HelmholtzZentrum münchen
German Research Center for Environmental Health

Kinderklinik München Schwabing

# Integrative analysis of microRNA and mRNA expression profiles in osteosarcoma cell lines

Stephanie Zillmer

Vollständiger Abdruck der von der Fakultät für Medizin der Technischen Universität München zur Erlangung des akademischen Grades eines

**Doktors der Medizin**

genehmigten Dissertation.

| | |
|---|---|
| Vorsitzender: | Prof. Dr. E. J. Rummeny |
| Prüfer: | 1. Prof. Dr. M. Nathrath |
| | 2. Prof. Dr. S. Burdach |

Die Dissertation wurde am 26.05.2015 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 06.04.2016 angenommen.

## TABLE OF CONTENTS

**“Two roads diverged in a wood, and I -**
**I took the one less traveled by, and that has made all the difference.”**
(*The road not taken*, Robert Frost)

# 1. Introduction

## 1.1. Osteosarcoma

### 1.1.1. Definition and epidemiology

Osteosarcoma is a malignant bone tumor characterized by the presence of osteoid. This unminerealized bone matrix (osteoid) is assumed to derive from malignant mesenchymal cells (Klein and Siegal, 2006).

Although osteosarcoma is the most common primary bone tumor in childhood, with its incidence of 2-3 new cases per year per million, it still belongs to the rare cancer subtypes (Deutsches Krebsregister, 2009).

In adolescents this tumor entity represents the third most frequent neoplasia, in children still the sixth frequent. There are two age peaks for osteosarcoma: The first one arises in the adolescent age group, with the incidence being slightly higher in adolescent males. The second age peak appears in the fifth to sixth life decade (Bielack et al., 2002; Stiller, 2002). Osteosarcoma in older patients mostly appears as a secondary malignancy, e.g. in the line of Paget's disease or radiation-induced (Potratz et al., 2006).

In contrast to Ewing's sarcoma, osteosarcoma is most frequently located in the long tubular bones, with > 65 % occurring in the distal femur and proximal tibia (Isakoff et al., 2007).

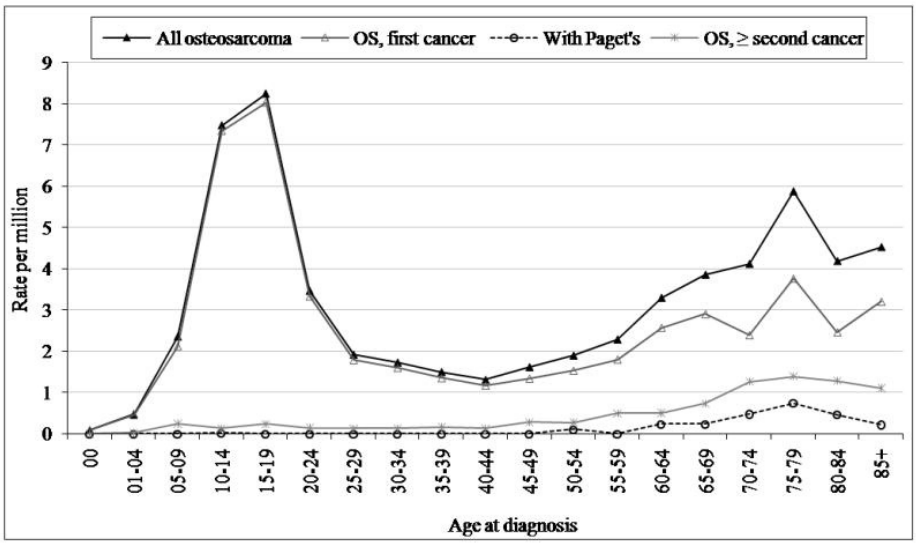



Figure 1: Osteosarcoma incidence by disease sequence, SEER 9 (1973-2004)

### 1.1.2. Etiology und pathogenesis

A definite etiological classification of osteosarcoma has not been possible so far (Ottaviani and Jaffe, 2010). Since osteosarcoma, in most of the cases, develops in the metaphyseal area of long bones, a close correlation to sceletogenesis has been assumed (Potratz et al., 2006). The accumulation at the time of growth spurt additionally supports this theory (Price, 1958).

No specific predisposing parameter has been identified so far; distinct risk factors exist in only 10% of the patients (Potratz et al., 2006). Ionizing radiation, for example, is known to be a cause for secondary osteosarcoma (Rosemann et al.; Tucker et al., 1987). Other environmental parameters, like chemicals, viral infection or repeated trauma to the affected bone have been discussed in several studies. Patient-related factors, beside a certain age or gender, that seem to promote osteosarcoma development are pre-existing bone abnormalities or diseases and black or hispanic ethnicity (Ottaviani and Jaffe, 2010).

The influence of an individual's height is an issue that has been controversially discussed (Longhi and Pasini, 2005; Troisi et al., 2006).

Osteosarcoma is known to be associated with several syndromal diseases, such as Li-Fraumeni- or Rothmund-Thomson-syndrome. Individuals with a mutation in the RB1-tumorsuppressor-gene even have a 500 times greater risk for developing osteosarcoma (Carrle D, Bielack, 2007; Ottaviani and Jaffe, 2010).

### 1.1.3. Molecular genetics

Comprehensive cytogenetic studies characterized osteosarcoma as a tumor with a high amount of numerical and structural chromosomal alterations (Bridge et al., 1997; Fletcher et al., 1994; Man et al., 2004; Ozaki et al., 2003; Smida et al., 2010) with aneuploidy being a hallmark typically to be found in this malignancy (Al-Romaih et al., 2003; Zoubek et al., 2006).

One of the best-described genetic defects associated with osteosarcoma is the mutation of the RB1 tumor suppressor gene, which is assigned to chromosome 13q14 (Araki, N Uchida, 1991; Friend et al., 1986). It has been shown that sporadic

osteosarcomas exhibit alterations in the retinoblastoma gene in up to 80 % of the cases (Benassi and Molendini, 1999; Miller et al., 1996; Sandberg and Bridge, 2003; Smida et al., 2010; Zoubek et al., 2006). As a cell-cycle regulator RB1 binds and, after phosphorylation by the CyclinD/CDK4 complex, activates the E2F-family of transcription factors. CDK4 (cyclin D kinase 4) itself is inhibited by the protein $p16^{INK4A}$ (Nevins, 2001). This protein is, as well as $p14^{ARF}$ and $p15^{INK4B}$, encoded by the CDKN2A (=INK4A) gene. All these components of the RB1 pathway positively or negatively regulate proliferation processes in osteosarcoma (Benassi and Molendini, 1999; Benassi et al., 2001; Nielsen et al., 1998). All the interrelations of this pathway are illustrated in figure 2.

P16 and p14 are known to be involved in the p53 pathway, as well. The associated tumor suppressor gene TP53 on chromosome 17p13 has been found altered in many osteosarcoma samples, where inactivation of p53 mostly occurs by allelic loss (70-80%); point mutations or rearrangements have been detected less frequently (van Dartel and Hulsebos, 2004; Overholtzer et al., 2003; Gokgoz et al., 2001).

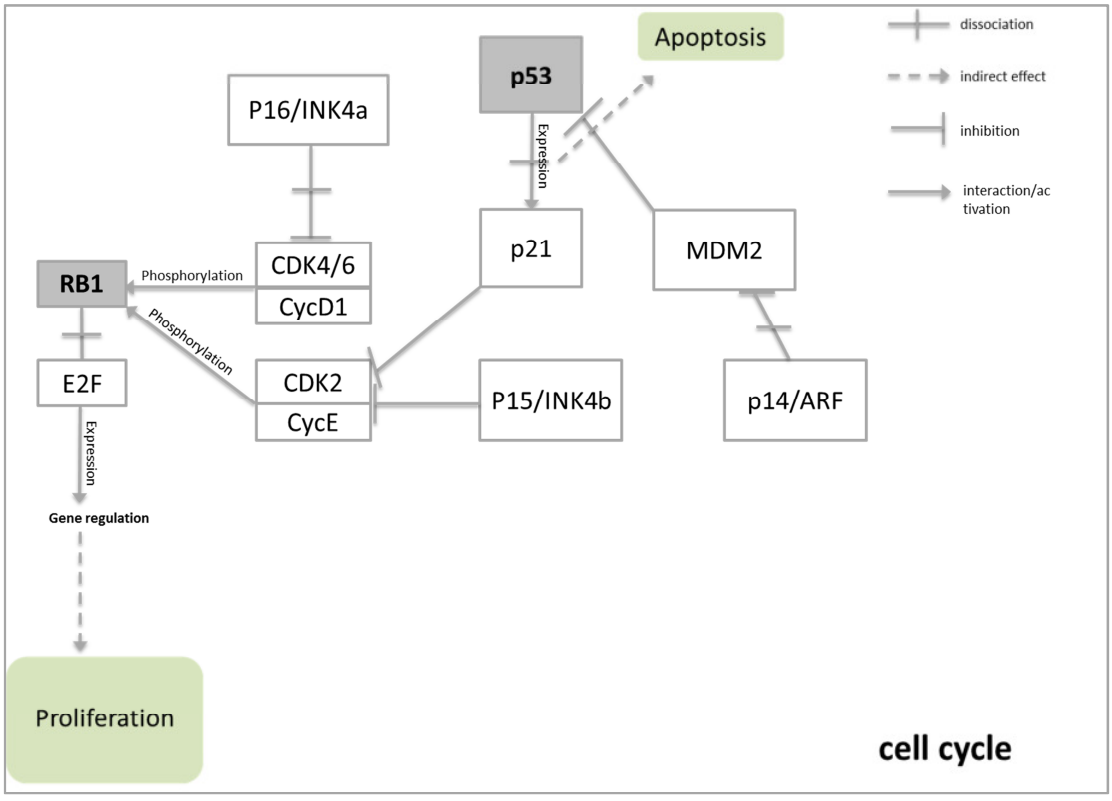



Figure 2: Important pathways in osteosarcoma; modified from: KEGG cancer pathways 2012

An inherited disease characterized by an autosomal-dominant mutation in p53 is Li-Fraumeni-syndrome, with osteosarcoma being the second-most common malignancy in those patients. MDM2-amplification has been identified in up to 16% of osteosarcomas and is accountable for p53 inactivation in these cases (Lonardo et al., 1997; Momand et al., 1998). The Mouse Double Minute 2 homolog, MDM2, is an E3 ubiquitin ligase that was described in 1991 in mice and later as a regulator of p53 in men (Fakharzadeh et al., 1991; Momand et al., 1992).

The oncogene Her-2/neu, the human epidermal growth factor receptor 2, has been found overexpressed in many different tumor types, above all in breast cancer (Ross and Fletcher, 1998). The role of Her-2 in osteosarcoma has been discussed controversially. Some studies described overexpression of its encoding ERBB2 gene as related to poor clinical outcome (Gorlick et al., 1999; Zhou et al., 2003). Others, including our group, could not find any correlation or even stated that HER-2-overexpression may have a favorable effect on clinical outcome (Baumhoer et al., 2011; Ma et al., 2012; Maitra et al., 2001; Scotlandi et al., 2005).

Other (onco-)genes that have been reported in relation to osteosarcoma pathogenesis include MYCN, RECQL4, MMP2, SAS, MET, FOS, GLI1 and RUNX2, MAPK, RANKL and the Wnt-pathway (Martin et al., 2012).

By using different cytogenetic methods (CGH, FISH, SKY) several studies identified chromosomal rearrangements in osteosarcomas involving chromosomal bands or regions 1p11-13, 1q11-12, 1q21-22, 11p14-15, 14p11-13, 15p11-13, 17p, and 19q13. Furthermore, gains on chromosome 1 and losses on chromosomes 9, 10 , 13 and 17 have been shown (Bayani et al., 2003; Boehm and Neff J.R., Squire J.A., Bayani J., 2000; Bridge et al., 1997; Mertens and Mandahl, 1993).

Generally, the molecular genetic changes in osteosarcomas commonly involve proteins of the cell cycle, e.g. those regulating the transition from G1 to the S-phase. Furthermore, as already described, genomic instability is an essential feature in osteosarcoma pathogenesis.

The general, conventional idea is that a “Cancer Genome” (Stratton, 2009), irrespective of its cancer type, is the result of gradual changes over time.

A few years ago a completely different model was proposed by Stephens et al.: They found such extended rearrangements in up to 25% of the examined

osteosarcomas (besides other cancer types examined) that they could only have occurred in a "single strike". They found indications that chromosomes were shattered into pieces and repaired and called the phenomenon "chromothripsis" (Stephens et al., 2011).

### 1.1.4. Histological classification

Osteosarcoma can be classified into numerous histological subtypes, which are listed in table 1 below (Carrle D, Bielack, 2007). Among them the conventional variant is the most common subtype in children and adolescents.

| Localisation in bone | Subtype | Frequency (in %) |
|---|---|---|
| Central OS | Conventional (osteoblastic, fibroblastic, chondroblastic) and teleangiectatic | 80-90 |
| | Small-cell | 1-4 |
| | Low-grade-central | 1-2 |
| Juxtacortical/surface OS | High-grade surface | <1 |
| | Periosteal | 2 |
| | Parosteal | 5 |
| Extrasceletal OS | | 2 |
| Secondary OS | | 8 |

Table 1: Histological types of osteosarcoma and their frequency of occurrence;
(WHO classification 2002, table modified from Bielack et al, ärztliches journal onkologie 3/2007)

### 1.1.5. Clinical signs and diagnosis

There are no typical clinical signs indicating osteosarcoma. The majority of patients present with localized swelling and load-dependent pain in the affected extremity (Schauwecker et al., 2006; Zoubek et al., 2006). Unfortunately, since symptoms like night sweats, weight loss or unexplained fever are usually missing, the bone tumor is often diagnosed at an advanced stage, several months after the first symptoms appeared (Potratz et al., 2006).

Diagnostic staging comprises, beside thorough clinical examination and investigation of medical history, detailed imaging of the local process and the searching for metastases is essential. Conventional X-ray and MRI are used to

display the extent of the primary tumor and to look for so-called skip-lesions. For detection of distant metastases conventional chest x-ray and lung CT is used in addition to whole body scintigraphy: 10-15% of the patients present with primary metastases, mostly in the lung (Carrle D, Bielack, 2007; Kager et al., 2003).

### 1.1.6. Therapy

Preoperative (neo-adjuvant) chemotherapy is of great importance in osteosarcoma and includes administration of methotrexate, doxorubicin (adriamycin), cisplatin (MAP) for 10 weeks prior to operation.

Until the end of June 2011 all patients (<40 years of age) with resectable high-grade osteosarcoma were included in the EURAMOS1/COSS clinical trial (Bielack et al., 2009). Main criterion of this therapy protocol was to take tumor response to neoadjuvant chemotherapy into account and by this means, optimize therapy accordingly. Histologic response was assessed according to the Salzer-Kuntschik-grading system, which is explained in table 2 (Salzer-Kuntschik and Delling, 1983).

| Histologic response assessment (Salzer-Kuntschik grading) | |
|---|---|
| Grading | Morphology |
| I | No viable appearing tumor cells |
| II | Single vital tumor cells or one vital cell cluster < 0,5 cm |
| III | Vital tumor < 10% |
| IV | Vital tumor 10 – 50% |
| V | Vital tumor > 50% |
| VI | No effect of chemotherapy |

Table 2: Salzer-Kuntschik response grading

By randomization within this trial, it was furthermore evaluated whether the addition of pegylated interferon (ifn) post-operative has an effect on “event-free-survival”- time for those who showed good response to neo-adjuvant chemotherapy. Additionally, the trial examined, whether initial poor responder

have a benefit from additional application of etoposide and ifosfamide (MAPIE), as far as event-free-survival was concerned.

The recruitment for the above mentioned trial has been closed in June 2011.

Until further notice, the study committee is recommending treatment with standard therapy MAP. A therapy adjustment according to histological response after pre-operative chemotherapy is no longer included. An overview of the actual treatment regimen can be seen in figure 3, page 14.

The first results regarding the good responders were officially presented in June 2013. It has been found that disease-free survival after 3 years post diagnosis has not been influenced by whether the patients were randomized to receive interferone or chemotherapy alone (77 vs. 74 %). The assessment of the poor responder was presented in 2014. The Euramos Coss Trial group showed that adding Ifosfamide and Etoposide to the therapy regimen does not have influence on outcome of osteosarcoma patients. In fact adding these drugs to standard therapy led to severe side effects (see EURAMOS-1 Poor Responders CTOS Presentation and MRC CTU Article, November 2014).

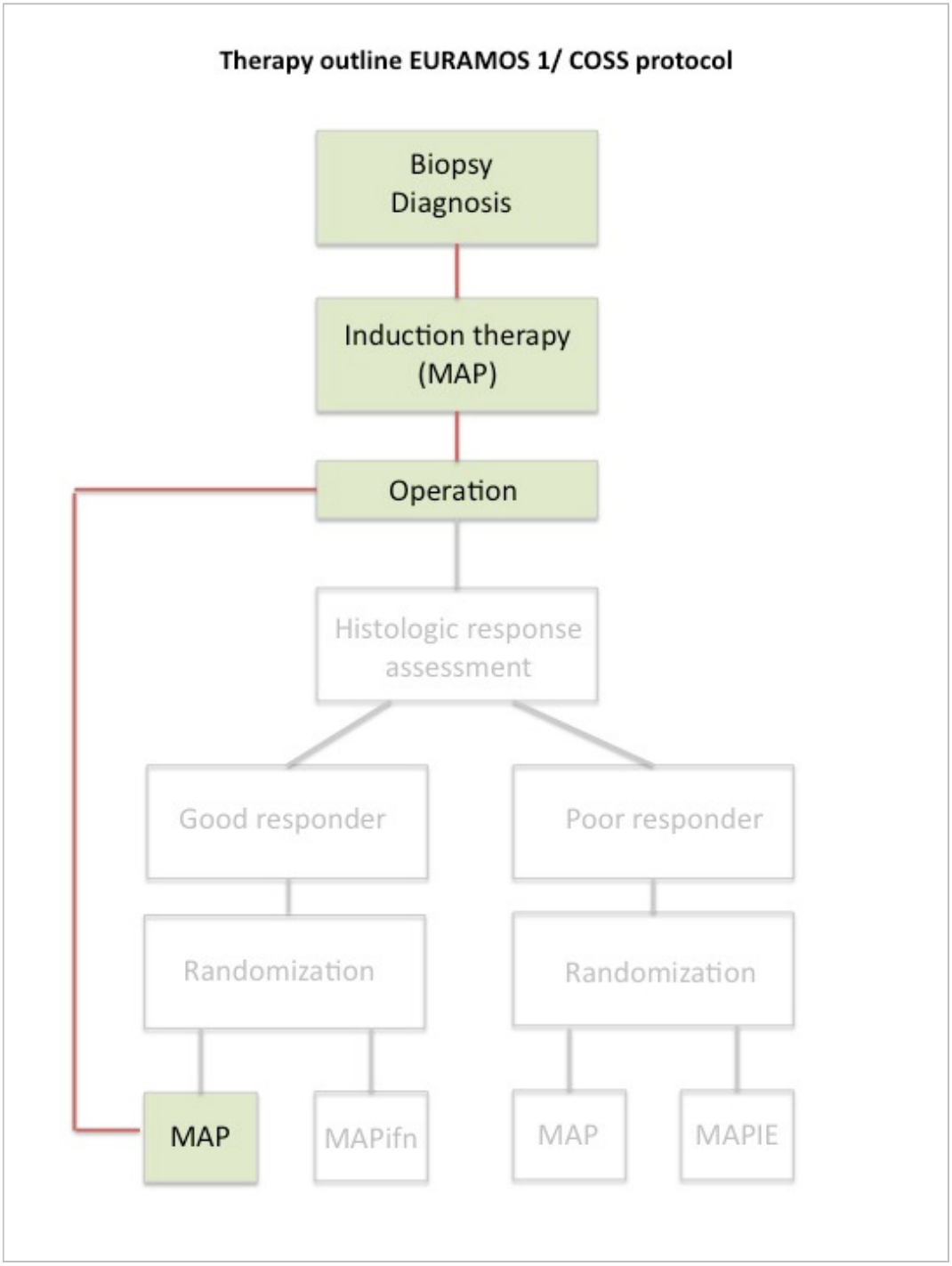



Figure 3: Therapy outline (according to EURAMOS1/COSS protocol); grey: therapy design until 30.06.2011; black/red: recommendations since 01.07.2011

### 1.1.7. Prognosis

Several parameters, as tumor localization and volume or response to adjuvant chemotherapy, determine the prognosis of osteosarcoma. Presence of primary metastasis at the time of diagnosis is still considered to have the strongest impact on prognosis (Carrle D, Bielack, 2007; Schauwecker et al., 2006).

