ASSET: Analysing and Striking the Sensitivities of Embryonal Tumours

ASSET WP 3 – microRNA expression in ETs: rRegulation of by ET oncogenes identification of targets & effects

Deliverable D 3.1 - miRNAs regulating ET Cell viability

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Dissemination Level		
PU	Public	
PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission	
CO	Confidential, only for members of the consortium (including the Commission Services)	X

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Introduction

MicroRNAs (miRNAs) are central connections between the genome, transcriptome and proteome. Their crucial roles in cancer are increasingly recognised. The objective of this deliverable, and task 3.2. was 'global approaches to identify critical miRNAs for embryoid tumor (ET) cell viability using high-throughput screening (HTS)'. VTT Medical Biotechnology is equipped to conduct high throughput screening in a 384 well plate format using a robotic HTS screening system (Figure 1). The objective was to make high-throughput cell-based miRNA transfection screens with a comprehensive library of >800 pre-mirs (Dharmacom miRIDIAN Mimics (N=810) As VTT has also a library of miRNA inhibitors (Dharmacom miRIDIAN inhibitors (N=896)) these were also screened. These screens will generate data concerning ET cell proliferation.

Methods

The pre-mir and inhibitor miRNA cell viability screens were performed in the selected cell lines and conditions per tumour type and conducted in at least two biological replicates.

The three model systems selected for the screens were:

- NB: N-myc-inducible NB (SY5Y) cells +/- induction (from Frank Westermann, DKFZ).
- MB: c-Myc-inducible MB (UW-228) cells +/- induction (from Alex Arcaro, UBERN).
- ESFT: conditional EWS-FLI1 knockdown ESFT (ASP#14) cells +/- induction (from Heinrich Kovar, CCRI).

The miRNA libraries used in the screens were:

miRNA libraries	miRNAs	Plates (n)
Dharmacon miRIDIAN® mimics	810	3
Dharmacon miRIDIAN® inhibitors	896	3
Total 384-plates (3 model systems +/- induction) x 2 repeats	--	72 (actual 84*)

* For SY5Y neuroblastoma model 3 repeats had to be made because the first screen was not successful (low cell viability due to transfection reagent lot differences and low MYCN induction).

ASP14 cells were grown in D-MEM (glucose 4.5 g/l) supplemented with L-glutamine, 10% tetracycline free FCS (Clontech) and pen/strep in the presence with 50 µg/ml Zeocin (Invitrogen) and 2 µg/ml Blasticidin (Sigma). To induce the expression of EWS-FLI1 shRNA, 1 µg/ml doxycycline (Sigma) was added to the cells. For ASP14 cells the screens were initiated 72 h after shRNA induction and 1700 cells were transfected using SilentFect (Bio-Rad, 0.07 µl/well).

SY5Y/6TR (EU)/pTrex-Dest-30/MYCN cells were grown in RPMI 1640 (without Hepes) supplemented with 10% FBS and pen/strep (100 µg/ml), blasticidin (7,5 µg/ml), G418 (200 µg/ml) and HEPES (25 mM). The screens were initiated in the absence and presence of tetracycline: 70% EtOH (solvent control) or Tetracycline (1 µg/ml) treatment was done as instructed for 1 day. 1500 cells were transfected using SilentFect (Bio-Rad, 0.07 µl/well).

UW228 Myc/ER cells were grown in D-MEM (glucose 4.5 g/l) supplemented with L-glutamine, 10% FCS and pen/strep in the presence with 1 µg/ml Puromycin. Tamoxifen (Sigma) treatment was done as instructed; Puromycin was removed from the growth medium 24 h before 4-OTH treatment (1 µM). The screens were initiated 24 h after 4-OTH treatment. 1500 cells were transfected using SilentFect (Bio-Rad, 0.07 µl/well)

The final concentration of pre-mirs (Dharmacon miRIDIAN Mimics) and anti-mirs (miRIDIAN inhibitors) was 19 nM and for siRNAcontrols (AllStars death, AllStars negative, PLK1 siRNA and KIF11 siRNA) 15 nM in a total volume of 50 µl. Cell viability was measured in response to 72 h transfection using CellTiterGlo reagent (Promega).
The raw data were normalized using a new, statistically robust, screening data normalization method, implemented in R *(reference:* R Development Core Team *2011)*, that down-weights outliers on the plate before calculating the loess fit, termed loess- log. After loess correction, data was divided by the median of negative controls and log2-transformed.

