Lass2 were subtle: the fold change difference between inhibition and over-expression in experiment at 3DIV was 1.074 fold ($P < 0.1$) and at 6DIV it was 1.61 fold ($P < 3.15e-05$). In addition to Lass2, another gene, Acta2, was chosen as an indicator of the effect of miR-124 transfection. Acta2 does not have a seed-matching site and may be an indirect target. However it showed bigger changes in expression (4.17 fold with $P < 1.36e-12$ at 3DIV, and 3.78 fold with $P < 2.48e-06$ at 6DIV), thus Acta2 was useful as an additional indicator of transfection efficiency.

To deduce if shorter incubation times were conducive to a bigger contrast in expression of targets in bidirectional miR-124 perturbation experiments, expression levels of Lass2 and Acta2 were assessed with qRT-PCR over a timecourse after transfection. For this, transfection of miR-124 was conducted at an intermediate developmental timepoint (4DIV) and total RNA was collected from cultures at 4 h, 12 h, 24 h and 36 h (see Methods, section 2.5). Subsequently, qRT-PCR with primers for Lass2 and Acta2 was performed as described in Methods (section 2.3). Differential expression of Lass2 and Acta2 was estimated using $\Delta\Delta$Ct method (Methods, section 2.3). Surprisingly, in contrast to results reported in the ES cells ((Hanina et al., 2010) and Matthew Davis, personal communication), the most significant differential expression of both Acta2 and Lass2 was observed at a relatively long incubation time of 36 h (Figure 4.10). This indicated that

a long post-transfection incubation time (36h) was likely to enable the most consistent detection of miRNA mediated effects.

In the next experiment, 36 h and 48 h incubations were compared. To achieve a better separation of differential expression values between 36 h and 48 h, transfection in this experiment was performed at 6DIV. The 6DIV timepoint was selected for this experiment, because results of the microarray profiling indicated a stronger miR-124 effect at 6DIV than at 3DIV (see above), and the qRT-PCR timecourse experiment at 4DIV confirmed that changes in expression of miR-124 targets were very subtle (Figure 4.10). Additionally, plating cells at a relatively lower cell plating density was tested in this experiment. For this experiment cells were plated at a density of 790 cells $\cdot$ mm^{-2}, which was over two times lower than the cell density used in experiments up to now (Methods, sections 2.5 and 2.3). The cell plating density was reduced, because in siRNA transfection experiments plating primary cultures at relatively low densities (400 to 800 cells $\cdot$ mm^{-2}) was found to increase the knock-down efficiency at the protein level (Esperanza Fernandez, personal communication).

In comparison between 36 h and 48 h post-transfection incubation, inhibition of the direct target, Lass2, was the strongest at 36 h: $\Delta\Delta$Ct was 0.924 and 0.584 at 36 h and 48 h respectively. Additionally, in agreement with the proposition of lower cell density to favour higher knock-down efficiency (Esperanza Fernandez, personal communication), lower cell plating density appeared to increase the differential expression contrast of Lass2 at mRNA level. If $\Delta\Delta$Ct at 36 h was converted into a fold change ($\Delta\Delta$Ct 1 $\approx$ 2 fold difference), then Lass2 inhibition in the lower density 6DIV experiment was bigger than that detected by microarrays in the 6DIV transfection experiment with the higher cell plating density (see above).

In summary, relatively short post-transfection incubation times (24h or less) were not identified to be significantly more efficient than longer incubation times at generating the expression contrast of miR-124 targets in bidirectional perturbation experiments. However, the results described in this section showed that several relatively minor changes to the transfection protocol could improve detection of direct miRNA targets. These changes were lowering the incubation time from 48 h to 36 h and reducing cell plating density. Therefore, these changes to the settings of the protocol were used for bidirectional per-

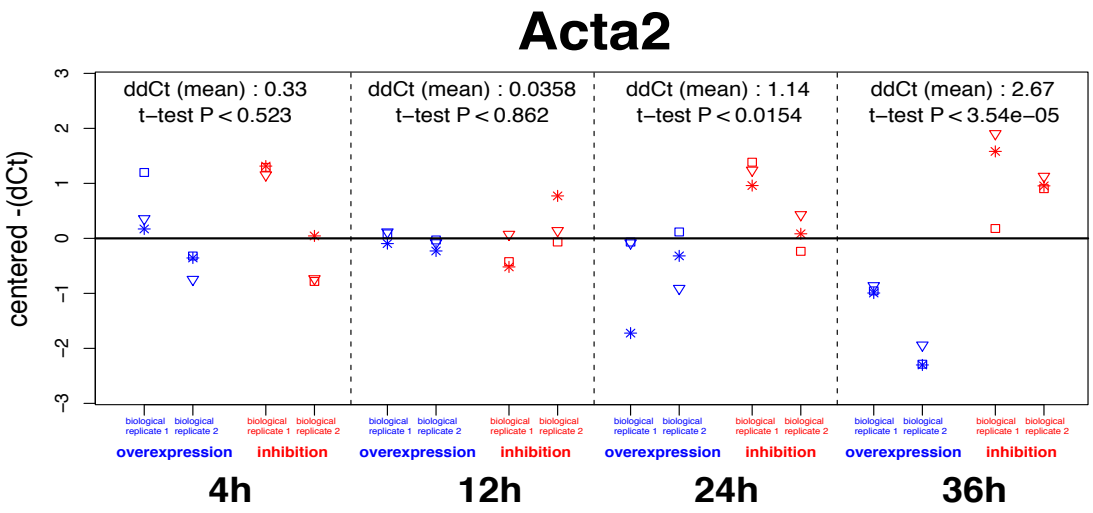
turbation experiments on the miRNAs selected for functional experiments[1] (the selection is described in Chapter 3, section 3.3).