With the above-mentioned multimodal therapy scheme an overall 5-year-survival rate of about 65%, in patients with localized disease, has been achieved. Although the therapy concept has been altered over the last decades, only about 31% of the patients with primary metastases survive the first 5 years after initial diagnosis (Bielack et al., 2002) .

The Kaplan-Meier curve in Figure 4 below illustrates the statistics for both localized and metastatic disease.

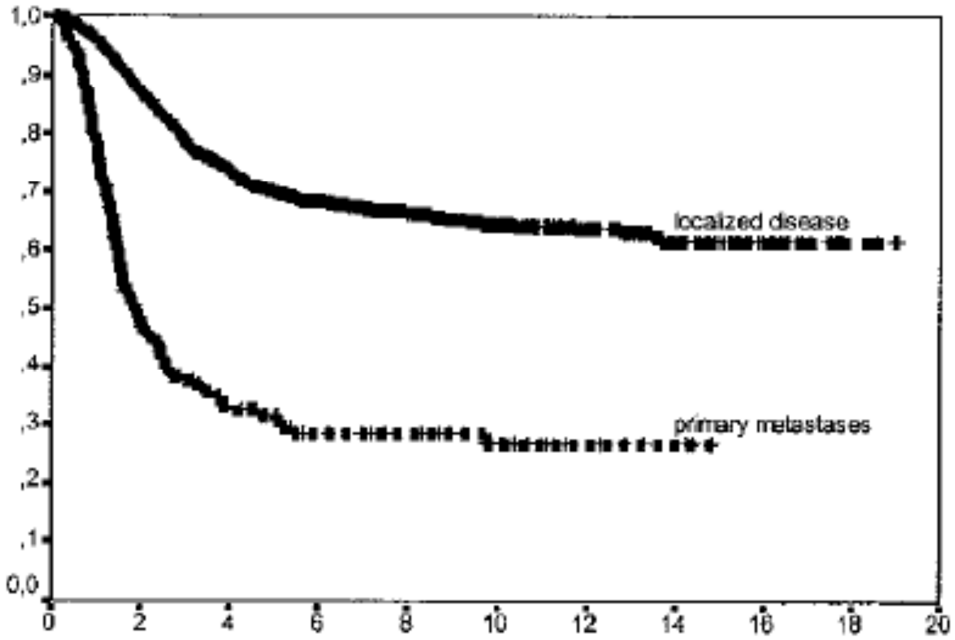



Figure 4: Kaplan-Meier curve of metastases at diagnosis (from: Bielack et al, JCO 2002)

## 1.2. MicroRNA

### 1.2.1. Definition and biogenesis

MicroRNAs are very short, non-coding RNAs of 20-24 nucleotides in length.

Lin-4 and let-7 were the first microRNA being described, discovered in the nematode *C. elegans* (Lee et al., 1993; Reinhart et al., 2000). With the identification of let-7-homologues in human genome in the year 2000, the microRNA research hit the next level (Pasquinelli et al., 2000). In the last two decades it has been discovered that microRNA play an important role in gene regulation (Ambros, 2004; Bartel and Chen, 2004; He and Hannon, 2004).

The expression of potential targets is controlled either by inducing mRNA-cleavage or by interfering with the protein translation (Bartel, 2004; Kong et al., 2008; Pillai et al., 2005). First step in the microRNA maturation is the transcription of the microRNA gene by means of RNA polymerase II. These microRNA-transcripts (pri-miRNA) are subsequently processed into the 70-nucleotide-long precursor-microRNA (pre-miRNA) by the RNASE III Endonuclease Drosha inside the nucleus (Lee et al., 2002, 2003). Secondly, after being transported into the cytoplasm, another RNAse III endonuclease (DICER) is responsible for the pre-miRNA processing into microRNA-duplexes, consisting of a mature and a complement microRNA-strand. In the following, these duplex is separated so that just one strand is introduced into the so-called RISC (RNA-induced silencing) - complex (He and Hannon, 2004; Kim, 2005). Usually the mature miRNA is incorporated, whereas the complementary strand is lost to degradation. Depending on the extent of complementarity to the target mRNA, the microRNA incorporated in the RISC-complex induces either translational repression or degradation of the mRNA (Grosshans and Filipowicz, 2008; Yekta et al., 2004). The whole biogenesis of microRNA and their processing is visualized in figure 5.

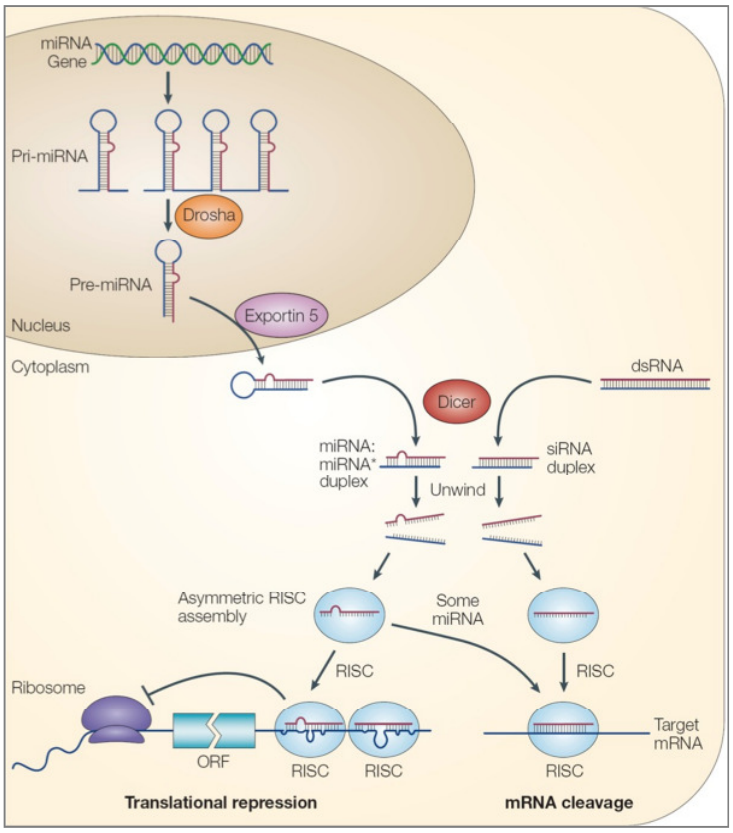



Figure 5: miRNA biogenesis and post-transcriptional processes (from He and Hannon, Nature 2004)

### 1.2.2. MicroRNA in cancer

MicroRNA expression profiling has been established as a method to unravel the significance of microRNA-involvement in malignancies. There are numerous microRNA, termed “oncomiRs”, that have been found differentially expressed in human cancers whereas some function as tumor suppressors and others act as oncogenes (Calin and Croce, 2006a, 2006b; Esquela-Kerscher and Slack, 2006a).

In the year 2002 the correlation between microRNA and cancer has been described for the first time. Calin et al. discovered that the miR-15 and miR-16 genes, both located in a region frequently deleted in patients with CLL (Chronic Lymphatic Leukemia), seem to function as tumor suppressors. In more than 2/3 of the CLL-cases under examination both miRNA-genes were down-regulated. In the following years the same group has shown that microRNA genes are commonly located in so-called fragile sites or other regions that are cancer-related (Calin et al., 2002, 2004).

Typical microRNA expression profiles have been identified for nearly all cancer subtypes. A study from 2005, for example, described a set of 15 different microRNA that managed to distinguish between normal and malignant breast tissue in 86 samples (Iorio et al., 2005). In the same year, Lu and others were able to classify numerous different cancer entities according to their microRNA expression profiling (Lu et al., 2005). Moreover, microRNAs have not only been found to regulate certain cancer-associated genes but to play a key role in most known cancer pathways. Certain let-7-family-members seem to be involved in regulating NRAS oncogenes. MiR-143 and miR-145 were proven to have a suppressing effect in colorectal cancer by targeting KRAS (Chen et al., 2009; Johnson et al., 2005; Michael et al., 2003).

MiR-21 has been characterized as having anti-apoptotic features in glioblastoma. In addition to that, miR-21 seems to be involved not only in breast cancer but also in colorectal, other gastrointestinal malignancies or lung cancer (Asangani et al., 2008; Frankel et al., 2008; Krichevsky and Gabriely, 2009). By targeting PDCD4, TPM1 or MAPK, respectively, miR-21 has influence on migration, invasion and proliferation representing cellular abilities that are uncontrolled in cancer. Furthermore, miRNA like miR-126, miR-1 or miR-146b, miR-182 and miR-183 have been recognized in this context, as well (Baranwal and Alahari, 2009).

Because of the multitude of miRNAs that has been linked to cancer it is almost impossible to list all comprehensively. MicroRNA dysregulation, by down- or up-regulation, seems to be a feature in all malignancies (Croce, 2009).

### 1.2.3. MicroRNA in osteosarcoma pathogenesis

There are several studies existing that examine microRNA expression in osteosarcoma regarding their impact on clinical parameters, its pathogenesis or their influence on certain target genes. The common point of all these studies is the search for biomarkers or potential therapeutic targets in osteosarcoma.

A number of studies analyzed microRNA expression focusing on its ability to discriminate between osteosarcoma and normal tissue (Maire et al., 2011; Namløs et al., 2012). Furthermore genome-wide microRNA profiling has been performed to

see how relevant certain microRNA are in osteosarcoma cell invasion, migration and proliferation, apoptosis, metastasis or chemoresponse (Gougelet et al., 2011; He et al., 2009; Song et al., 2010; Ziyan et al., 2011).

A database summarizing and evaluating all the data concerning microRNA expression and their targeted genes has been established, in cooperation with our group, just recently (Poos et al., 2014). On osteosarcoma-db.uni-muenster.de a comprehensive overview about what is known so far about miR involvement in osteosarcoma (81 microRNA-entries, 911 target genes as of 12/ 2014) can be found. Based on this database and literature, the most relevant microRNA (as measured by number of appearance, at least repeated once) in osteosarcoma are:

miR-9, miR-16 (16-5p), miR-17-92 cluster (17,18a, 19a, b and 20a, miR-92a and miR-93), miR-21, miR-29a and b, miR-31, miR-34a, b, c, miR-133a and b, miR-134, miR-140, miR-143, miR-145, miR-148a, miR-183, miR-195, miR-199a-3p, miR-223, miR-335, miR-382, miR-451a.

To what extent this work can add new microRNA relevant in osteosarcoma or strengthen existing data will be subject of the discussion later on.

# 2. Thesis objectives and design

## 2.1. Thesis objectives

Patients with osteosarcoma are in need of new therapy strategies. That microRNAs seem to play an important role in osteosarcoma pathogenesis has been outlined before. By approaching the topic “microRNA and its relevance in osteosarcoma pathogenesis” from both cellular and genomic level, I intended to give a comprehensive answer to the following questions:

- Can microRNA or a subset of microRNA be identified helping to distinguish between osteosarcoma and normal tissue?
- Is it possible to connect the miRNA and mRNA expression patterns to real biologic effects in the cells? Which are likely targets of these miRNA?
- Can microRNA deregulation help to explain the typical malignant features (invasion, uncontrolled proliferation, migration) in osteosarcoma?
- Is it possible to locate more microRNAs as key players in canonical pathways of osteosarcoma? Are there more miRNAs responsible than already identified in osteosarcoma (for example miR-21, miR-34)?
- Will the “usual suspects” in osteosarcoma as RB1, c-myc or CDKN2A be connected to candidate miRNA or will new potential target genes be found?
- Will the integration of miRNA and mRNA data help creating “new networks” to explain how osteosarcoma is forming? Could the findings be connected to existing networks (as bone differentiation or proliferation)?

## 2.2. Study design

To investigate microRNA and their pathogenic relevance in osteosarcoma this work was structured as follows (see figure 6, page 21):

- Eight established commercially available osteosarcoma cell lines (listed in table 3, page 23), one human ostoblastic (hFOB1.19) and one mesenchymal stem cell line (L87.4) were analyzed for genome wide expression of microRNA (miRCURY™ LNA Array; miRbase version 15.0) and mRNA (Affymetrix 1.0 ST arrays; estimated number of genes 28.869). Additionally the osteosarcoma cell lines were characterized using in vitro (proliferation, migration and invasion) cell assays.
- The expression of osteosarcoma cells and the reference cell lines were compared for both microRNA and mRNA separately for identifying microRNA and mRNA differentially expressed in osteosarcoma versus progenitor cell lines.

  Moreover, using conventional association testing, deregulated microRNA and their potential target genes significantly correlating with the osteosarcoma cell lines' potential to proliferate, migrate and invade, respectively, were identified. In another, more advanced analytical approach, the expression matrices of both microRNA and mRNA were utilized to find gene regulatory networks. Hereby the focus lay exclusively on those microRNA-mRNA-couples that showed differential expression between the phenotype groups (migration/invasion and proliferation as indicator for degree of aggressiveness). To identify "real" mRNA-miRNA-modules the couples in the prediction database mirdb were validated and only the "most likely" (prediction score>80) kept for further analysis. Finally, by means of the IPA (Ingenuity Pathway Analysis) software the microRNA-mRNA regulatory modules (proliferation and migration/invasion) have been evaluated in regard to their part in canonical pathways in cancer and disease.
- As common points were found in the analyses (deregulation of the same microRNA identified by different approaches) primary validation experiments were performed in the cell lines, e.g. transfection of siRNA.

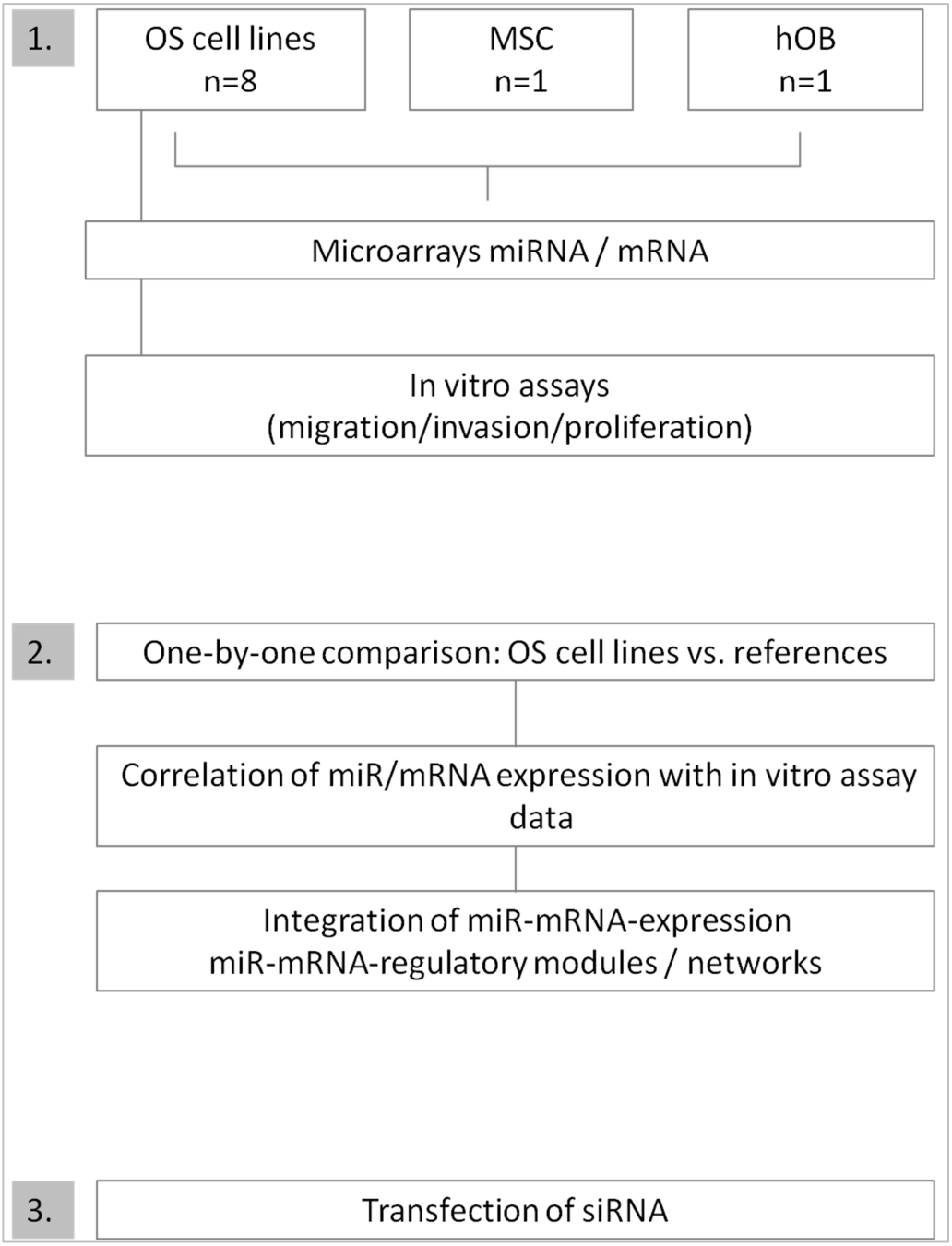



Figure 6: Workflow of the thesis; part 1: expression profiling microRNA/mRNA genome wide and assays in vitro; MSC-mesenchymal stem cell line; hFOB: human osteoblast cell line; part 2: analysis of data by different analytical methods; part 3: validation

# 3. Material and methods

## 3.1. Osteosarcoma cell lines

Pre-therapeutic patient material of osteosarcoma is limited due to the treatment trial design. Therefore tumor derived cell lines represent the best available model for investigating the cells properties in a comprehensive manner without wasting valuable patient samples. The immortal cell lines utilized in this study are well-described adherent cell lines purchased from ATCC or other partner institutes (Heide Siggelkow, Nelson Lab). An overview of the cell lines is given in table 3 on the following page.

| Cell line | Origin | Age | Sex | Race | Reference |
|---|---|---|---|---|---|
| MG-63 | ATCC | 14 | Male | Caucasian | (Billiau and Edy, 1977; Heremans et al., 1978; Ottaviano et al., 2010; Ozaki et al., 2003) |
| U2OS | ATCC | 15 | Female | Caucasian | (Heldin et al., 1986; Ottaviano et al., 2010; Ozaki et al., 2003; Ponten and Saksela, 1967) |
| SaOS-2 | ATCC | 11 | Female | Caucasian | (Fogh et al., 1977; Ottaviano et al., 2010; Ozaki et al., 2003) |
| SJSA-01 | ATCC | 19 | Male | Black | (Oliner et al., 1992; Ozaki et al., 2003; Roberts et al., 1989) |
| HOS | ATCC | 13 | Female | Caucasian | (McAllister et al., 1971; Ottaviano et al., 2010; Ozaki et al., 2003; Rhim et al., 1975b, 1975c) |
| MNNG-HOS | ATCC | 13 | Female | Caucasian | (Ottaviano et al., 2010; Ozaki et al., 2003; Rhim et al., 1975a) |
| HOS-58 | Siggelkow | 21 | Male | Caucasian | (Siggelkow et al., 1998) |
| ZK-58 | Jundt / Schulz | 21 | Male | Caucasian | (Ottaviano et al., 2010; Ozaki et al., 2003) |
| hFOB 1.19 | ATCC | Fetus | NA | - | (Subramaniam et al., 2002) |
| L-87 | Nelson Laboratories | 70 | Male | - | (Thalmeier and Meissner, 1994) |

Table 3: Cell line characteristics and references

## 3.2. Cell cultivation

### 3.2.1. Cell culture conditions

The osteosarcoma cell lines SaOS-2, SJSA-01, MG-63, U2OS, HOS, HOS-58, ZK-58 und MNNG-HOS, as well as the human osteoblast cell line hFOB 1.19 and the stem cell line L-87.4 were cultivated under sterile conditions in a humidified atmosphere (37°C and 5% $CO_2$ ) .The medium used for all cell lines was RPMI 1640 + L-Glutamine (PAA), supplemented each with 10% FCS. No antibiotics or antimycotics were added.