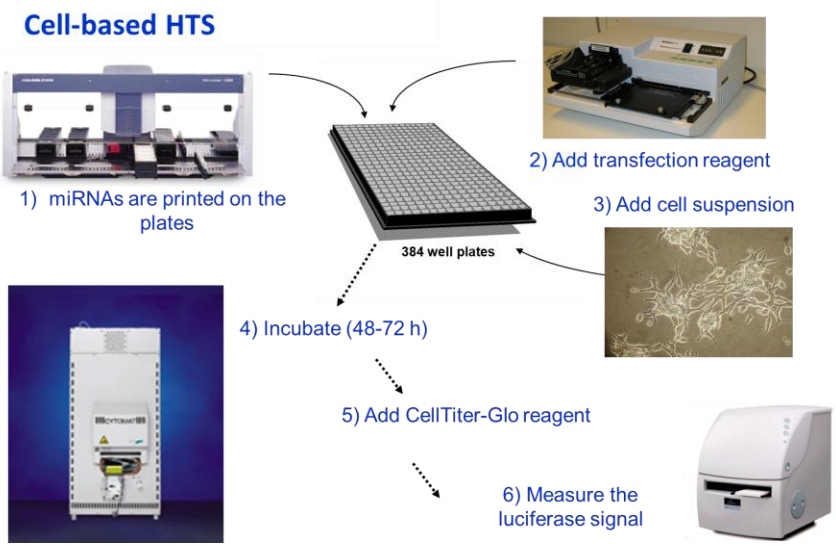


Figure 1. Scheme of the high throughput screening system in place at VTT Medical Biotechnology consisting of robotics, plate fillers, incubators and plate readers.

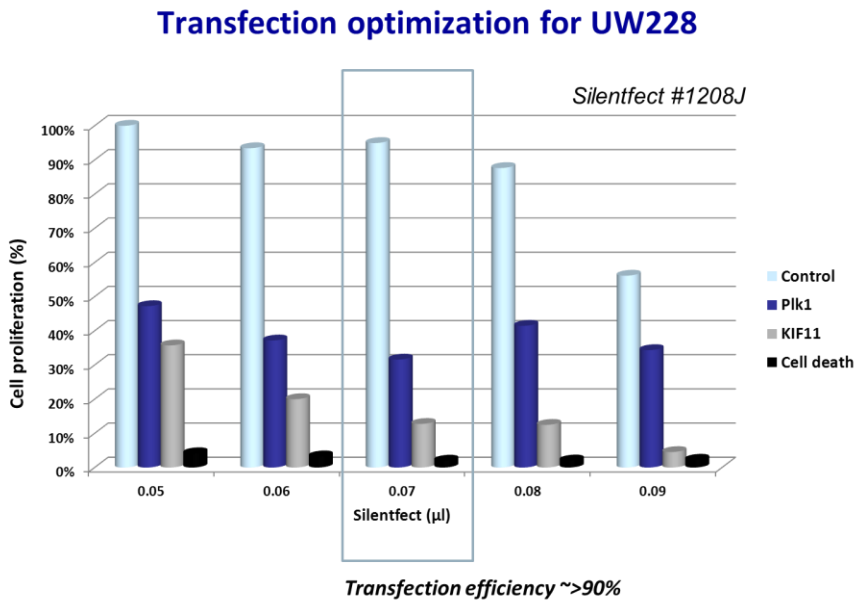
Results

Transfection optimization

Before HTS screens the cell number, transfection efficacy and oncogene / shRNA induction was optimized/ tested for 3 model cell lines in 384-well format. An example of the transfection optimization is shown below in figure 2 for medulloblastoma UW228 cell line and in figure 3 for neuroblastoma and Ewings sarcoma cell lines.

Transfection efficiency was between 60% and 90% depending on cell line. This was well acceptable for screening.

Figure 2. Transfection optimization for medulloblastoma UW228 MYC/ER with Silentfect transfection reagent.