Summary of section 4.2

The detection of miR-124 mediated inhibition of gene expression was more efficient when transfections with the mimic were compared to transfections with the inhibitor, rather than to mock transfections. Following a robust identification of miR-124 mediated effects in miR-124 transfection experiments, series of these experiments were used to improve upon the original transfection protocol. A drastic reduction of post-transfection incubation time did not significantly increase detection of miR-124 mediated inhibition of its targets, however an improvement was detected upon a 12 h reduction of post-transfection incubation time and reduction of cell plating density. Therefore the bidirectional strategy and the adjusted protocol were used to identify targets of all other selected miRNAs (with the exception of cel-miR-67, for which the inhibition was not available), which is described in the next chapter.

[1] Apart from miR-124, for which 48 h incubation and higher cell density worked satisfactorily (section 4.2.1), and cel-miR-67 that was not present in the mouse genome and its inhibition was not logistically possible.

(a)



(b)

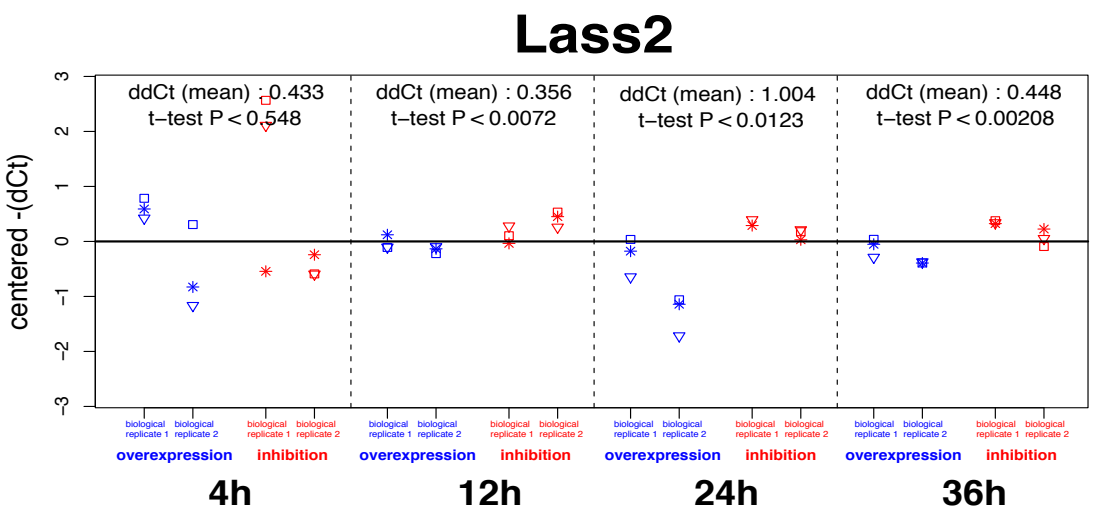
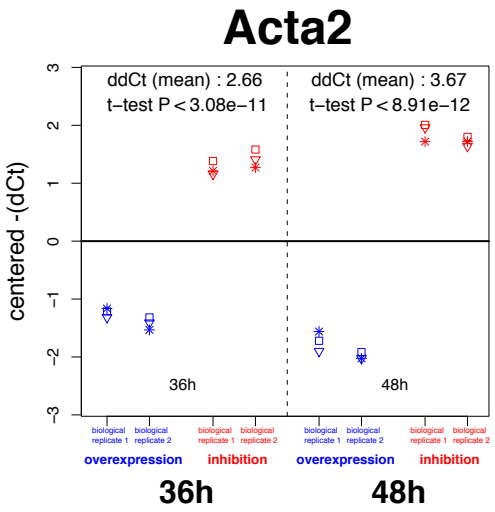


Figure 4.10: qRT-PCR profiling of the effect of miR-124 transfection in a high cell plating density timecourse at 4DIV.

The x-axes correspond to biological replicates of transfected samples (*overexpression* - with the miR-124 mimic, *inhibition* - with the miR-124 inhibitor. The y-axes correspond to inverse ΔCt values centered around the experimental medians. The styled points correspond to ΔCt values for technical replicates per one biological replicate. The text gives the mean of ΔΔCt values and the t-test P-value for the differential expression. The ΔΔCt method is described in the Methods (section 2.3).

(a)



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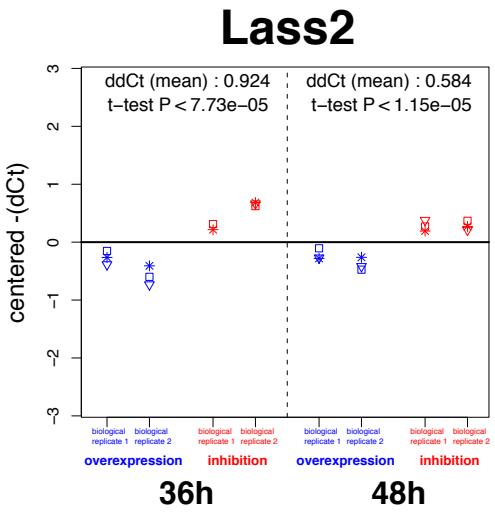

Figure 4.11: qRT-PCR profiling of the effect of miR-124 transfection in a low cell plating density timecourse at 6DIV.
See Figure 4.10 for description.