### 3.2.2. Cell counting

Cell counting was performed using the Beckman Cell Coulter Z1. After trypsinization of adherent cells, the reaction has been stopped by adding RPMI Medium. After that 0,5µl of this solution was added to 19,5ml sodium chloride solution obtaining a dilution factor of 1:40. The Beckman Coulter then assessed cell number by counting all particles exceeding a diameter of 7 µm.

### 3.2.3. Wash cells and medium changing

Culture medium was changed every 2-3 days, depending on each cell line's requirements. The color change of the medium indicated the nutritional status in the culture flasks. First the old culture medium was removed very carefully with a single-use-pipette. The adhering cells were washed once with PBS to remove any residua of medium or cell debris. The washing buffer has again been aspirated with a single-use-pipette. Subsequently 2,5 ml (or 6ml for T75-flasks) of fresh medium was added to the culture flask.

### 3.2.4. Sub-culturing

When showing confluence under the light microscope, cells were sub-cultured. The initial procedure was the same as previously described: old medium was completely removed; attached cells were washed with PBS and aspirated again. Doing this it was made sure to leave no residua of medium, which could diminish the effect of

trypsinization. Then (depending on size of the culture flask) around 0,4 ml trypsin (or 1ml for T75) was added to culture flask. After a short incubation time (2-5 min, differing according each cell lines adhesive characteristics) at 37°C on a hot plate, culture flask was checked under an inverted microscope to see the amount of cell detachment. The tenfold amount of cell culture medium RPMI (compared to the amount of trypsin used) was added to stop the Trypsin effect.

After repeated re-suspension of this cell suspension a small amount (around 5-10%) was transferred into a new culture flask. Culture medium was added to cell suspension and the cells were incubated again.

## 3.3. Cell culture assays

### 3.3.1. General outline

For evaluation of the proliferative, migrative und invasive properties of our osteosarcoma cell lines, assays already established in literature were used. Every assay was performed under the same conditions for all cells. RPMI 1640 (10% FCS) was used as the culture medium for all cell lines. Furthermore only cells showing around 60-80% confluence were used for the assays.

### 3.3.2. Proliferation assay

For growth determination of the cell lines $1x10^5$ cells were seeded in $25cm^2$ cell culture flasks. This was done for every cell line in duplicate. Over a time period of 7 days cells were counted using an automated cell counter (Beckman Coulter). Therefore, cells were harvested after 24h, 48h, 72h, 96h and 168h hours by trypsinization. The mean cell numbers for every cell line were calculated and plotted into a growth curve with logarithmic scaling (see figure 7). In logarithmic phase doubling time ($t_d$) has been calculated. This has been done for each cell line using the following equation:

$t_d = \ln 2 / \mu$

$[\ \mu = \text{growth constant} = (\ln x_t - \ln x_0) / (t - t_0)\ ]$

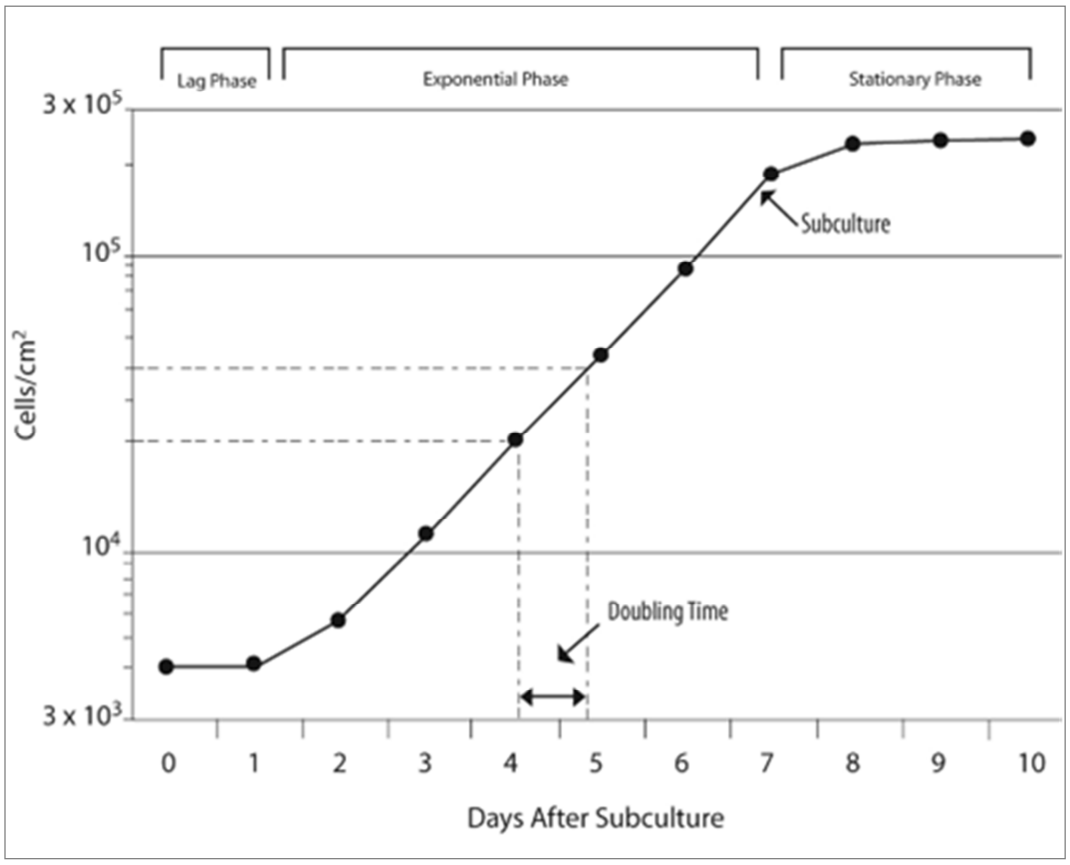



Figure 7: Standard growth curve (log scale) for calculating dt (doubling time), ref. see ATCC cell culture protocol basic cell culture: A practical approach (J.M. Davis); ATCC Cell Culture Technical Resource, www.lgcstandards-atcc.org, version 02/2010

### 3.3.3. Migration assay

For evaluation of each cell lines' migrative potential the migration assay from BD Biosciences has been conducted. This was done according to the manufacturer's instructions. All experiments were performed in duplicate. Plastic cell culture inserts, purchased from BD Biosciences in addition to the protocol, were used to perform the experiments. Its membrane comprises pores of 8µm in size, which are randomly located over the complete membrane surface (see also figure Invasion assay). The experiment was conducted as follows:

The osteosarcoma cell lines were harvested and counted as previously described.

In each well 900µl cell culture medium (RPMI 1640), supplemented with 10% FCS, was put in to act as a chemo-attractant. After that the inserts were placed into the wells. A cell suspension of $2{,}5x10^4$ cells in 0,2% FCS containing RPMI was added making sure that the amount of fluid did not extent 350µl. Subsequently the migration chambers (24-well-plate with inserts) have been incubated for 24 and 48 h at 37 °C (5% $CO_2$). After that incubation time the inserts were transferred into a clean 24-well-plate. They were washed once with 600µl PBS each. Afterwards the upper side of the membrane was swabbed with a cotton tip twice to remove all cells that have not been migrating through. Later the inserts were put in methanol for 2 minutes to fixate the invaded cells on the lower side of the membrane. A short washing procedure in aqua (Ampuwa) was performed after that. To stain the cells on the lower membrane side, the inserts were placed into a 24-well-plate containing 2% Toluidine-blue. The staining was performed for 10 minutes. The color residuals were then washed in water. Again cotton swabs were used to clean the membrane's upper side from all color residuals. Subsequently the membranes were dried for 1 h at 37°C. To allow light-microscopic analysis, the membranes were then cut using a fine cannula. The membranes were fixated under a cover slip. For each cell line (and duplicates) 10 visual fields (magnification 10x) were analyzed, counting the stained cells.

### 3.3.4. Invasion assay

For evaluation of each cell lines' invasive potential the Biocoat™ Matrigel™ Invasion Assay (BD Biosciences) has been conducted. The BD Biocoat™ Matrigel™ Invasion chamber is built similarily to the migration insert but additionally a Matrigel® membrane coats the bottom of the cell culture insert. Please consult figure 8 on page 29 for visualization. Matrigel® is a gelatin-like substance that derived from mouse sarcoma cells (EHS). Since this protein mixture contains collagen Type IV, laminin or heparan sulfate proteoglycan, it is supposed to simulate the basement membrane (Kleinman et al., 1986). The method has basically been performed analogous to the migration assay. Difference was that the chambers' storage in -20°C was necessary because of matrigel® coating present in these cell culture

inserts. Additionally, before starting the assay the matrigel-coated inserts have been warmed up at room temperature for 20 minutes. In the following, according to the manufacturer's instructions, hydrogenating of the membrane with the basic medium (RPMI 1640) was performed. The inserts were placed into the 24-well-plates and incubated for 2 h at 37°C and 5% $CO_2$.

Differently from the Migration assay a total of max. 500µl fluid for the inserts and a total of 750µl chemo-attractant were used for the wells. This was a recommendation by the manufacturer BD Biosciences. Subsequently the invasion chambers (24-well-plate with inserts) have been incubated for 48 h at 37 °C (5% $CO_2$) and evaluation of invaded cell number was done only after 48 h.

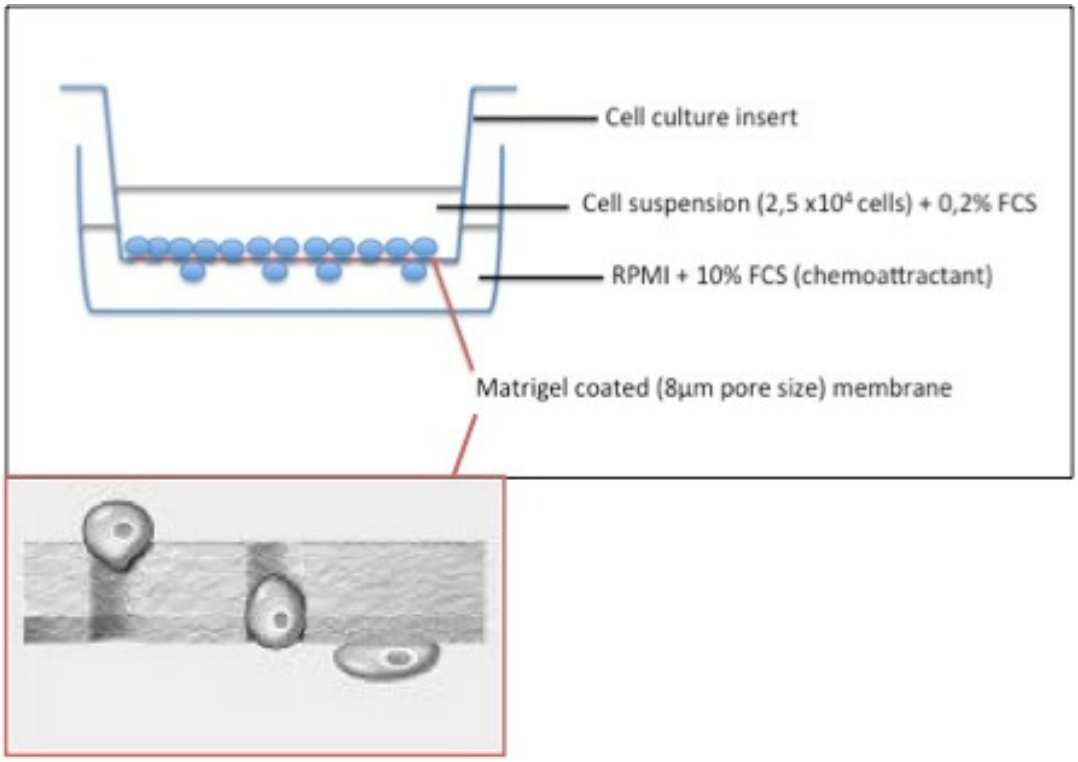



Figure 8: Principle of Matrigel™ Invasion chamber, lower picture with red margin: Matrigel® coated membrane in detail; modified from: BD Biosciences®

## 3.4. Transient transfection

### 3.4.1. Method definition

Transfection is a method to implement nucleic acid, such as siRNA, into human cells. By transfecting microRNA mimics or inhibitors probable targets of particular microRNAs can be identified. MicroRNA mimics are chemically synthesized

microRNAs which, after being transfected into the cell, mimic naturally occurring microRNAs. MicroRNA inhibitors are single-stranded modified RNAs which, after transfection, specifically inhibit miRNA function. Reduced gene expression after transfection of a microRNA mimic or increased expression after transfection of a microRNA inhibitor provides evidence that the miRNA under study is involved in regulation of that gene. Alternatively, the role of miRNAs in various pathways can be studied by examination of a specific phenotype following microRNA mimic or inhibitor transfection (see guidelines for miRNA mimic and miRNA inhibitor experiments, Quiagen®).

### 3.4.2. Optimization of transfection efficiency

For evaluating transfection efficiency the AllStars Hs Cell Death Control siRNA ® (purchased from Quiagen®) was used. AllStars Hs Cell Death Control® is a siRNA mix targeting human genes responsible for cell survival. Transfection of this control leads to a knockdown of these genes and subsequently a high amount of cell death.

The transfection control experiments were conducted according to the protocol provided by Quiagen® (for detailed protocol description see Appendix)

After 48-96 hours post transfection its efficiency was observed by light microscopy. Transfection conditions that resulted in the greatest degree of cell death in comparison to transfection with a negative control were maintained in future experiments. For finding the appropriate amount of transfection reagent, as well as the right microRNA-mimic/inhibitor ratio necessary for the final transfection of our cell type a number of optimization trials were conducted. Based on suggestions given by the manufacturer different ratios of HiPerFect® reagent and siRNA were pipetted together with the AllStar Hs Cell Death siRNA® as a positive control.

Analogue to the recommendations of Quiagen the 10fold amount of microRNA-inhibitor compared to mimic was used for transfection.

### 3.4.3. Transfection of miRNA-181a and miRNA-let-7f

As a first part of the validation experiments both the mimicry miRNA and the inhibiting microRNA of let-7f and miR-181a were transfected. These specific

microRNAs were chosen exemplarily to find out if the conclusion drawn from the expression profiling and the cell culture assays can be validated.

Therefore 2µM of miRNA-181a- and the let-7f-mimic were transfected into 6 cell lines (MNNG, SJSA, MG-63, HOS, SaOS and MNNG; duplicates for each) according to the manufacturers protocol (see appendix for further information on the protocol). Additionally 20µM of the inhibiting siRNA (anti-miR-181a and anti-let-7f) were transfected. The transfection was started one day after seeding the cells with a number of $10^5$ – $10^6$/well on 12-well plates to obtain an optimal confluence and adequate physiological conditions for the osteosarcoma cells. These conditions were chosen according to the traditional protocol, also provided by Quiagen® (see appendix). In the next step only one of them (miR-181a) was transfected to see the effect more clearly and to have a comparison between all osteosarcoma cell lines. These first experiments should enable to find the appropriate amounts of inhibiting or mimic miRNA and to verify which would be a safer choice for further experiments. Since the positive control (AllStar Hs Cell Death Control®) was already applied before, for this transfection only negative controls (same cells in media only with transfection reagent) were used. According to the manufacturers recommendations cells were seeded in 12-wells plates with 100.000 cells/well 24 hours prior to transfection. Transfection with 181a-miR-inhibitor (Anti-hsa-miR-181a miScript miRNA inhibitor, mature miRNA sequence: 5'AACAUUCAACGCUGUCGGUGAGU), 181a-miR-mimic (syn-hsa-miR-181a miScrpt miRNA mimic, mature miRNA sequence: 5' AACAUUCAACGCUGUCGGUGAGU), let-7-inhibitor (anti-hsa-let-7f miScript miRNA inhibitor, mature miR-sequence: 5'UGAGGUAGUAGAUUGUAUAGUU) and let-7-mimic ( Syn-hsa-let7f miScript miRNA mimic, mature miR-sequence: 5'UGAGGUAGUAGAUUGUAUAGUU) was performed using HiPerfect® Reagent. All reagents and oligonucleotides were purchased from Quiagen®. For each replicate 3µl (=75ng) of the siRNA and 6µl of the Transfection reagent HiPerfect was used.

After transfection of the osteosarcoma cell lines the in vitro assays (described in chapter 3.3.2 - 3.3.4) to measure the growth activity and the potential to migrate and invade were performed again. By this means the changes in phenotype after transfection were evaluated.

We decided to focus only on the miR-181a-inhibitor to monitor its influence in proliferation potential. Therefore all osteosarcoma cell lines were again transfected with this inhibiting microRNA and a growth curve for the transfected cells was conducted.

## 3.5. Molecular genetic material and methods

Since the microRNA expression arrays and the gene expression profiling were performed by a cooperative department of the core facility or a service by a company the methods will be described only for a general understanding but not in detail.