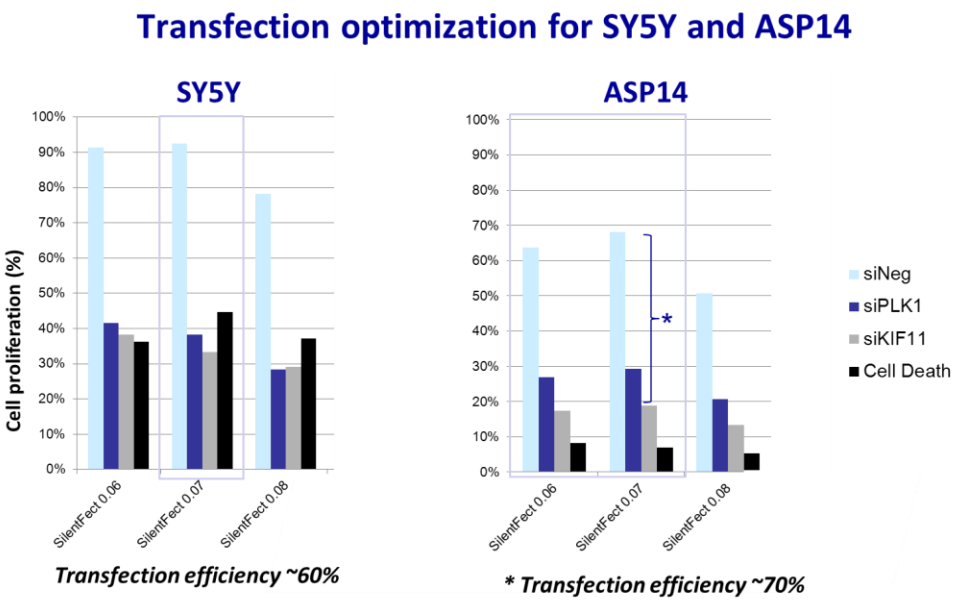


Figure 3. Transfection optimization for Neuroblastoma SY5Y/6TR (EU)/pTrex-Dest-30/MYCN and ASP14 Ewings sarcoma with Silentfect transfection reagent

The miRNA high throughput screening results

The pre-mir and inhibitor miRNA cell viability screens were performed in the selected cell lines and conditions per tumour type and conducted in at least two biological replicates as described previously. In total this yielded 84 plates in 384-well format. Cell proliferation data was normalized for analysis to get rid of potential artefacts

caused by evaporation in the edge wells. Statistics with three standard deviations below the mean values of the screen was employed to find miRNAs that affect cell proliferation during oncogene induction or with no induction. Positive controls such as All Stars Cell death (Qiagen) or PLK1 and KIF11 siRNAs were proliferation reducing hits regardless of the induction, and thus proved that the transfection worked well during the screens for all cell lines. An example for this is shown in Figure 4 for **ASP14**. Also the EWS-FLI1 silencing worked well; EWS-FLI1 was silenced by 90%. The hsa-mir-491 was found as a proliferation reducing hit specifically in the absence of EWS-FLI1 in both screens (reduce cell viability selectively in the presence of doxycycline) (data not shown). More PreMir hits, namely nine, were found in the presence of EWS-FLI1 (Figure 5). Thus decrease in EWS-FLI1 expression reduced the number of identified hits. Hits identified only in the presence of EWS-FLI1 could indicate oncogene induced vulnerabilities; we proposed hsa-miR-631,hsa-miR-421, hsa-let-7f-2*, hsa-miR-9* and hsa-miR-552 for validation

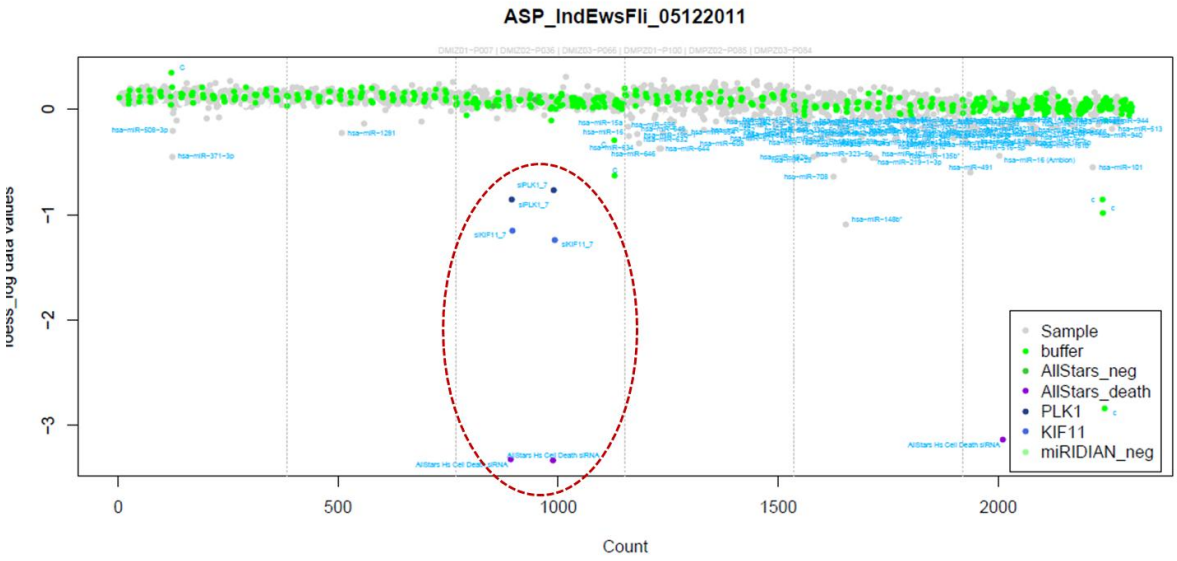


Figure 4. Cell viability results of pre-mir and anti-mir screens performed with ASP#14 Ewing´s sarcoma cells with induction: EWS-FLI1 shRNA expression on (1 µg/ml doxycycline). The pre-mirs (Dharmacom miRIDIAN Mimics) were distributed in three 384 well plates (on left hand side) and the anti-mirs (miRIDIAN inhibitors) in another three 384-well plates. The results from wells derived from different plates are separated in the figure using dashes lines. The positive controls (AllStars death, PLK1 siRNA and KIF11 siRNA) can be clearly identified due to their growth inhibitory effect whereas the majority of negative controls (buffer, AllStars negative control, miRIDIAN) are located close to zero (no effect on cell viability), indicating that the HTS has functioned as expected. The red ellipse denotes the positive cell death controls in the screen that work well.