### 3.5.1. RNA extraction and sample preparation

Isolation of total RNA was conducted by using the Ambion miRVana® Extraction Kit and performed according to the manufacturer's instructions. RNA-concentration and -purity have been assessed by measuring UV absorbance. All samples showed a ratio of 1,8 – 2,1 (Absorbance ratio $A_{260nm}$ /$A_{280nm}$ ) indicating highly pure RNA. RNA quantification was performed by Nanodrop measurement. A total of 20 samples (each cell line in duplicate, different passages) were submitted to Exiqon®. There RNA's high quality and therefore suitability for further microRNA micro array analysis was confirmed.

### 3.5.2. MicroRNA expression array (miRCURY LNA-Array)

The performance of microRNA arrays was done by Exiqon® (Vedbaek, Denmark) as follows: The samples were labeled using the miRCURY™ Hyr3/Hy5 Power Labeling Kit and hybridized on the miRCURY™ LNA Array (5th Generation Array). This array contained capture probes targeting all human microRNAs listed in the miRBase 15.0 version (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008). The normalization of the quantified, background corrected signals was accomplished using the global Lowess Algorithm (Ritchie et al., 2007). The microRNA data were provided as an excel spread sheet file containing the log2 ratio expression matrix of the microRNA array probes. Those marked with "NA" showed insufficient quality. Only probes with valid expression values (n=255 probes) in all cell lines were kept for further analysis.

Technical duplicates of the cell line microRNA expression data showed an overall good correlation (> 70%).

### 3.5.3. Gene expression profiling (Affymetrix 1.0 Gene Chip Array)

The array data for the 10 cell lines (8 osteosarcoma, 2 progenitor cell lines) were conducted in cooperation with the Institute of Experimental Genetics at HMGU.

The Affymetrix 1.0 Gene array is a whole-transcript-approach covering an estimated number of 28.869 genes. An average of 26 probes per gene and only perfect match probes (set of controls for background subtraction) were used. Around 58% of the probe sets are supported by the databases RefSeq, Ensembl and GenBank, another 32% only by Ensembl. Around 100-150mg of total RNA was amplified and labeled according to the WT Sense Target Labeling Assay. Labeled single stranded DNA was hybridized to the above-mentioned array chip. Scanning of the chips was performed using the Affymetrix GenChip Scanner 3000 7G. QC (quality control) and RMA (robust multichip average) data were generated using the Affymetrix expression console including annotation.

## 3.6. Data analysis

### 3.6.1. In vitro assay analysis

The assaying of the cell lines regarding their biological behavior in vitro provided a way of distinguishing the osteosarcoma cell lines according to their phenotype. Groups of similarities (fast and slow growing, migrating and non-migrating as well as invading and non-invading cell lines) were created. The osteosarcoma cell lines were assigned to be either negative or positive in the three characteristics.

This knowledge concerning the phenotype was later used to associate biological appearance with the expression patterns in microRNA and mRNA.

### 3.6.2. MicroRNA target prediction

For this work the prediction data from the website miRDB 15.0 (http://mirdb.org/miRDB/) has been used. A prediction score is utilized to weigh

the miRDB prediction results. Only targets with a prediction score > 80 are very likely to represent real microRNA targets of the miRNA of interest, so that only those were included in target analysis (Griffiths-Jones, 2004; Griffiths-Jones et al., 2008; Wang and El Naqa, 2008).

Two reference cell lines, one human osteoblast (FOB 1.19) and a mesenchymal stem cell line (l-87) were used for analysis. The linear miRNA and mRNA expressions of the tumor cell lines have been divided by those of the reference cell lines and the resulting ratios were log2-transformed. Genes and microRNA were regarded as differentially expressed when the log2-fold change was less than 0,8 (down-regulation) or greater than 1,2 (up-regulation). Since in one-by-one comparison statistical testing is not possible it was determined by sign-testing. Basis was the null hypothesis that unchanged microRNA expression is reflected by unchanged mRNA expression and hence by an equal distribution of positive and negative log2-ratios around 0. The sign-test assigns a +1 to all positive log2-ratios and a -1 to all that are negative regardless of the absolute value of the log2-ratio. Null hypothesis is rejected when a microRNA molecule with a positive log-fold change leads to significantly more negative microRNA log2-ratios in comparison to an equal distribution of negatives and positives and vice versa. The results of this analysis were summarized in an excel spreadsheet containing lists of microRNAs and genes. The gene lists were then used to feed the online analysis tool DAVID (http://david.abcc.ncifcrf.gov/) for generating DO term and pathway enrichment analysis in order to get an idea of the functional impact of the genes (Huang et al., 2009).

### 3.6.3. Integration microRNA and mRNA expression in correlation to phenotype

The endpoint assay data for migration/invasion and cell growth were used to classify (two groups for each phenotype) the cell lines as positive or negative regarding these certain characteristics. For both the microRNA and mRNA datasets differentially expressed microRNA and mRNA were identified using the R package limma. The expression data were fitted to a linear model using the function lmfit and the contrasts, including estimated coefficients and standard errors, were calculated between the groups using the function contrast.figt and the moderated

t-statistics, moderated f-statistic and log-odds of differential expression computed by empirical Bayes shrinkage of the standard errors. The results were presented as lists generated by the function toptable (see table 4; sorted by the log-FC and includes only genes with p-values smaller than 0,05) containing the following values:

| Abbreviation | Explanation |
|---|---|
| Gene list | One or more columns of probe annotation, if genelist was included as input |
| LogFC | Estimate of the log2-fold change corresponding to the effect or contrast |
| CI.025 | Left limit of confidence interval for logFC |
| CI.975 | Right limit of confidence interval for logFC |
| AveExpr | Average log2-expression for the probe over all arrays and channels |
| t | Moderated t-statistic |
| F | Moderated F-statistic |
| p-value | Raw p-value |
| Adj.p-value | Adjusted p- or q-value |
| B | Log-odds that the gene is differentially expressed |

Table 4 : Legend for toptable

### 3.6.4. Integrative analysis of microRNA and mRNA expression using correlation networks

For integrative analysis of the groups using correlation networks the following approach was used (Peng et al., 2009a), see figure 9:

The matrices of mRNA and microRNA expression were tested for negative correlation (Pearson) based on the assumption that microRNAs inhibit the expression for their target mRNAs. Hereby, the information I obtained from the in vitro assays was utilized as the expression matrices of the fast and slow proliferative and migrative/non-migrative (identical for invasion) cell lines were compared separately.

Two matrices resulted from this analysis, a correlation coefficient for each mRNA-microRNA pair and a p-value. The miRDB prediction database (version 15.0) was used to assign a “1” to real and a “0” to relationships that are unlikely to be real. Only correlation coefficients and p-values from predicted microRNA-mRNA-relationships were used for further analysis. To determine significant microRNA-

mRNA relationships a p-value of 0,05 was used as a threshold. However, due to multiple testing error (>20.000 tests) the false discovery rate had to be determined for a range of correlation coefficient thresholds (-1 to 0,15; see figure 10, following page). A threshold of 0,82 (FDR 0,02) was chosen. At this threshold 2% of significant results (p<0,05) can be expected to be false-positive. A possible explanation for the FDR being lower in our data compared to the results Peng et al. provided is the fact that in this study a different, more stringent miRNA prediction database has been used.

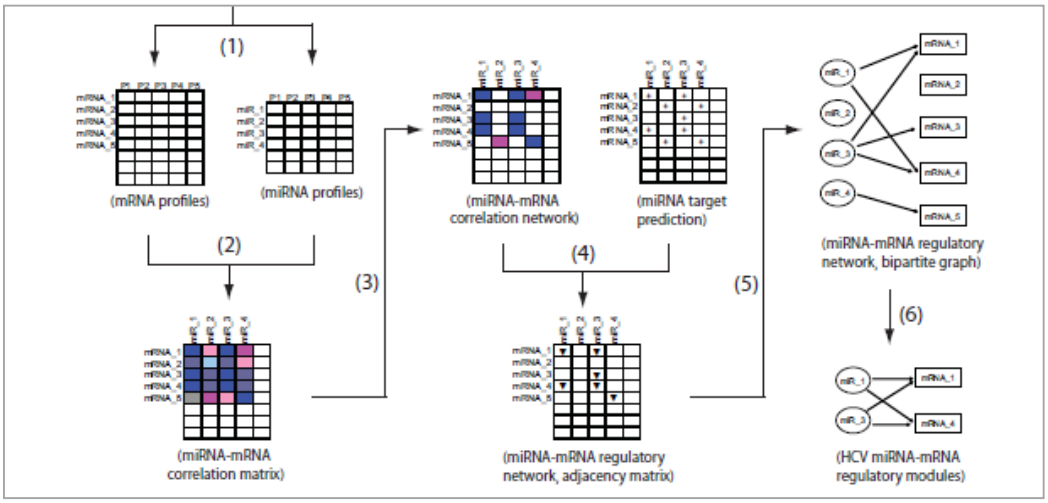



Figure 9: Workflow (see Peng et al, 2009) for identification of regulatory miR-mRNA-modules

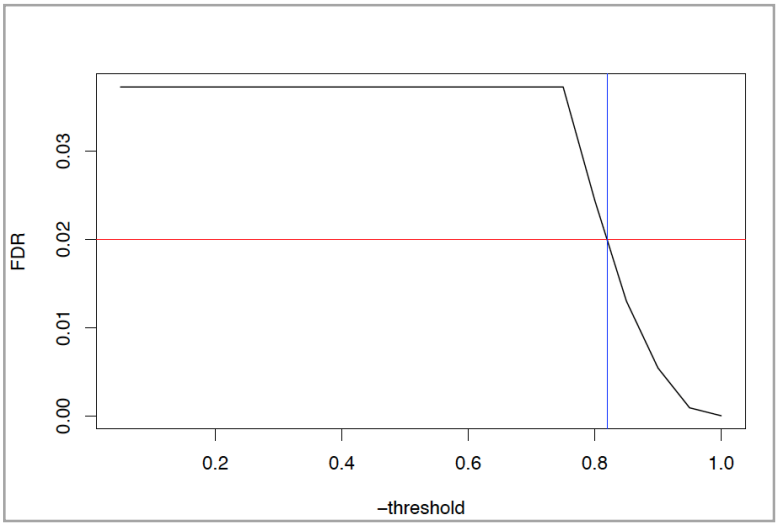



Figure 10: Correlation coefficient thresholds (from: Peng et al.; 2009); range of coefficient thresholds -1 to 0,15; FDR (false discovery rate) 0,02; i.e. at a threshold 0,82 2% of the results reaching significant level (p<0,05) can be expected to be false-positive

For both proliferation and migration or invasion, respectively, 2 larger and a few smaller bipartite networks were identified. Genes and microRNA from these networks were fed into the Ingenuity Pathway Analysis software IPA, version 9.0, (Ingenuity Pathways Analyses, Ingenuity Systems, Mountain View, CA, see www.ingenuity.com). IPA is a web-based software application for analyzing data derived from gene or microRNA expression based on the Ingenuity Pathways Knowledge Base. It helps to visualize and understand the impact the set of deregulated miRNA amd mRNA identified in this study might have in the context of canonical pathways (on basis of the actual literature). By uploading the microRNA-mRNA-network lists (proliferation and migration networks) the IPA-software groups the data according to the biological function or disease they seem to play a role in. This is accomplished by a certain algorithm creating scores that show their significance based on the number of genes/molecules that map to a biological function, pathway, or network. Genes were overlaid onto a global molecular network developed from information in the Ingenuity Pathways Knowledge Base. Networks of these genes were then algorithmically generated based on their connectivity. The IPA software creates networks rated by scores, which represent the negative exponent of a p-value calculation and indicate the number of eligible genes within a network. The higher the number of network eligible genes in a network, the higher the score. By setting a particular threshold during analysis set up IPA ignores values less than 2 fold up or down, differentiating the samples.

Based on these lists the program generates a graph displaying the connectivity of certain genes or miRNA, whereas a number of 35 (for smaller networks) and 70 (merged networks) molecules were chosen as maximum in order to keep it easier to visualize. For legend and further explanations consult graphs 20 (page 67) and 23 (page 70) in the results section.

# 4. Results

## 4.1. MicroRNA expression profiling

### 4.1.1. Overview and unsupervised hierarchical clustering

The microRNA expression data provided by Exiquon® have been visualized in heatmaps. Figure 11 shows the result of the two-way hierarchical clustering of microRNAs (top 100 microRNA) and samples. The comparison of all samples and their distinct microRNA expression reveals a variety of differentially expressed microRNA and shows subgroups among the complex data. The osteosarcoma cell lines HOS, HOS-58 and ZK-58 for instance show a high level of similarity in their expression patterns. Furthermore, the control cell lines (L87.4 and hFOB 1.19) show similar expression when compared to the osteosarcoma cell lines.

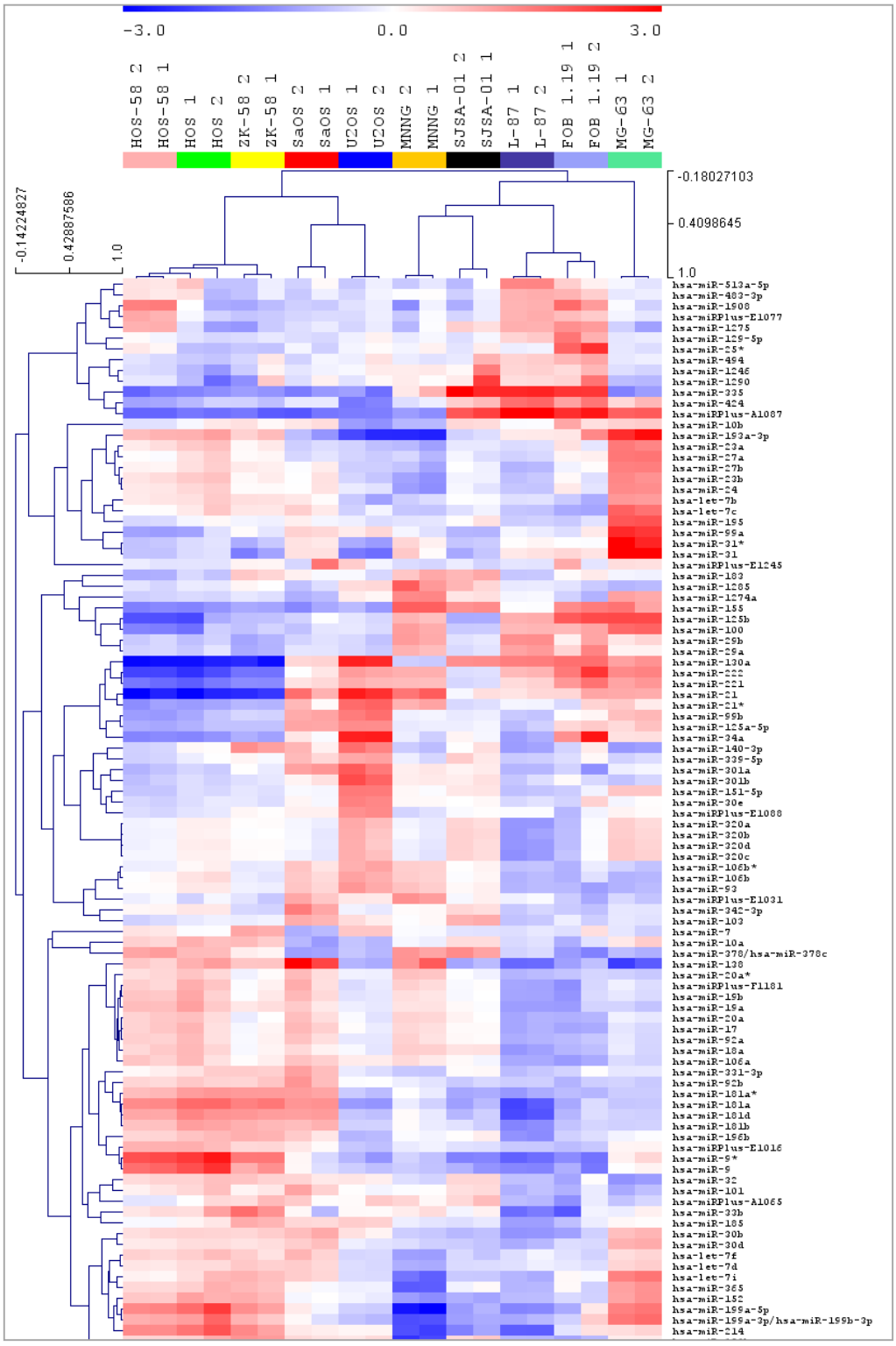



Figure 11: Heatmap: unsupervised hierarchical clustering of all samples and microR top 100; each row represents a microRNA and each column represents a sample, every sample is shown in duplicate; microRNA clustering tree shown on the left; color scale at the bottom illustrates the relative expression level (-3 to+3) of a microRNA across all samples; red color: expression level above mean, blue: expression level lower than mean

The PCA plot in figure 12 shows that the duplicates of the cell lines cluster together illustrating a high overall correlation of the expression data (>70%). With correlation estimates (Pearson) between 0,74 and 0,77 the cell lines SaOS-2 , SJSA-01 and hFOB 1.19 were the ones with the lowest correlation. The controls FOB1.19 and L-87.4 , already described as similar according to their expression patterns, are clustering together in the PCA plot, as well. Additionally, the plotting reveals that cell line passage (the duplicates) is a minor factor compared to cell line origin. As noted before HOS, HOS-58 and ZK-58 form a tight cluster which leads to the assumption that they are biologically similar. For this reason I decided to keep only one of these cell lines, HOS-58, for further analysis.

In Figure 13A and B the overall correlation of microRNA (A) and mRNA (B) expression in between the respective cell lines is visualized as a heat map using the Pearson correlation method. Both heatmaps show a high level of similarity.