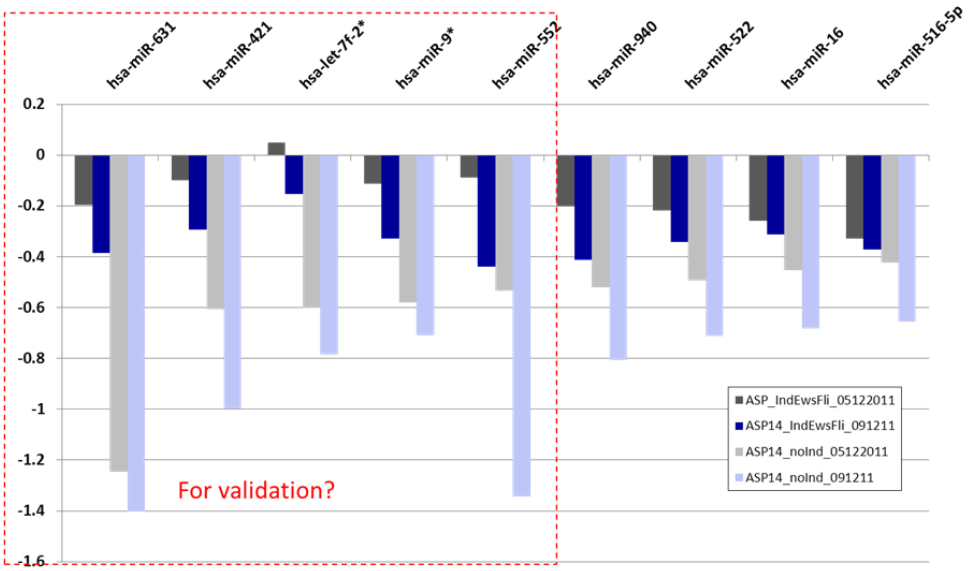


Figure 5. Cell viability results of pre-mir and anti-mir screens performed with Ewings sarcoma model ASP14. Shown are proliferation-reducing-hits in the presence of EWS-FLI1 (no doxycycline). Two of the first columns with darker shades are with doxycycline induction and two of the latter columns with lighter shades are without doxycycline thus in the presence of EWS-FLI1 oncogene. Boxed area shows best hit miRNAs where difference between no induction and doxycycline induction seemed biggest in the two replicate screens.

For **UW228** with inducible c-Myc construct five precursor miRNAs (hsa-miR-509, hsa-miR-644, hsa-miR-339-3p, hsa-miR-148a*, hsa-miR-892a) and two miR inhibitors (mir-891b and mir-106b) were identified to reduce cell viability only in the 4-OTH treated thus c-Myc expressing UW228 Myc/ER medulloblastoma cells (Figure 6).

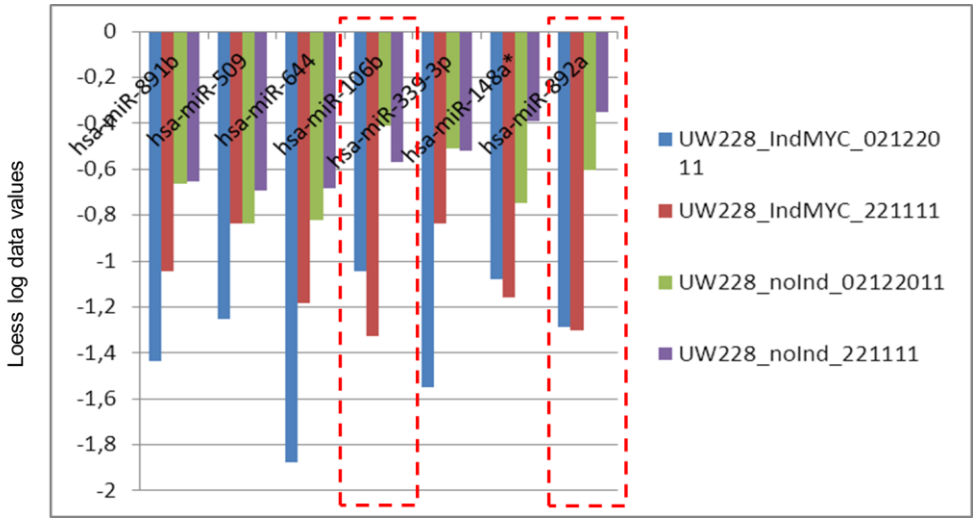


Figure 6. Cell viability results of pre-mir and anti-mir screens performed with UW228 medulloblastoma model with tamoxifen inducible c-Myc. Boxed are most clear proliferation reducing hits.