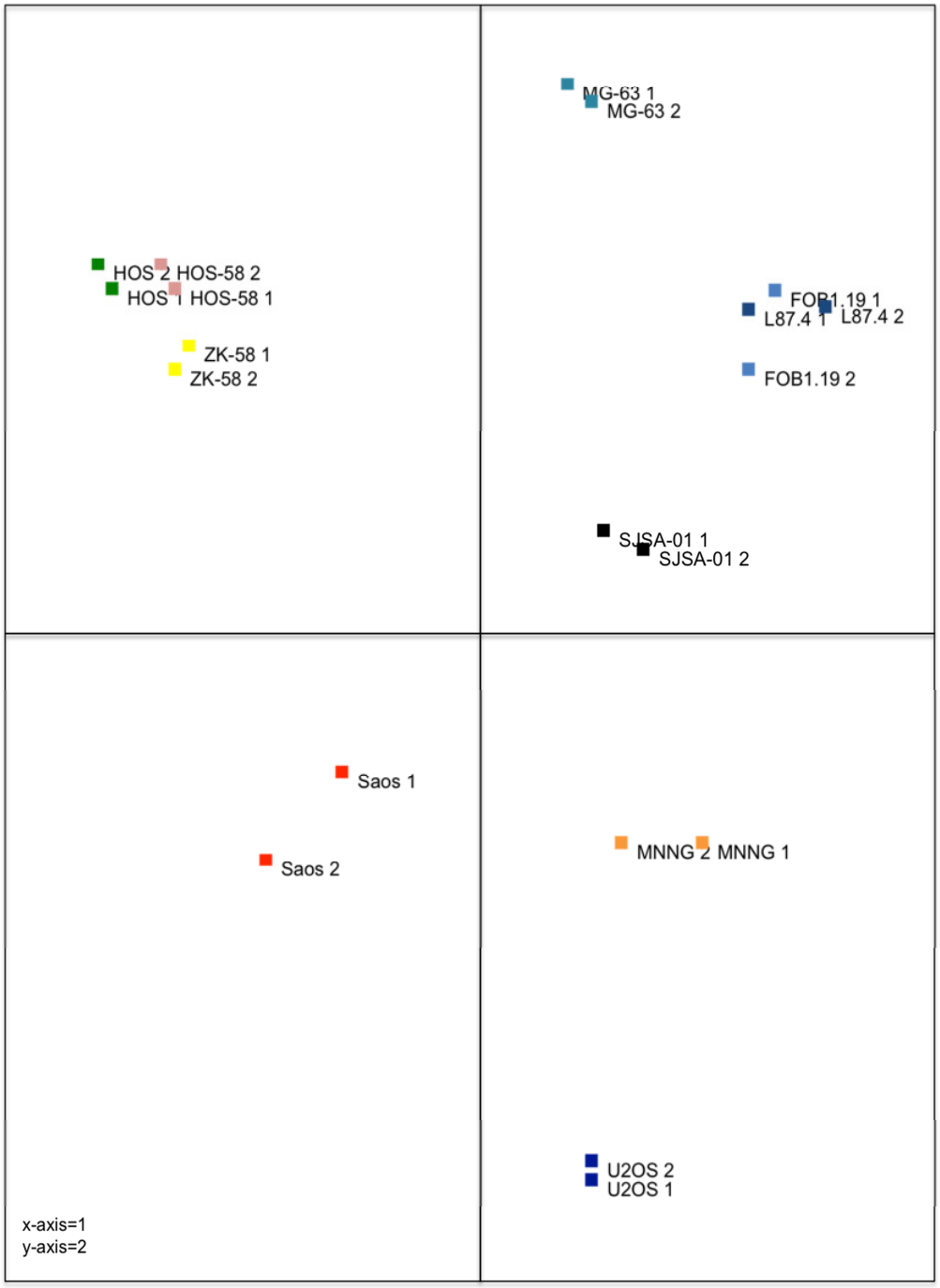



Figure 12: PCA plotting of all cell lines, clustering of biological replicates (1 and 2), duplicates of all cell lines are each represented with the same colored dots; references hFOB 1.19. and L87.4 cluster together closely; osteosarcoma cell lines ZK-58, HOS-58 and HOS form a cluster, as well; the cell lines with the lowest Pearson correlation coefficient SaOS, hFOB 1.19 and SJSA-01 are more distant from their duplicates

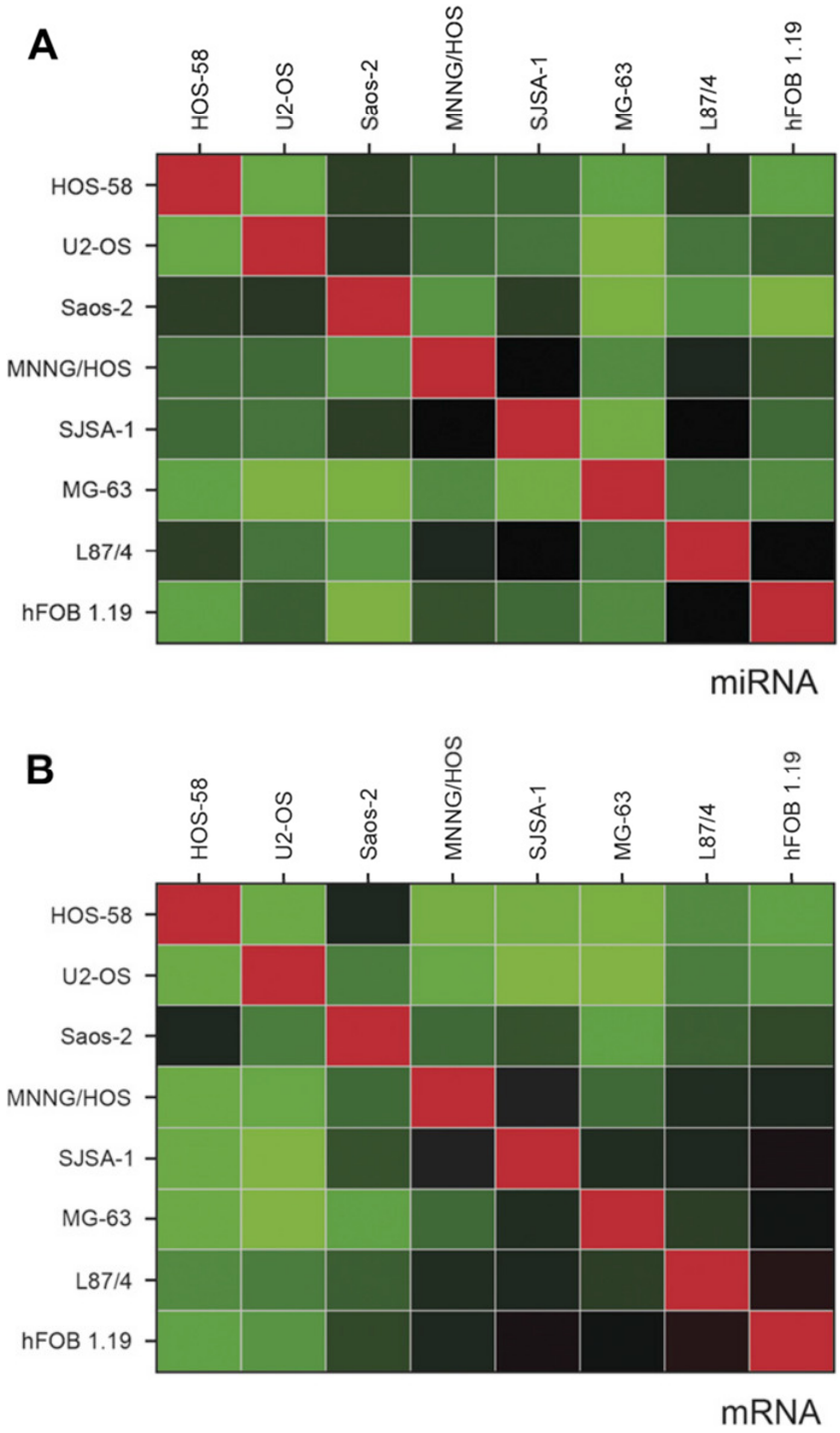



Figure 13: Pearson correlation heat maps, microRNA (A) expression and mRNA (B) expression between cell lines; red: positive correlation → high level of similarity; green: negative correlation→ low level of similarity

### 4.1.2. Osteosarcoma cell lines vs. progenitor cell lines

In order to find differentially expressed microRNA each osteosarcoma cell lines' expression pattern was compared separately to the expression levels of the reference cell lines (L87-4 and hFOB 1.19).

Focusing only on the miRNA showing a deregulation (up/down) repeatedly, i.e. in ≥ 4 of 6 osteosarcoma cell lines, I found a number of 15 miRNA with constant alteration in comparison to the progenitor cell lines. An overview is given in table 5. Of these 15 miRNA, 7 (miR-17-5p, miR-18a, miR-30b, miR-93, miR-106a and b, miR-301a) were constantly up-regulated in all affected cell lines in comparison to both osteoblasts (hFOB) and mesenchymal stem cells (L-87.4). In this set of miRNAs, 5 belong to the well-described oncogenic miR- 17-92 cluster.

A repeated down-regulation, when referred to the progenitor cell lines, was noted for the miRNAs 29a, miR-335, miR-424 and miR-1275.

The miRNAs 125b, miR-193-3p and miR-193b showed differential regulation between the individual osteosarcoma cell lines, meaning that miR-125b and 193a-3p were up-regulated only in the cell line MG-63 and miR-193b was down-regulated only in MNNG. One microRNA (miRNA-34a) was differential expressed within the two progenitor cell lines, i.e. it presented up-regulated when compared to the stem cell line and was down-regulated in 5/6 of the osteosarcoma cell lines when compared to the osteoblasts

| Ref | miRNA | HOS | SaOS | MG63 | MNNG | SJSA | U2OS | AFC |
|---|---|---|---|---|---|---|---|---|
| L-87 | hsa-miR-17 | up (1.62) | up (1.68) | - | up (1.4) | up (1.04) | - | 1,44 |
| hFOB | hsa-miR-17 | up (1.67) | up (1.73) | - | up (1.45) | up (1.09) | - | 1,49 |
| L-87 | hsa-miR-18a | up (1.84) | up (1.47) | - | up (1.74) | up (1.51) | - | 1,64 |
| hFOB | hsa-miR-18a | up (1.49) | up (1.12) | - | Up (1.39) | up (1.16) | - | 1,29 |
| L-87 | hsa-miR-29a | down (-1.56) | down (-1.6) | - | - | down (-1.52) | down (-1.67) | -1,56 |
| hFOB | hsa-miR-29a | down (-1.53) | down (-1.37) | - | - | down (-1.44) | down (-1.6) | -1,49 |
| L-87 | hsa-miR-30b | up (1.78) | up (2.16) | up (2.07) | - | - | up (1.51) | 1,88 |
| hFOB | hsa-miR-30b | up (1.33) | up (1.71) | up (1.62) | - | - | up (1.06) | 1,43 |
| L-87 | hsa-miR-34a | - | up (1.17) | up (1.41) | - | up (1.06) | up (3.7) | 1,84 |
| hFOB | hsa-miR-34a | down (-3.56) | down (-2.08) | down (-1.84) | down (-2.59) | down (-2.19) | | -2,45 |
| L-87 | hsa-miR-93 | up (1.11) | up (1.43) | - | up (1.51) | - | up (1.97) | 1,51 |
| hFOB | hsa-miR-93 | up (1.24) | up (1.56) | - | up (1.64) | up (1.11) | up (2.1) | 1,53 |
| L-87 | hsa-miR-106a | up (1.62) | up (1.66) | - | up (1.37) | - | up (1.24) | 1,47 |
| hFOB | hsa-miR-106a | up (1.7) | up (1.74) | - | up (1.44) | up (1.07) | up (1.32) | 1,45 |
| L-87 | hsa-miR-106b | up (1.24) | up (1.39) | - | up (1.41) | - | up (1.87) | 1,48 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| hFOB | hsa-miR-106b | up (1.01) | up (1.16) | - | up (1.18) | - | up (1.64) | 1,25 |
| L-87 | hsa-miR-125b | down (-2.4) | down (-1.21) | up (1.19) | - | down (-1.94) | down (-1.33) | -1,14 |
| hFOB | hsa-miR-125b | down (-3.48) | down (-2.29) | - | down (-1.23) | down (-3.02) | down (-2.42) | -2,49 |
| L-87 | hsa-miR-193a-3p | - | down (-1.24) | up (2.45) | down (-2.83) | - | down (-2.59) | -1,05 |
| hFOB | hsa-miR-193a-3p | - | down (-1.94) | up (1.75) | down (-3.53) | down (-1.42) | down (-3.28) | -1,68 |
| L-87 | hsa-miR-193b | up (1.64) | up (1.26) | up (1.08) | down (-1.42) | up (1.4) | up (1.22) | 0,86 |
| hFOB | hsa-miR-193b | up (1.74) | up (1.35) | up (1.18) | down (1.33) | up (1.49) | up (1.32) | 0,96 |
| L-87 | hsa-miR-301a | - | up (2.04) | - | up (1.11) | up (1.05) | up (2.63) | 1,71 |
| hFOB | hsa-miR-301a | - | up (2.05) | - | up (1.11) | up (1.05) | up (2.63) | 1,71 |
| L-87 | hsa-miR-335 | down (-3.97) | down (-3.95) | down (-3.88) | down (-1.94) | - | down (-3.95) | -3,54 |
| hFOB | hsa-miR-335 | down (-3.77) | down (-3.75) | down (-3.68) | down (-1.74) | - | down (-3.75) | -3,34 |
| L-87 | hsa-miR-424 | down (-2.43) | down (-2.02) | down (-1.09) | down (-2.05) | down (-1.05) | down (-3.28) | -1,99 |
| hFOB | hsa-miR-424 | down (-2.06) | down (-1.65) | - | down (-1.67) | - | down (-2.91) | -2,07 |
| L-87 | hsa-miR-1275 | down (-1.64) | down (-1.24) | down (-1.74) | down (-1.31) | - | down (-1.28) | -1,44 |
| hFOB | hsa-miR-1275 | down (-1.96) | down (-1.56) | down (-2.06) | down (-1.64) | - | down (-1.61) | -1,77 |

Table 5: Differentially expressed miRNA in ≥ 4 out of 6 cell lines (n=15); each microRNA looked at separately for expression level in osteosarcoma versus progenitor cell lines (leftmost column); abbreviations: ref: reference cell lines; AFC: Average Fold Change

## 4.2. Differentially expressed microRNA and likely targets

### 4.2.1. One-by-one comparison against the background of progenitor cell lines

For predicting likely target genes for the subset of differentially expressed microRNA the miRDB (http://mirdb.org) database was used. By means of a certain prediction score (>80) up to 207 target transcripts and 155 target genes have been identified per microRNA. The expression of all genes of the osteosarcoma cell lines targeted by the 15 previously determined microRNAs were subsequently compared separately between the osteosarcoma cells and both reference cell lines. When a microRNA and its likely target mRNA did show an inverse expression pattern, i.e. the microRNA up- and mRNA down-regulated (and vice versa), its deregulation was assumed to be an effect of the microRNA. In table 6 the genes that show proper regulation in ≥4/12 comparisons are presented. Since a comparison of 6 cell lines and 2 reference cell lines was done, one gene had the possibility to present with appropriate regulation by one microRNA in up to 12 comparisons.

RGMB, known as RGM domain family member B, for example showed a proper regulation by miR-93 in 8/12 comparisons, herewith marking the combination of microRNA/mRNA that show “correct” regulation in the highest number of comparisons. The miR-93 as well as the other top-listed microRNA in table 6 (miR-106a, b and miR-17) are members of the 17-92 cluster.

| OGS | Gene name | Reg | miRNA | F |
|---|---|---|---|---|
| RGMB | RGM domain family, member B | down | hsa-miR-93 | 8 |
| | | | hsa-miR-106a | 7 |
| | | | hsa-miR-106b | 7 |
| | | | hsa-miR-17 | 6 |
| PDCD1LG2 | Programmed cell death 1 ligand 2 | down | hsa-miR-106b | 7 |
| | | | hsa-miR-93 | 7 |
| | | | hsa-miR-106a | 6 |
| | | | hsa-miR-17 | 5 |
| FAM70A | Family with sequence similarity 70, member A | up | hsa-miR-424 | 7 |
| NT5E | 5'-nucleotidase, ecto (CD73) | down | hsa-miR-30b | 6 |
| CCNE1 | Cyclin E1 | up | hsa-miR-424 | 6 |
| LIMA1 | LIM domain and actin binding 1 | down | hsa-miR-106a | 5 |
| | | | hsa-miR-106b | 5 |
| | | | hsa-miR-93 | 5 |
| F3 | Coagulation factor III (thromboplastin, tissue factor) | down | hsa-miR-93 | 5 |
| | | | hsa-miR-17 | 4 |
| POLR3G | Polymerase (RNA) III (DNA directed) polypeptide G (32kD) | down | hsa-miR-93 | 5 |
| SPTLC2 | serine palmitoyltransferase, long chain base subunit 2 | down | hsa-miR-93 | 5 |
| | | | hsa-miR-17 | 4 |
| CAMK2N1 | calcium/calmodulin-dependent protein kinase II inhibitor 1 | down | hsa-miR-106a | 4 |
| | | | hsa-miR-106b | 4 |
| | | | hsa-miR-17 | 4 |
| | | | hsa-miR-93 | 4 |
| FLI1 | Friend leukemia virus integration 1 | up | hsa-miR-193b | 4 |
| MYBL1 | v-myb myeloblastosis viral oncogene homolog (avian)-like 1 | down | hsa-miR-301a | 4 |
| LRRC17 | leucine rich repeat containing 17 | down | hsa-miR-30b | 4 |
| NRXN1 | neurexin 1 | up | hsa-miR-335 | 4 |
| CASK | calcium/calmodulin-dependent serine protein kinase (MAGUK family) | up | hsa-miR-424 | 4 |
| MGAT4A | Mannosyl(alpha-1,3-)-glycoproteinbeta-1,4-N-acetylglucosaminyltransferase, Isozyme A | up | hsa-miR-424 | 4 |

Table 6: Target genes of deregulated miRNA osteosarcoma vs. reference cell lines, 4/12 comparisons; OGS=official gene symbol; F=frequency of adequate comparisons

## 4.3. Characterization of the phenotype

### 4.3.1. Proliferation analysis

After assaying the cell lines proliferative characteristics, it was possible to discriminate the osteosarcoma cell lines in a slow and a fast proliferating group on the basis of the doubling time. Based on findings in literature, 30 hours was used as a cutoff. The cell lines with the fastest doubling time, calculated in log-phase (see figure 7, chapter 3, page 27), were MNNG, SJSA-01, MG-63 and U2OS. The other 4 cell lines (HOS, HOS-58, ZK-58 and SaOS) were grouped as slow proliferating because they showed distinctively higher doubling times. The average doubling times for each osteosarcoma cell line (out of repetitive proliferation experiments) are demonstrated in figure 14 below.

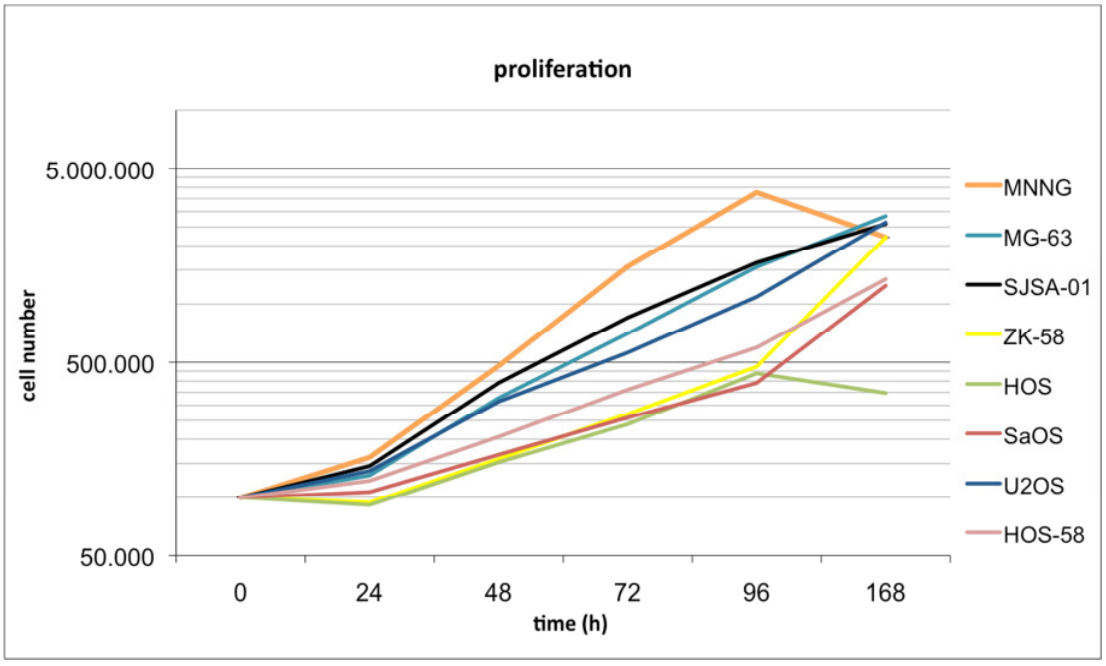



Figure 14: Exponential growth curves for all osteosarcoma cell lines (n=8); y-axis: cell number log.scale; x-axis: time

### 4.3.2. Migration and invasion analysis

As already described in chapter 3 I used a transwell approach with a Boyden chamber for assaying the migrative and invasive potential. In figure 15 A-D below it is apparent that the cell lines MNNG, SJSA-01 and U2OS were by far those with the highest migrative potential. Their cell numbers migrating to the lower membrane surface after 24 hours were significantly higher (cut off: average number per field >125 cells) when compared to the other five cell lines. After 48 hours the distribution of the cell lines differed only by the fact, that SJSA-01 now presented as the osteosarcoma cell line with the highest number of migrated cells (instead of U2OS). In figures 15 B and C for each time point (24h and 48h) a corresponding microscopic picture is displayed to exemplarily show low (MG-63), moderate (MNNG) and high (SJSA-01) migrative potential.

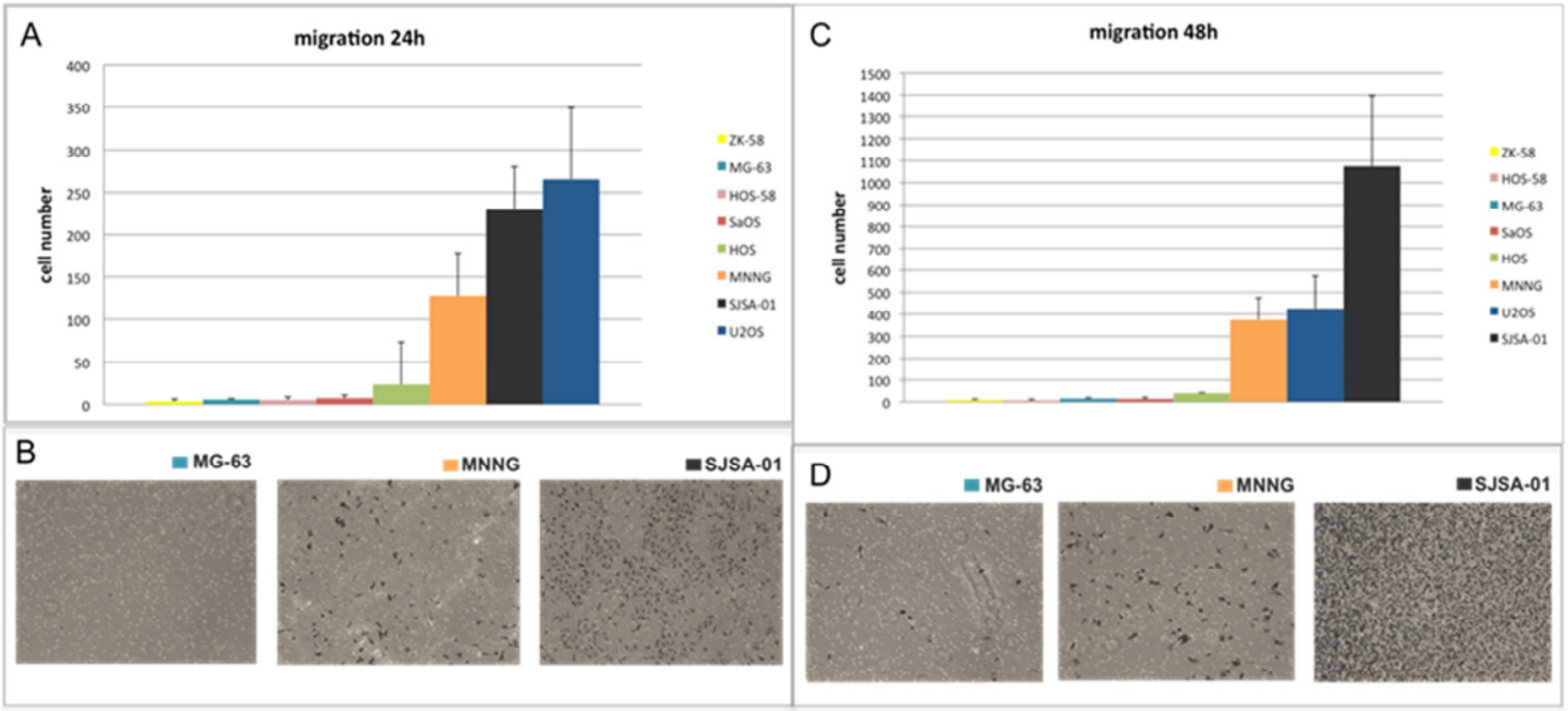



Figure 15: (A) Migrated cells for each osteosarcoma cell line (n=8) ; $t_1$=24h; x-axis: cell line names; y-axis: average number of migrated cells/membrane, (B) corresponding microscopic pictures (10x magnification) of 3 exemplarily chosen cell lines, left: MG-63, showing <25cells/24h migrating; middle: moderately migrating cell line MNNG): showing ~125 migrated cells/24h per membrane; right: highly migrative cell line (SJSA_01) with ~ 225 cells/24h ; (C) MIgrated cells for each osteosarcoma cell line (n=8) after second timepoint ($t_2$ =48h); (D) again corresponding microscopic pictures (10x magnification) of cell lines, MG-63, showing <25cells/48h migrating; MNNG: showing ~375 migrated/cells per membrane; highly migrative cell line (SJSA-01) with up to 1100/cells per membrane after 48 hours incubation

As far as the invasive properties are concerned, the osteosarcoma cell lines showed a similar pattern. The methodical difference for the invasion assay consists of the matrigel coating of the membrane to form a barrier simulating the cell membrane. After 48h (visible in figure 16 below) the highly migrative candidates MNNG (orange), U2OS (blue) and SJSA-01 (black) have also beeen identified as the most invasive ones with average cell numbers from >250 up to 650 on the lower membrane side. In contrast to these numbers, the non-invasive group presented with average cell numbers < 50 cells/field.

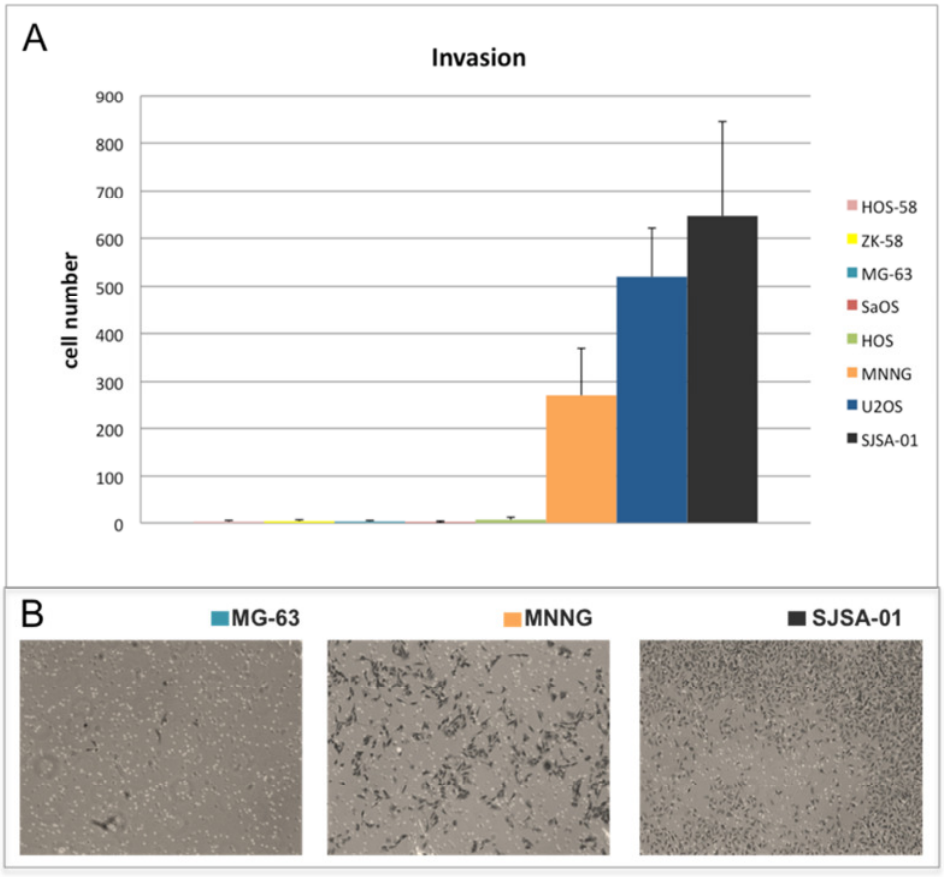



Figure 16: Number of invaded cells for each osteosarcoma cell line (n=8), t=48h; x-axis: cell line names; y-axis: number of invaded cells/field; (B) corresponding microscopic pictures (10x magnification) of 3 exemplarily chosen cell lines, left: MG-63, showing <5 invasive cells/48h; middle: moderately invading cell line MNNG: showing approx. 275 cells per membrane; right: cell line (SJSA-01) with the highest potential for invasion with >600 cells/48h and membrane

### 4.3.3. Grouping according to phenotype

The grouping of the cell lines according to their phenotype is displayed in table 7.

I assigned the osteosarcoma cell lines to a fast or slow growing, highly migrative or invasive subgroup to put the expression data into a functional biologic context. As visible, the migration and invasion groups were identical.

| Cell line | Proliferation | Migration | Invasion |
|---|---|---|---|
| HOS-58 | negative | negative | negative |
| HOS | negative | negative | negative |
| ZK-58 | negative | negative | negative |
| U2OS | positive | positive | positive |
| SaOS | negative | negative | negative |
| MNNG | positive | positive | positive |
| SJSA-01 | positive | positive | positive |
| MG-63 | positive | negative | negative |

Table 7: Phenotypic characterization of all 8 osteosarcoma cell lines
(negative - non-proliferating/migrating/invading; positive - highly proliferative/migrative/invasive)

## 4.4. Correlation of microRNA expression with cell lines' phenotype

### 4.4.1. Differential microRNA expression of proliferative cell lines

The expression data and the proliferation assay results were correlated using the R-package limma. This method has been created to analyze comprehensive microarray data by fitting them to a linear model. A detailed description of the approach can be found in chapter 3, pages 34-35. The differentially expressed microRNAs that have been found by this means are visualized in table 8.

Four members of the miRNA-181 family were identified to be of importance.

I found microRNA-181a, b, d and miR-181* (p=0,0033 and 0,0017) to be significantly down-regulated in highly-proliferative cell lines. The miRNA-186 (p=0,0266) also showed a differential expression when fast and slow growing osteosarcoma cell lines were compared. In figure 17 the results are displayed in boxplots to show the differences and variability between the fast and slow proliferating groups.

| miR-ID | logFC | AveExpr | t | P-value | Adjusted p-value |
|---|---|---|---|---|---|
| hsa-miR-181a | -151.3 | 103.0 | -957.3 | 4,72E+09 | 0.0033 |
| hsa-miR-181d | -123.2 | 106.0 | -117.2 | 1,36E+09 | 0.0017 |
| hsa-miR-181a* | -120.4 | 103.8 | -1.205 | 1,14E+09 | 0.0017 |
| hsa-miR-181b | -105.6 | 11.4 | -945.2 | 5,10E+09 | 0.0033 |
| hsa-miR-186 | -0.751 | 119.0 | -63.7 | 5,22E+04 | 0.0266 |

Table 8: miRNA distinguishing between fast and slow proliferating cell lines

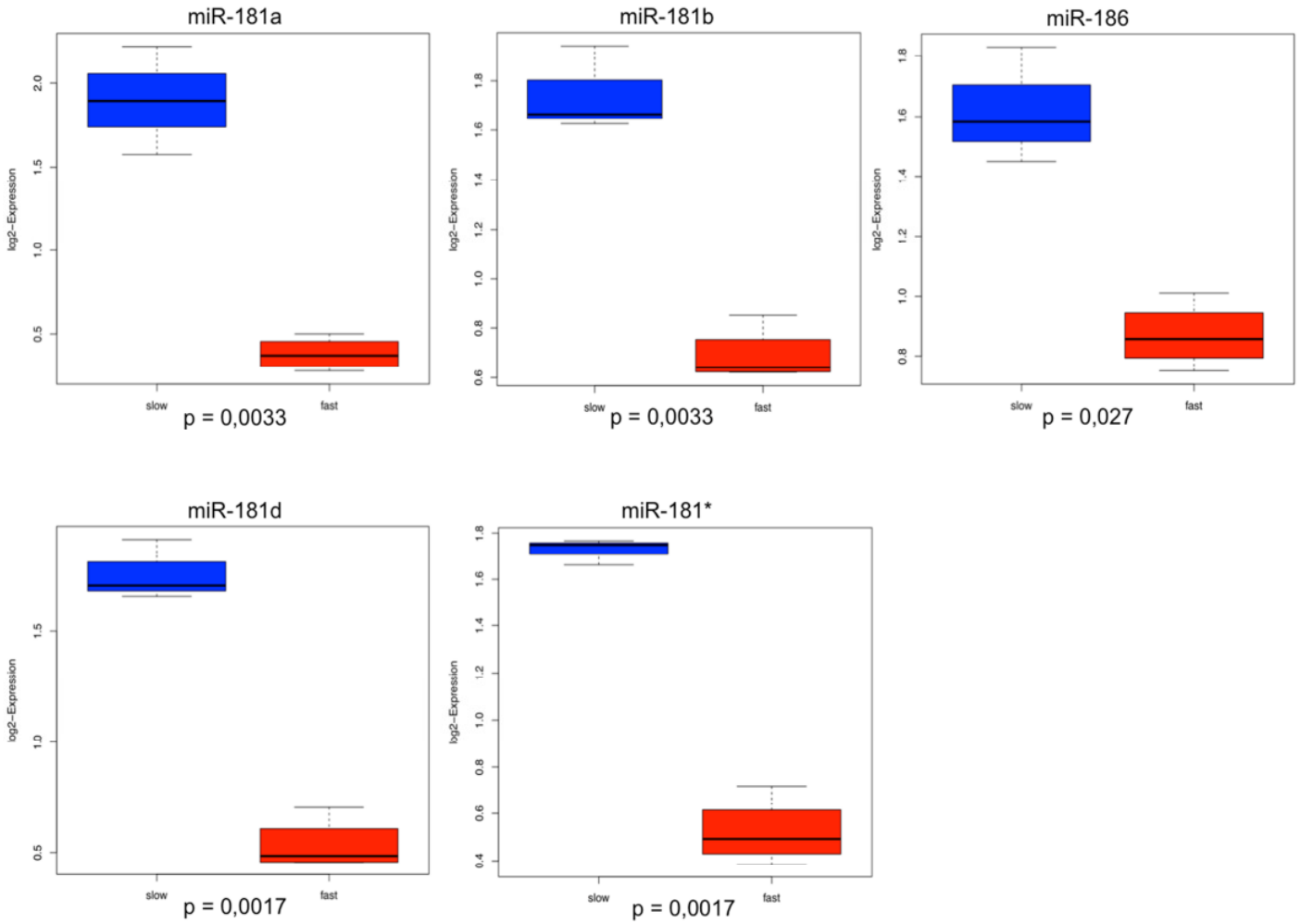



Figure 17: Correlation boxplots for all miRNA with significant p-value after comparison of slow and fast proliferating cell lines (181a, b, d and 181*, miR-186), overexpression in slow proliferating cell lines (blue), expression lower than average (red) in fast proliferating lines.

### 4.4.2. Differential microRNA expression in migrative and invasive cell lines

Correlation of the migration and invasion assay data has been done analogue to the proliferation analysis. Comparing the expression levels of both groups (invasive and migrative were identical) and the microRNAs hsa-let-7d and let-7f were found to be differentially expressed. I have noted a significant down-regulation (p=0,0295) in cell lines that were characterized by distinct migrative and invasive potential.

Table 9 and figure 18 below show the ability of the miRNAs let-7d and f to distinguish between migrative/invasive and non-migrative/non-invasive cell lines.