Twelve cell viability reducing hit miRNAs were found only in the absence of 4-OTH treatment in UW228 Myc/ER medulloblastoma cells. These were precursor miRNAs: hsa-miR-600, -21*,-516b*-3p-2, -516a-3p-2, -377, -383, -129-1, -193b*, -183, -182, -181b-1, -216b.

SY5Y/6TR (EU)/pTrex-Dest-30/MYCN neuroblastoma cells were the most difficult ones to transfect as compared to other ET cells. The transfection efficiency was around 60% (Figure 3.) and therefore we had to

make three screens as the first one did not perform optimally. But the transfections in the 2nd and 3rd HT-miRNA screens worked: PLK-1 and KIF11 positive control siRNAs could be identified (see figure 7). MYCN induction was about 4-6 fold during screens.

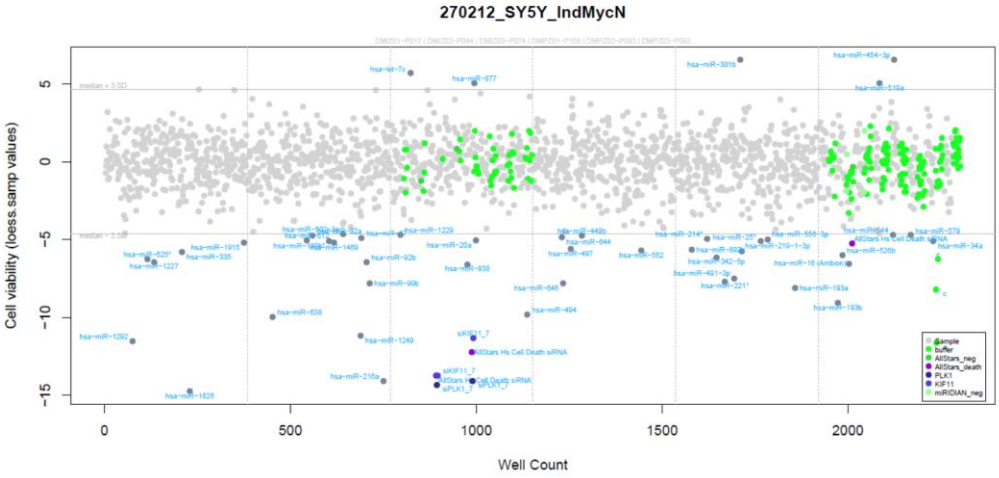


Figure 7. Cell viability results of pre-mir and anti-mir screens (3rd screen) performed with SY5Y/6TR (EU)/pTrex-Dest-30/MYCN neuroblastoma cell model with NMYC expression induced with tetracyclin.

The 3rd screen gave biggest amount of proliferation hits and had the best induction. However, between the two screens the proliferation differences were small and not well repeating, therefore not many common repeating hits were found. Based on 2nd and 3rd screens five miRNAs were identified to selectively reduce cell viability in Tet treated SY5Y/6TR (EU)/pTrex-Dest-30/MYCN cells in both screens. Pre-mirs hsa-miR-552 and hsa-miR-221* were the strongest MYCN specific hits if Loess log was used as normalization method. These should be validated. Two pre-miRs inhibited cell viability regardless of induction in both screens (hsa-miR-193b and hsa-miR-193a). Several hits were seen only based on the 3rd screen but these could be also validated if resources permitting.

Validation

Medulloblastoma (UBERN)

With the aim of identifying critical miRNAs involved in medulloblastoma (MB) cell proliferation and survival, the medulloblastoma cell line UW228 expressing an inducible c-Myc construct, was used as cellular model for this study. The HTS (high-throughput screening) analysis (VTT) identified 7 miRNA candidates involved in cell proliferation in c-Myc over-expressing MB cells. First, we validated the selected miRNAs by performing a cell viability proliferation test (MTS) under the same conditions as in the HTS screen. The analysis was initiated 24 h after 4-OTH treatment and 1500 cells were transfected using SilentFect (Bio-Rad, 0.07µl/well). As described above, the most prominent cell proliferation reduction in c-Myc-overexpressing MB cells was observed after inhibition of hsa-miR-106b or induction of hsa-mir-892a. The impact of the miRNA mimics and antagomirs on expression of the endogenous miRNAs in UW228 cells was validated by quantitative RT-PCR. To understand the role of the miRNA candidates in MB cell proliferation and survival in more details, we performed several bioinformatics analysis in order to identify their potential targets involved in this process. Via GeneGo/Metacore analysis and in collaboration with UCD, we identified a potential regulatory role of hsa-miR-106b in TGF□, IL-6, MAPK and JAK-STAT pathways, all known to be involved in MB pathogenesis. More precisely, has-miR-106b is known to inhibit PTEN and Smad3 and interact with MAPK9. According to the literature, miR-106b is up-regulated in MB compared to the adult subtype and is mostly expressed in the anaplastic subtype of MB. It is also known to regulate neuronal stem/progenitor cells proliferation and differentiation in primary cultures of stem/progenitor cells of mice (Brett et al. 2011, Wang et al 2012, Peck and Schulze 2011). Furthermore, studies on endometrium cancer demonstrated that myc-dependant Apoptosis inhibition was induced in miR-106b dependant manner (Zhao et al 2012). Very few published data are available on the second candidate hsa-miR-892a. After bioinformatics analysis we identified a potential role in PKA and GRPC signalling networks and also an involvement in the cancer cell metabolism.

Concerning the other candidates identified in the HTS screen:

Hsa-miR-509-5p: The bioinformatics analysis predicts a role in CRMPs signalling and histamine metabolism. When up-regulated, a decrease of apoptosis and increase of proliferation was observed in renal cancer (Hidaka et al 2012).

Hsa-miR-891b: The bioinformatics analysis predicts a role in insulin and TGF□□signalling.

Hsa-miR-644: GeneGo analysis revealed only the androgen receptor as a potential target

Hsa-miR-148a: GeneGo analysis revealed PTEN, PI3K p55□ and Bim as potential targets. The bioinformatics analysis also predicts a role of Hsa-miR-148a in TGF□ and Wnt signalling.

Hsa-miR-148a is up-regulated in the WNT subtype of MB and has a tumour suppressive effect in MB (Gokhale et al. 2012).

Hsa-miR-339-3p: the bioinformatics analysis predicts a role in the Hh and WNT pathways. According to the literature, Hsa-miR-339-3p was reported to be most uniformly expressed in primary MB specimens.

We are currently focusing on the analysis of the downstream targets of hsa-miR-106b in MB cells. In addition, we are studying the role of the selected miRNAs in the TGF□ pathway, since this pathway has been postulated to be a potential avenue to develop new therapies in c-Myc-over-expressing MB.

Ewings sarcoma (CCRI)

Validation of two microRNAs identified by VTT in WP3 to reduce growth of the Ewing sarcoma cell line model Asp14 only in the presence but not in the absence of EWS-FLI1 (hsa-mir-631 and hsa-mir-552) is ongoing. So far we demonstrated that miR-552 reproducibly induces a G1 arrest while miR-631 elicits a G2 cell cycle arrest in the model cell line. None of the two microRNAs was found to be expressed in Ewing sarcoma cell lines, neither in the presence nor in the absence of EWS-FLI1, nor were they found to be present in mesenchymal stem cells that serve as a relevant common control for Ewing sarcoma.

Neuroblastoma (DKFZ) -Part 1.

miRNA high-throughput functional screening identifies several potential tumour suppressive miRNAs in MYCN-overexpressing neuroblastoma: 1p revisited

Background and results: So far, most of the functional studies on miRNA in NB have been based on the miRNA expression data (i.e. from RT-qPCR, array platforms or small RNA library sequencing) and have been so far aimed at single miRNA species. In this project, we established together with the VTT large scale functional screening of NB miRNAome using the gain-of-function (with miRNA mimics library) and the loss-of-function (with miRNA inhibitors library) approaches. Phenotypic miRNA high-throughput functional screening data were generated at the VTT using MYCN-inducible SY-5Y cells and were analyzed first for putative synthetically lethal miRNAs with high MYCN. However, only few candidates, which fit this criterion, were retrieved. Therefore, the potential of miRNA screening was not confined to the concept of synthetic lethality, because new tumor suppressive miRNAs might be identified as well. Using these criteria, we selected 180 miRNA species from the original large scale miRNA functional screening. The selected miRNAs were mapped to the human genome. Interestingly, this analysis retrieved five miRNAs from 1p, which showed striking growth inhibitory potential. This set included well-known mir-34a. Remarkably, the remaining four candidates have not yet been functionally characterized in NB. Moreover, dozens of growth inhibitory and growth promoting miRNAs were mapped to two