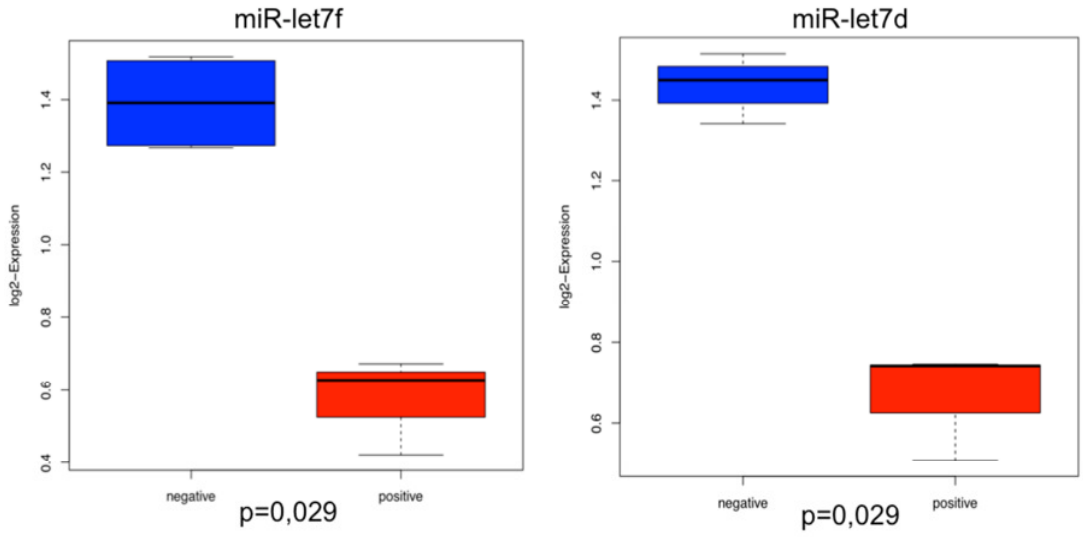



Figure 18: Correlation box plot miRNA let-7f and let-7d migration/invasion negative and overexpressed (blue) and migration/invasion positive with let-7f low expression levels (red); Fold Change: -0,82 and -0,77; adjusted p-value: 0,029 for both miRNA; more details see table 9

| miR-ID | logFC | AveExpr | t | P.Value | adj.P.Val |
|---|---|---|---|---|---|
| hsa-let-7f | -0.82 | 10.4 | -728.0 | 2,31E+04 | 0.029 |
| hsa-let-7d | -0.77 | 110.8 | -807.3 | 1,25E+04 | 0.029 |

Table 9: miRNA distinguishing between migrative/invasive and non-migrative/non-invasive cell lines

## 4.5. Correlation of mRNA expression and cell lines' phenotype

### 4.5.1. Differential gene expression in highly proliferative vs. slow proliferating cell lines

In table 10 the genes differentiating between the fast and slow proliferating osteosarcoma cell line are displayed, sorted by p-value. All these 60 genes show significant (p-value < 0,05) up- or down-regulation in the fast- vs. slow growing cell lines of this study. In the list 37/60 (61,6%) genes are down-regulated when the groups of proliferative and non-proliferative cell lines are compared. Only 23/60 genes (38,3%) showed an up-regulation in the fast growing group.

| gene name | regulation | logFC | AveExpr | T | P.Value | adj.P.Val |
|---|---|---|---|---|---|---|
| C4orf31 | down | -6,62 | 8,63 | -26,58 | 7,82E-09 | 0,00008 |
| PANX3 | down | -6,46 | 8,99 | -27,92 | 5,39E-09 | 0,00008 |
| S100A16 | up | 5,22 | 9,20 | 22,88 | 2,44E-08 | 0,00017 |
| HIST1H2BM | up | 4,94 | 9,67 | 16,85 | 2,45E-07 | 0,00125 |
| LRRC15 | down | -4,11 | 9,09 | -14,32 | 8,22E-07 | 0,00279 |
| DCP1B | up | 2,57 | 7,68 | 14,53 | 7,40E-07 | 0,00279 |
| ANO5 | down | -5,51 | 7,48 | -12,57 | 2,16E-06 | 0,00628 |
| IFITM5 | down | -4,10 | 8,05 | -11,79 | 3,46E-06 | 0,00881 |
| JAKMIP2 | down | -3,42 | 6,98 | -11,30 | 4,71E-06 | 0,00884 |
| VGLL3 | up | 3,04 | 7,87 | 11,39 | 4,45E-06 | 0,00884 |
| CCDC3 | down | -2,74 | 7,93 | -11,28 | 4,77E-06 | 0,00884 |
| ARHGAP29 | up | 4,27 | 7,84 | 11,08 | 5,43E-06 | 0,00922 |
| PXDN | up | 4,90 | 8,78 | 10,92 | 6,05E-06 | 0,00949 |
| ALPL | down | -4,82 | 9,20 | -10,46 | 8,22E-06 | 0,01118 |
| MAP1A | down | -2,79 | 8,01 | -10,54 | 7,82E-06 | 0,01118 |
| CCND1 | up | 3,03 | 9,07 | 10,36 | 8,82E-06 | 0,01124 |
| ROBO2 | down | -2,65 | 7,48 | -10,05 | 1,10E-05 | 0,01323 |
| DLX5 | down | -2,88 | 8,32 | -9,93 | 1,20E-05 | 0,01358 |
| SERPINE1 | up | 3,07 | 8,07 | 9,60 | 1,53E-05 | 0,01641 |
| CHN2 | down | -3,94 | 8,60 | -9,40 | 1,78E-05 | 0,01724 |
| ADRA1D | down | -1,89 | 7,81 | -9,44 | 1,72E-05 | 0,01724 |
| FAT3 | down | -3,82 | 7,56 | -9,20 | 2,06E-05 | 0,01914 |
| NME4 | up | 3,73 | 9,45 | 9,03 | 2,35E-05 | 0,02086 |
| PTPRZ1 | down | -3,52 | 6,88 | -8,80 | 2,84E-05 | 0,02411 |
| SCIN | down | -4,22 | 7,85 | -8,42 | 3,87E-05 | 0,03001 |
| NOTUM | down | -2,74 | 8,01 | -8,39 | 3,97E-05 | 0,03001 |
| XPR1 | down | -1,75 | 9,75 | -8,46 | 3,74E-05 | 0,03001 |
| CNTN4 | down | -3,77 | 7,34 | -8,31 | 4,23E-05 | 0,03079 |

| | | | | | | |
|---|---|---|---|---|---|---|
| LEPREL1 | up | 3,24 | 7,61 | 8,26 | 4,43E-05 | 0,03114 |
| WISP1 | down | -2,91 | 8,01 | -8,22 | 4,58E-05 | 0,03114 |
| ANGPT1 | down | -2,95 | 7,33 | -8,14 | 4,87E-05 | 0,03207 |
| MAB21L2 | down | -2,49 | 7,42 | -8,06 | 5,25E-05 | 0,03246 |
| LPAR4 | down | -2,36 | 6,25 | -8,08 | 5,13E-05 | 0,03246 |
| EMP1 | up | 3,28 | 8,92 | 8,02 | 5,42E-05 | 0,03252 |
| TM4SF1 | up | 4,13 | 8,46 | 7,95 | 5,75E-05 | 0,03341 |
| ARRB1 | up | 1,94 | 7,77 | 7,92 | 5,90E-05 | 0,03341 |
| CDKN2A | down | -3,95 | 8,08 | -7,88 | 6,12E-05 | 0,03373 |
| DLX3 | down | -3,24 | 7,65 | -7,82 | 6,48E-05 | 0,03386 |
| ADRA1A | down | -1,96 | 6,47 | -7,82 | 6,43E-05 | 0,03386 |
| CDK6 | up | 3,99 | 8,17 | 7,61 | 7,78E-05 | 0,03623 |
| CTSZ | up | 2,40 | 8,00 | 7,63 | 7,68E-05 | 0,03623 |
| APOBEC3F | up | 2,06 | 7,60 | 7,63 | 7,63E-05 | 0,03623 |
| NUDT4 | down | -1,80 | 6,80 | -7,64 | 7,55E-05 | 0,03623 |
| ARAP3 | up | 1,28 | 7,05 | 7,61 | 7,82E-05 | 0,03623 |
| GPR133 | down | -3,00 | 7,53 | -7,42 | 9,26E-05 | 0,04055 |
| TIMP3 | up | 2,57 | 9,55 | 7,43 | 9,14E-05 | 0,04055 |
| FOXP2 | down | -1,88 | 5,80 | -7,41 | 9,34E-05 | 0,04055 |
| SPRED2 | up | 1,55 | 9,20 | 7,33 | 1,00E-04 | 0,04263 |
| LMO3 | down | -3,37 | 7,58 | -7,31 | 1,03E-04 | 0,04275 |
| C1orf118 | down | -1,26 | 6,58 | -7,23 | 1,10E-04 | 0,04505 |
| JUP | up | 1,72 | 7,74 | 7,16 | 1,18E-04 | 0,04718 |
| DENND2C | down | -2,57 | 6,86 | -7,04 | 1,33E-04 | 0,04746 |
| CSAG1 | down | -2,22 | 6,67 | -7,10 | 1,25E-04 | 0,04746 |
| CDH15 | down | -2,03 | 7,20 | -7,06 | 1,30E-04 | 0,04746 |
| ETV6 | up | 1,73 | 8,49 | 7,06 | 1,30E-04 | 0,04746 |
| MAGEA2 | down | -1,50 | 7,67 | -7,12 | 1,22E-04 | 0,04746 |
| KIAA0182 | down | -1,34 | 9,21 | -7,05 | 1,31E-04 | 0,04746 |
| KLHL29 | up | 2,17 | 7,04 | 6,98 | 1,40E-04 | 0,04907 |
| AFF2 | down | -1,71 | 6,91 | -6,96 | 1,42E-04 | 0,04907 |
| RAB8B | up | 1,56 | 9,20 | 6,95 | 1,44E-04 | 0,04907 |

Table 10: Differentially expressed mRNA in comparison of fast and slow proliferating cell lines (n=60); only genes with p-value<0,05 are shown

To evaluate the real biological impact of the differential expressed genes, a query of all 60 genes in the DAVID (Database for Annotation, Visualization and Integrated Discovery) annotation tool was done. A functional enrichment analysis (GOTerm BP is demonstrated in table 11 (only top-listed results with p-value <0,05). It revealed that a significant number of genes (10 genes, see table) is involved in the biological processes (BP) regulation of cell proliferation (p-value: 0,0017) , regulation of cell

differentiation (p-value: 0.00616) or positive regulation of cell proliferation (p-value: 0,0129). The annotation clustering tool of DAVID furthermore showed a significant (p-value 0,0011) clustering of 4 genes (CDK6, CDKN2A, CCND1 and Serpine1) involved in the p53 pathway.

| Category / Go-Term | Count | p-value | Gene list |
|---|---|---|---|
| Regulation of cell proliferation | 10 | 0.00117 | CCND1, CDKN2A, DLX5, SCIN, SERPINE1, ADRA1A, CDK6, MAB21L2, ADRA1D, FOXP2 |
| Organ development | 15 | 0.00143 | ALPL, CDK6, AFF2, TIMP3, FOXP2, DLX3, CCND1, CDKN2A, DLX5, SERPINE1, ANGPT1, CNTN4, ROBO2, MAB21L2, EMP1 |
| Response to vitamin | 4 | 0.00145 | ALPL, CCND1, ANGPT1, TIMP3 |
| Regeneration | 4 | 0.00165 | CCND1, SERPINE1, ANGPT1, TIMP3 |
| Regulation of developmental process | 9 | 0.00179 | CCND1, CDKN2A, DLX5, SCIN, SERPINE1, CDK6, ROBO2, CNTN4, ARAP3 |
| Multicellular organismal development | 19 | 0.00442 | ALPL, PTPRZ1, CDK6, AFF2, TIMP3, FOXP2, DLX3, CCND1, CDKN2A, FAT3, DLX5, SERPINE1, SPRED2, ROBO2, ANGPT1, CNTN4, MAB21L2, EMP1, ADRA1D |
| Developmental process | 20 | 0.00519 | ALPL, PTPRZ1, CDK6, AFF2, TIMP3, FOXP2, DLX3, CCND1, CDKN2A, FAT3, DLX5, SERPINE1, SPRED2, ROBO2, ANGPT1, CNTN4, ETV6, MAB21L2, EMP1, ADRA1D |
| Regulation of cell differentiation | 7 | 0.00616 | CCND1, CDKN2A, DLX5, SCIN, CDK6, ROBO2, CNTN4 |
| System development | 16 | 0.00842 | ALPL, PTPRZ1, CDK6, AFF2, TIMP3, FOXP2, DLX3, CCND1, CDKN2A, DLX5, SERPINE1, ANGPT1, CNTN4, ROBO2, MAB21L2, EMP1 |
| Regulation of cell-substrate adhesion | 3 | 0.01065 | C4ORF31, CDKN2A, CDK6 |
| Response to nutrient | 4 | 0.01192 | ALPL, CCND1, ANGPT1, TIMP3 |
| Positive regulation of cell proliferation | 6 | 0.01288 | CCND1, DLX5, CDK6, MAB21L2, ADRA1D, FOXP2 |
| Regulation of multicellular organismal process | 9 | 0.01289 | CCND1, CDKN2A, DLX5, SCIN, SERPINE1, CDK6, ROBO2, CNTN4, ADRA1D |
| Cell-cell adhesion | 5 | 0.01418 | JUP, CDH15, FAT3, ROBO2, CNTN4 |
| Central nervous system development | 6 | 0.01429 | PTPRZ1, ROBO2, AFF2, CNTN4, TIMP3, FOXP2 |
| Negative regulation of biological process | 13 | 0.01599 | MAP1A, CDK6, APOBEC3F, TIMP3, FOXP2, CCND1, CDKN2A, SERPINE1, SCIN, ADRA1A, CNTN4, ARAP3, ADRA1D |
| Anatomical structure development | 16 | 0.01742 | ALPL, PTPRZ1, CDK6, AFF2, TIMP3, FOXP2, DLX3, CCND1, CDKN2A, DLX5, SERPINE1, ANGPT1, CNTN4, ROBO2, MAB21L2, EMP1 |
| Growth | 4 | 0.02420 | SERPINE1, TIMP3, EMP1, FOXP2 |
| Regulation of epithelial cell proliferation | 3 | 0.02424 | DLX5, CDK6, FOXP2 |
| Tissue development | 7 | 0.02438 | ALPL, CDKN2A, DLX5, SERPINE1, TIMP3, EMP1, FOXP2 |

| | | | |
|---|---|---|---|
| Response to steroid hormone stimulus | 4 | 0.02739 | ALPL, CCND1, ANGPT1, TIMP3 |
| Axonogenesis | 4 | 0.02775 | PTPRZ1, DLX5, ROBO2, CNTN4 |
| Nervous system development | 9 | 0.02899 | PTPRZ1, DLX5, CDK6, ROBO2, AFF2, CNTN4, MAB21L2, TIMP3, FOXP2 |
| Response to nutrient levels | 4 | 0.02925 | ALPL, CCND1, ANGPT1, TIMP3 |
| Response to inorganic substance | 4 | 0.03236 | PXDN, CCND1, S100A16, SERPINE1 |
| Multicellular organismal process | 22 | 0.03354 | ALPL, PTPRZ1, MAP1A, CDK6, AFF2, TIMP3, FOXP2, DLX3, CCND1, CDKN2A, FAT3, ARRB1, DLX5, SERPINE1, SPRED2, ADRA1A, ROBO2, CNTN4, ANGPT1, MAB21L2, EMP1, ADRA1D |
| Response to external stimulus | 8 | 0.03367 | ALPL, CCND1, ARRB1, SERPINE1, ROBO2, ANGPT1, TIMP3, FOXP2 |
| Cell morphogenesis involved in neuron differentiation | 4 | 0.03399 | PTPRZ1, DLX5, ROBO2, CNTN4 |
| Neuron projection morphogenesis | 4 | 0.03565 | PTPRZ1, DLX5, ROBO2, CNTN4 |
| Positive regulation of biological process | 13 | 0.03620 | RAB8B, CDK6, TIMP3, FOXP2, C4ORF31, CCND1, CDKN2A, DLX5, SCIN, ANGPT1, ROBO2, MAB21L2, ADRA1D |
| Response to extracellular stimulus | 4 | 0.03867 | ALPL, CCND1, ANGPT1, TIMP3 |
| Positive regulation of cellular process | 12 | 0.04257 | C4ORF31, CCND1, CDKN2A, RAB8B, DLX5, SCIN, CDK6, ROBO2, MAB21L2, TIMP3, ADRA1D, FOXP2 |
| Anatomical structure morphogenesis | 9 | 0.04710 | CCND1, PTPRZ1, DLX5, SERPINE1, ROBO2, ANGPT1, CNTN4, MAB21L2, TIMP3 |
| Interphase of mitotic cell cycle | 3 | 0.04787 | CCND1, CDKN2A, CDK6 |
| Response to estrogen stimulus | 3 | 0.04954 | CCND1, ANGPT1, TIMP3 |
| Cell morphogenesis involved in differentiation | 4 | 0.04995 | PTPRZ1, DLX5, ROBO2, CNTN4 |

Table 11: GoTerm enrichment of table 10 gene list (n=60), genes that were deregulated in the comparison between proliferative and non-proliferative group of genes; sorted by p-value (only displayed p<0,05)

### 4.5.2. Correlation of gene expression and migrative/invasive properties

The expression pattern of the transciptome was compared between the groups assigned as migration/invasion positive and negative.

The following table 12 displays the genes that were found to be differentially expressed in comparison of the migrative/invasive and non-migrative/non-invasive group of cell lines. From the number of 10 genes listed in table 12 only one, TMEM119, shows a significant p-value (<0,05). The other 9 genes range between a p-value of 0,07 and 0,09. TMEM119 expression is significantly down-regulated (p-value 0,00295) in the migration positive versus the non-migrative/non-invasive cell lines. Among the other genes 4 are up-regulated and 5 downregulated when the groups of migrative and non migrative and invasive/non-invasive are compared, respectively.

| gene name | regulation | logFC | AveExpr | t | P.Value | adj.P.Val |
|---|---|---|---|---|---|---|
| TMEM119 | down | -4,70 | 9,04 | -18,15 | 1,45E-07 | 0,00295 |
| TP53 | up | 5,04 | 7,95 | 9,69 | 1,46E-05 | 0,07226 |
| KRT8 | up | 3,79 | 9,52 | 9,88 | 1,26E-05 | 0,07226 |
| FRMD5 | up | 2,86 | 7,02 | 8,99 | 2,48E-05 | 0,07226 |
| TRPS1 | down | -2,33 | 9,80 | -9,14 | 2,21E-05 | 0,07226 |
| XYLT1 | down | -2,11 | 7,66 | -9,50 | 1,68E-05 | 0,07226 |
| KIAA0907 | down | -1,64 | 9,56 | -9,18 | 2,14E-05 | 0,07226 |
| UNC5B | down | -2,20 | 8,66 | -8,53 | 3,60E-05 | 0,08549 |
| ARHGEF2 | down | -1,77 | 8,76 | -8,47 | 3,77E-05 | 0,08549 |
| C11orf41 | up | 1,93 | 7,06 | 8,27 | 4,46E-05 | 0,09102 |

Table 12: Differentially expressed mRNA migration/invasion (pos vs. neg migration/invasion groups), p-values <0,1 are shown

The top-table list was fed into the DAVID Annotation tool for analysis and the database recognized all 10 gene IDs. When considering the GO Term enrichment in the Annotation Clustering Analysis the genes ARHGEF2, UNC5B, KRT8 and TP53 show significant relation to the biological processes (GOTerm BP fat) apoptosis and programmed cell death (p-values 0,00082 or 0,000856 respectively).

Furthermore a subset of 3 genes out of these 10 (ARHGEF2, TRPS1, TP53) is significantly associated to the processes intracellular protein transport, protein transport in general, establishment of protein localization or related terms.

This again fits to the comparison of expression levels between migrative and non-migrative or invasive versus non-invasive cell lines that I performed.

## 4.6. Integrative analysis of microRNA and their target genes in correlation to phenotype

By using a modified approach described by Peng et al. (2009) regulatory microRNA-mRNA modules being associated to the features proliferation and invasion/migration have been identified. The expression matrices of the cell lines were used for testing mRNA and microRNA for negative correlation. It was assumed that microRNAs disable the expression of their target mRNA. I fed all data/microRNA-mRNA bipartite networks into the Ingenuity Pathway Analysis tool for illustrating the biological context of the respective microRNA-mRNA-coupling. The database created networks that include the up-/down-regulated molecules. The networks visualize the connection of the deregulated mRNA and microRNA to other molecules from canonical pathways derived from literature. The IPA software combines in its core analysis all microRNA that share the same seed sequence (identical Entrez Gene name) and terms those microRNA with one family name (e.g. miR-181a-5p and other miR w/seed ACAUUCA) or an asterisks (see for example miR-30*). To find a detailed description of the analytical approach, please consult chapter 3, pages 35 - 37.

### 4.6.1. Proliferation network

By means of the IPA (Ingenuity Pathway Analysis) software networks for microRNA and mRNA relationships assumingly involved in proliferation of osteosarcoma cells have been created. Five networks (2 larger and 3 smaller) have been identified; see screenshot in figure 20 for an overview of the networks.

In the following figure 19 the top-rated networks for proliferation are displayed ($scores 19 and 37).They include functional associations to Connective Tissue

Disorders (13 molecules), Inflammatory Disease and Response (13 molecules), Cellular Development (18 molecules), Cellular Growth and Proliferation (17 molecules) and Cancer.