largest miRNA clusters in the human genome, C19MC at chromosome 19 and 14q32 miRNA cluster. This finding is particularly interesting, because these clusters have been shown to play a role in pathogenesis of PNET and gliomas (Li et al., 2009; Lavon et al., 2010). Further, out of 180 candidates, four top candidates that demonstrated the most prominent growth inhibitory effect, depending on or regardless of MYCN expression effect, have been selected for "targeted validation". In subsequent experiments we tried to gain insight into the molecular machinery behind the growth inhibition. We utilized TET21N, SY-5Y-MYCN as well as SH-EP and IMR32 cells and found that all four miRNA candidates were able to activate p53 pathway. The strongest effect on p21 and BAX expression was rendered by "chromosome 1 candidate" miR-552.
Perspectives: We plan to assess the phenotype of miRNA-fransfected cells with several biochemical methods, i.e. colorimetric assaying for senescence and FACS-based analysis of apoptotic markers (sub-G1 peak, PS externalization and caspase-3/7 activity). The important point in our validation will be the characterization of the response of NB cells with modulated expression of candidate miRNAs to anti-cancer drugs. Finally, we plan to characterize the targets of selected miRNAs using SILAC method.

Neuroblastoma (DKFZ) -Part 2:

miR-873 is a putative tumor suppressor that can participate in spontaneous regression program in NB

Background: Agents that are able to reactivate wild type p53 or employ p53-independent tumor surveillance pathway in TP53-mutated background are of particular interest. miRNAs may have great potential in reactivating both of these networks in neuroblastoma. Several known and novel miRNA loci located within consistent regions of chromosomal losses in neuroblastoma were detected in our previous study; and one of the candidate miRNAs, miR-885-5p, was found to reactivate p53 pathway in TP53-wild type neuroblastoma via inhibiting CDK2 leading to growth arrest and senescence in neuroblastoma cells (Afanasyeva et al., 2008; Afanasyeva et al., 2011).
Results: *miR-873* at 9p21.3, a highly conserved mammalian miRNA, proximal to FRA9C gene, *LINGO2,* was cloned from 4S/4 NBs. In a small portion of primary NB, *miR-873* is codeleted together with the *INK4a/ARF* locus. *miR-873* expression correlates with good prognosis in NB. NB cell lines are presented with low steady state *miR-873* expression level. Very recently, *miR-873* has been identified as one of top miRNAs which are downregulated in glioblastoma vs normal brain (Skalsky et al., 2011). Further on, we found that enforced *miR-873* expression reduces viability and anchorage independent growth in p53 wild type and p53-disabled neuroblastoma. miR-873 enforced expression partially restored G1-arrest upon doxorubicin treatment in SK-N-BE(2)c and SH-EP cells (Figure 8). Although caspase-3 was activated by miR-873 enforced expression, and several proapoptotic Bcl-2 family proteins, BAX, PUMA and BID, were upregulated, classical apoptotic death was not induced; and the cells remained arrested. Together, these results prompted us to test miR-873 potential in switching differentiatory program. Retinoic acid is known to differentiate NB cells (i.e. SK-N-BE(2)c) into neurons; and BrdU induces glial differentiation. We revealed that BrdU treatment of SH-EP and SK-N-BE(2)c induced *miR-873* expression. Vice versa, treatment with retinoic acid downregulated *miR-873* in SH-EP and SK-N-BE(2)c. Moreover, the upregulation of glial markers, GFAP and CNPase, was detected upon miR-873-enforced expression. Together, these data indicate that miR-873 induces glial rather than neuronal differentiation features. Four NB-relevant miRNA candidate targets were retrieved from microrna.org and starbase databases: BMI1, CHK1, HNRNPA0 and HNRNPK. Two predicted miR-873 targets, CHK1 and BMI1, are particularly interesting, BMI1 downregulation in SK-N-BE(2)c is known to lead to glial differentiation (Cui et al., 2006). CHK1 downregulation leads to growth arrest in neuroblastoma (Cole et al., 2011). We found that all four candidates are downregulated in the cells with miR-873–enforced expression.
Perspectives: The impact of glial differentiation program to NB regression is known (Gershon et al., 2009). We plan to validate this in our project by using NB cells with inducible miR-873 expression as xenografts in nude mice. The second focus is recapitulation of miR-873-induced phenotype using siRNAs to its targets, BMI1, CHK1, and possibly, to HNRNPA0/K. The third focus is to dissect regulation of miR-873 expression, particularly, the role of PHOX2b and its transcriptional target TLX2 whose binding site is located in the vicinity of *miR-873*).

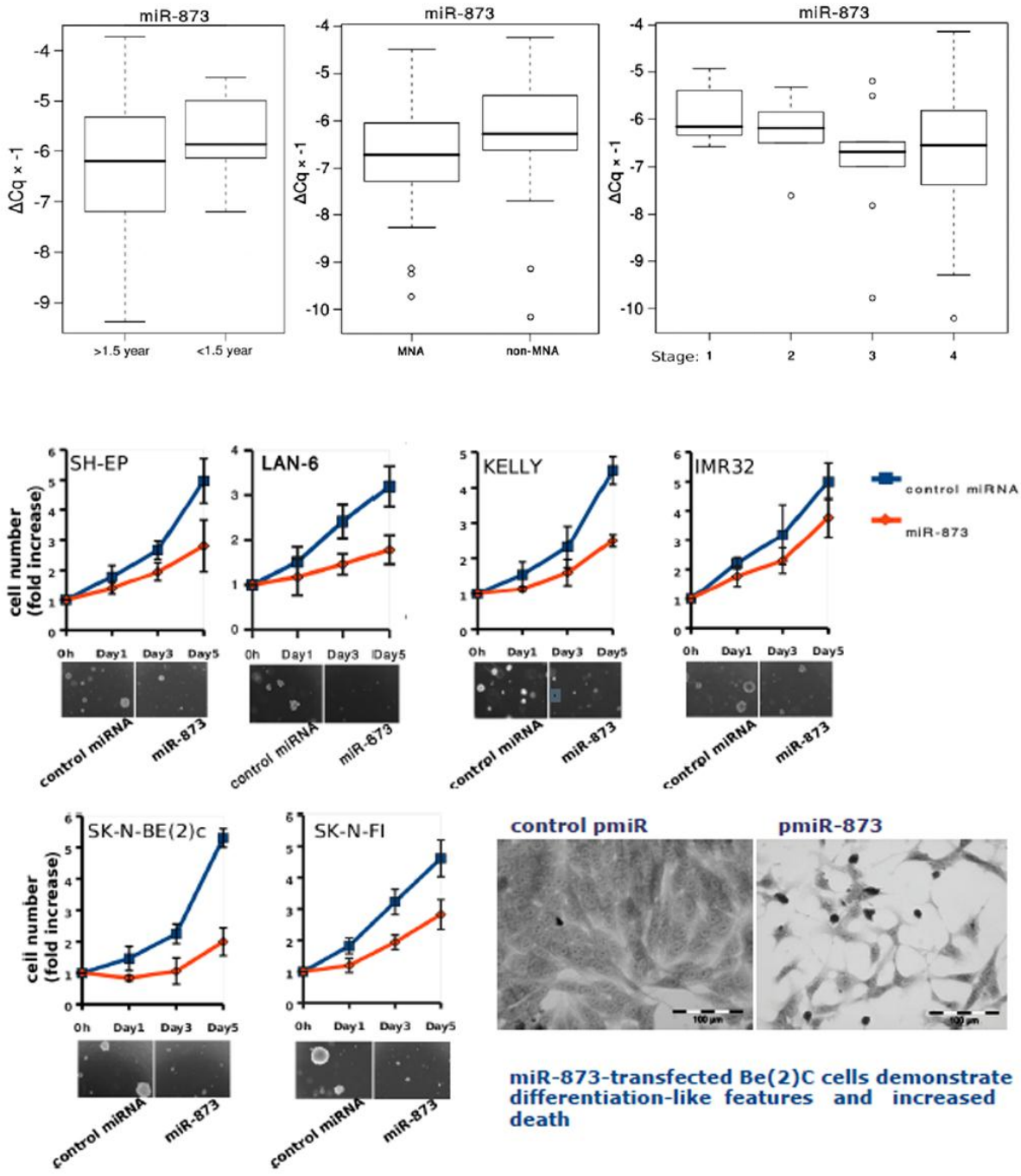


Figure 8: *miR-873* is differentially expressed in primary NB. Enforced expression of miR-873 reduces viability of p53 wild type and p53 disabled NB.

Conclusions and recommendations

This deliverable was aimed at the analysis of the miRNAs regulating ET Cell viability. Three pediatric model systems were selected, namely medulloblastoma, neuroblastoma and Ewings sarcoma. Each model system was presented with a model cell line with inducible or silenced oncogene. High-throughput screening of the miRNAome using the gain-of-function (with miRNA mimics library) and the loss-of-function (with miRNA inhibitors library) approaches were applied to the model systems and reduction in proliferation was used as read-out to analyse ET cell viability. Screens resulted in some putative synthetically lethal miRNAs for each oncogene and more generally in the cell viability reducing miRNAs irrespective of the oncogene expression. Validation of these findings is now underway, with some interesting findings already present and reported in this deliverable. The validation process for miRNAs is a complex one as it usually includes validation of the cell viability effect and more thorough search for the miRNA targets. Each miRNA can target either directly or indirectly several hundreds of targets. Therefore the validation process will take time beyond of this deliverable reporting period. This deliverable 3.1 has been successful in producing lists of miRNAs regulating ET Cell viability as well as a set of putative synthetically lethal miRNAs for each oncogene. Next deliverable 3.2 miRNAs regulated by the key oncogenes in ET cells will certainly continue to validate these putative synthetically lethal miRNAs during next years with the next deliverable due in month 48.

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