Constantly up-regulated (highlighted in red) molecules in the 2 largest networks are the genes AHR, CBX7, CAMK2N1, CEP97, DUSP6, TSHZ3, METAP1, KIF3A and ARSJ as well as the miRNA-21, miR-30c, miR-130, miR-155

The genes GJA1, FRS2, DYNC1I1 and the miR-9, miR-17-(family), miR-19b, miR-23, miR-30, miR-101, and miRNA 181a and b and miR-186, miR-374 present as constantly down regulated molecules in our analysis.

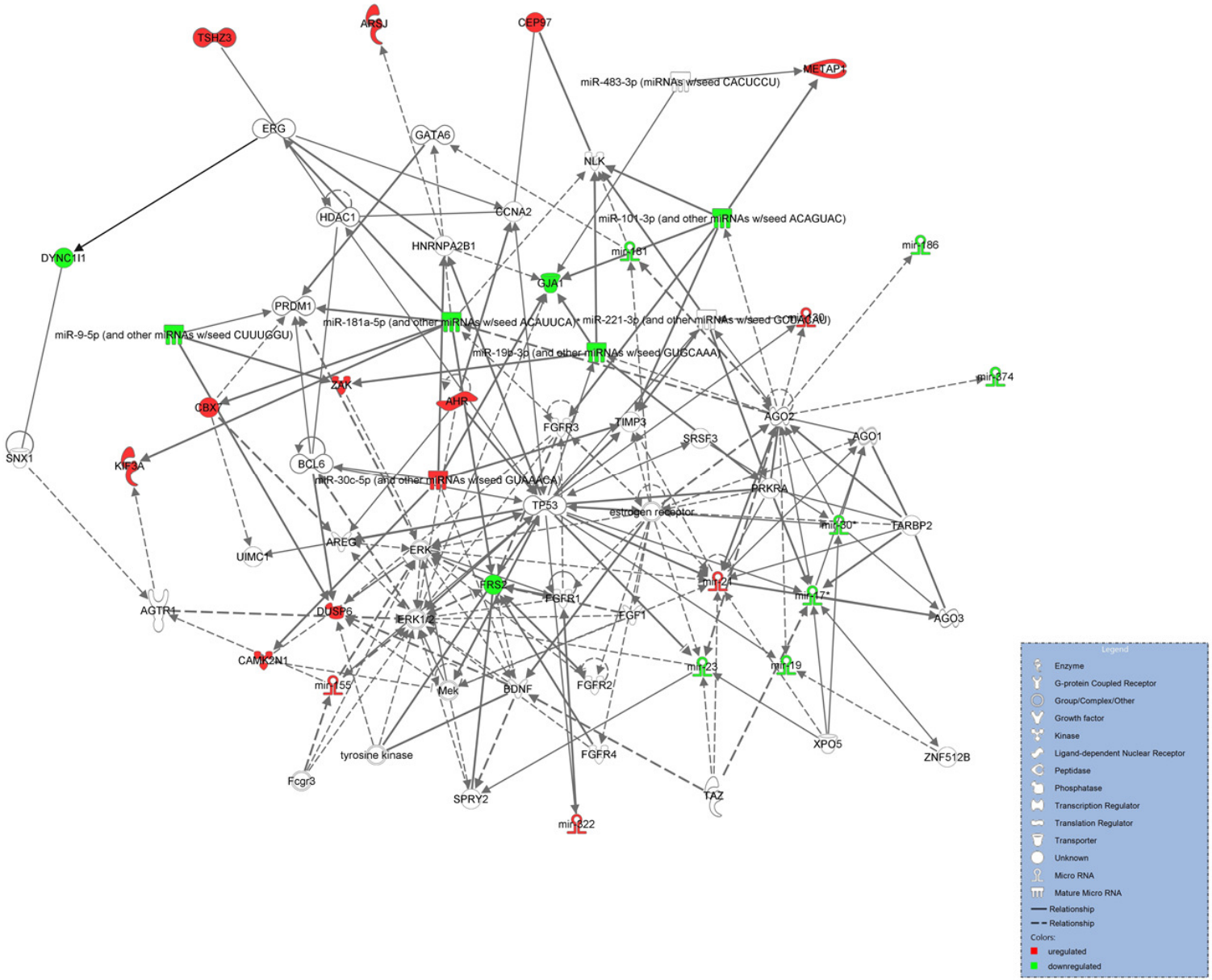



Figure 19: Proliferation network, generated by integration of miRNA and mRNA expression patterns of proliferative and non-proliferative group; IPA-top-score networks 1: Connective Tissue Disorders, Inflammatory Disease and Response (score 37) and 2: Cellular Development, Cellular Growth/Proliferation and Cancer (score 19); red: constantly up-regulated; green: constantly down-regulated; legend Edge Types on lower left corner; further info: IPA pathway analysis manual on quiagen.com/ingenuity. IPA combines all microRNA that share the same Entrez gene name (i.e. identical seed sequence), so that miR-21-5p = miR-21; miR-374=miR-374a; miR-17*=miR-106a, miR-20a and miR-17; miR-19b-3p=miR-19b, miR-19= miR-19a)

**Top Networks**

| ID | Associated Network Functions | Score |
|---|---|---|
| 1 | Connective Tissue Disorders, Inflammatory Disease, Inflammatory Response | 37 |
| 2 | Cellular Development, Cellular Growth and Proliferation, Cancer | 19 |
| 3 | Cellular Assembly and Organization, Cell Cycle, DNA Replication, Recombination, and Repair | 2 |
| 4 | Cell Morphology, Digestive System Development and Function, Endocrine System Development and Function | 2 |
| 5 | Cell Cycle, Reproductive System Development and Function, Embryonic Development | 2 |

Figure 20: Summary of analysis for proliferation, excerpt, see top-rated networks IDs 1 and 2, visualized in figure 19

**Top Networks**

| ID | Associated Network Functions | Score |
|---|---|---|
| 1 | Cancer, Organismal Injury and Abnormalities, Reproductive System Disease | 24 |
| 2 | Cellular Movement, Organismal Injury and Abnormalities, Cellular Development | 19 |
| 3 | Cell Morphology, Digestive System Development and Function, Endocrine System Development and Function | 2 |
| 4 | Cell Cycle, Reproductive System Development and Function, Embryonic Development | 2 |
| 5 | Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance | 2 |

Figure 21: Summary of migration analysis, top networks 1 and 2 have been merged and displayed in figure 22

### 4.6.2. Migration/invasion network

With the same approach again 5 networks (2 large and 3 smaller) have been created, this time integrating the expression data for both microRNA and mRNA in migrative/invasive osteosarcoma cell lines.

The 2 top-rated networks (scores 24 and 19, see screenshot figure 21) for the integrative analysis microRNA-mRNA-relationship can be seen in figure 22. Associated functions of these networks are Cancer (28 molecules), Organismal Injury and Abnormalities (23 molecules), Reproductive System Disease (20 molecules) as well as Cellular Development (13 molecules) and Cellular Movement (8 molecules).

The miR-181a, miR-186, miR-199a, miR-19b, miR-23a, miR-374b, miR-30c belong to the constantly down-regulated as well as DYNC11, FRS2 and GJA1, MEGF9.

ARSJ, CBX7, KIF3A, METAP1, PODXL, DUSP6, miR-19, miR-23, miR-30 and miR-181*

The miR-7a, miR-17, miR-21, miR-130a, miR-155 show up-regulation repeatedly.

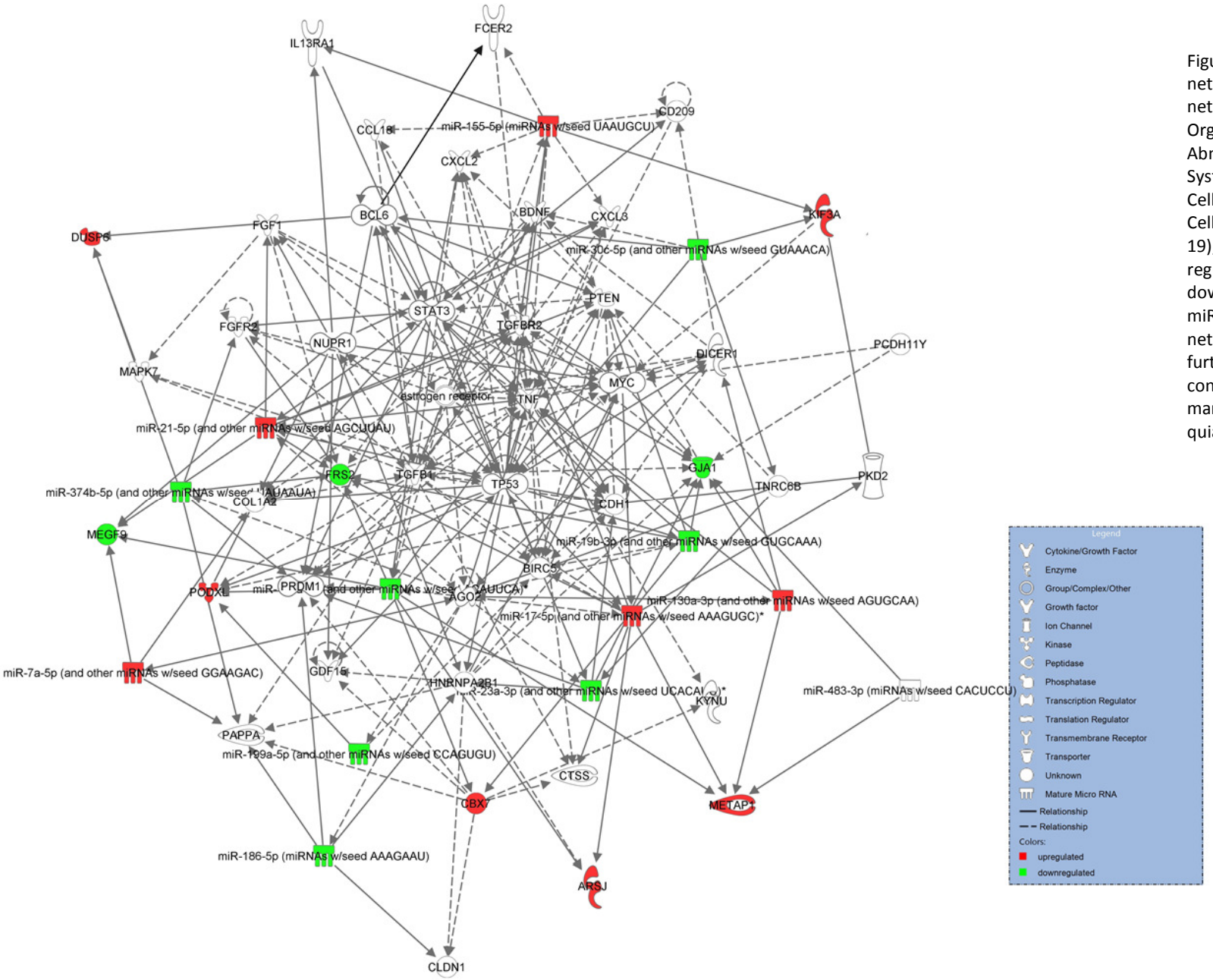



Figure 22 : Migration networks 1 and 2 merged: network 1: Cancer, Organismal Injury and Abnormalities, Reproductive System Disease (score 24);2: Cellular Development and Cellular Movement (score 19), red: constantly up-regulated; green: constantly down-regulated; terms for miR analogue to proliferation network, figure 19; for further information please consult: IPA pathway analysis manual on quiagen.com/ingenuity

## 4.7. Transfection of the miR-181a inhibitor

In paragraphs 4.4.1 and 4.6.2 I described the miR-181-family and miR-186 as significantly down-regulated in highly proliferative osteosarcoma cell lines. Moreover, both microRNA formed part of the regulatory network for migration and proliferation outlined in the preceding section. To assess if this observation really has a biological impact, I decided to select one of the microRNAs (miR-181a here exemplarily chosen) for transfection experiments. Thus, the miRNA-181a-inhibitor was transfected into 6 of the osteosarcoma cell lines. Subsequently, the osteosarcoma cell lines' growth (expressed in the doubling time value) was measured again. The doubling times for all cell lines can be seen in figure 23.

A comparison between the transfected cell lines and the controls (without inhibiting miRNA) is displayed as growth curves. Furthermore the doubling times for each transfected cell line (coloured bars) and the controls (empty bars) are demonstrated in a vertical bar chart. It is visible that the cell lines SJSA-01 and SaOS show a slight increase of the doubling time in the transfected cells (19 vs. 24 hours for SJSA-01; 34 vs. 41 for SaOS) but not reaching statistical significance. All the other osteosarcoma cell lines show equal distribution of doubling times, respectively, after being transfected with miR-181a-inhibitor.

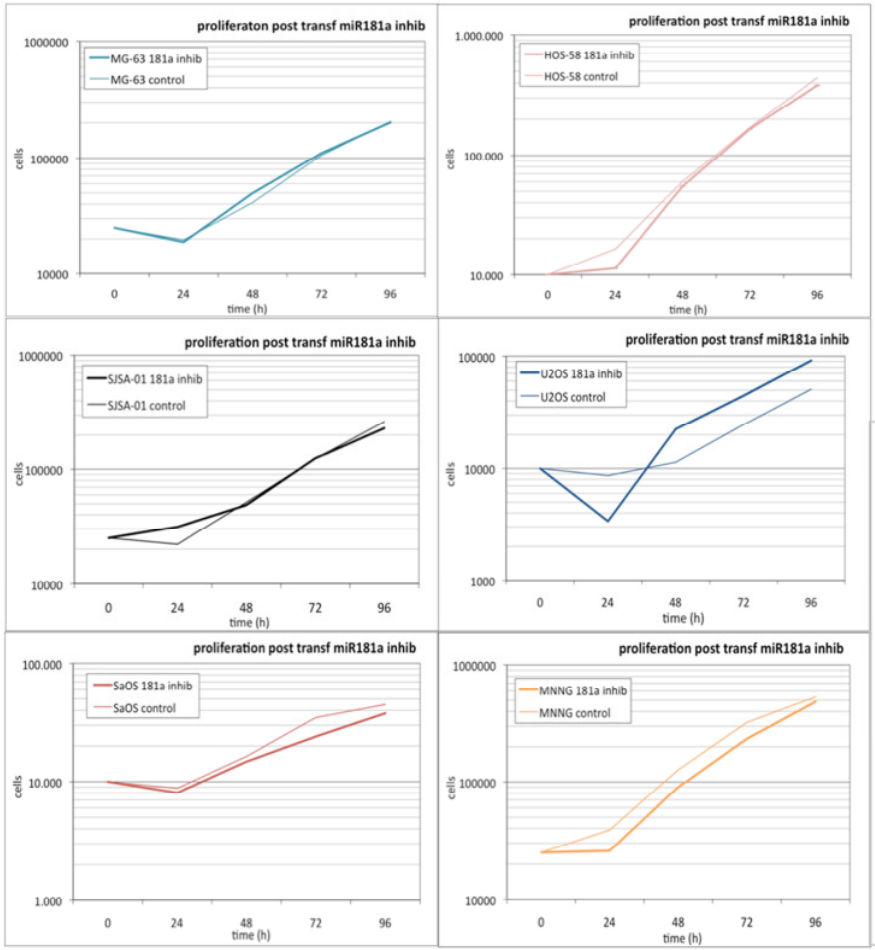
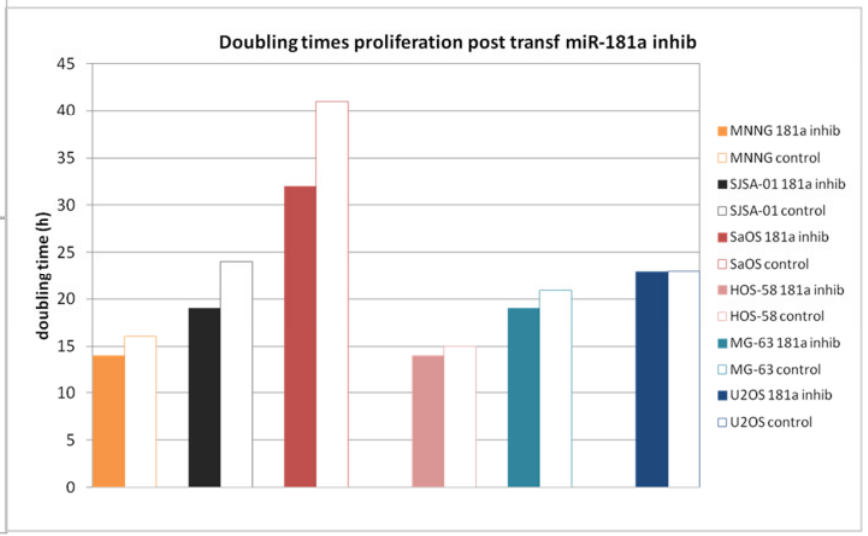



Figure 23: Proliferation assays conducted after transient transfection of miR-181a-inhibitor in the 6 cell lines MG63, HOS-58, SJSA-01, U2OS, SaOS and MNNG; left side shows cell growth of transfected cell lines and controls; right side: comparison of doubling times of transfected cells and controls; no statistical difference in transfected and non-transfected cell lines



# 5. Discussion

## 5.1. Summary and evaluation of methods

By combining different analytical methods I evaluated the microRNA and mRNA expression data that were generated for 8 established osteosarcoma cell lines and 2 progenitor cell lines. I found three of the cell lines (HOS, HOS-58 and ZK-58) to be very similar with only minor differences in their assay results and expression profiles. An authentication test revealed that the three cell lines originate from the same cell line. Hence, to maintain the discriminative power of the data, I decided to keep only HOS-58 for further analysis.

First expression of 6 osteosarcoma cells and the reference cell lines for both microRNA and mRNA was compared separately. Later on, using conventional association testing, deregulated microRNA and their potential target genes were correlated with the osteosarcoma cell lines' potential to proliferate, migrate and invade, respectively. Finally by integration of the expression matrices of both microRNA and mRNA, networks have been identified that might play a role once again in the proliferation or migration/invasion of osteosarcoma cells.

Goal of this work was to approach the topic microRNA deregulation in osteosarcoma both from a genomic and cellular level. By evaluating the data in conventional statistical methods (association testing, one-by-one-comparison) and integrative approaches I aimed to achieve more solid results. As I could identify common points in both approaches (e.g. miR-181a deregulation), a first validation experiment in the cell lines was initiated.

## 5.2. Study material

Generally, tumor cell lines are a mean for gaining an insight into tumor biology in vitro in a comprehensive manner without using valuable primary tumor material and with the option of numerous repetitions. Due to the treatment regime and the nature of osteosarcoma, primary patient material is rare and has to be dealt with carefully. Therefore I decided to use osteosarcoma cell lines for studying the microRNA expression in osteosarcoma.

This allowed research without material limitations. Moreover the cell lines can be used as a proximate model for studying osteosarcoma pathogenesis (Greshock et al., 2007; Mohseny et al., 2011). Certainly, cell lines, like these chosen in this thesis, have undergone mutations during the immortalization, so that their similarities to the original osteosarcoma might change over time of cultivation. Therefore, the significance of this study's findings for the osteosarcoma pathogenesis has to be validated in primary tumor samples.

## 5.3. Differentially expressed miRNA and their target genes in osteosarcoma cell lines versus normal tissue

*The oncogenic cluster miR-17/92 is deregulated in osteosarcoma cell lines*

In this study 15 deregulated microRNA (see table 5 in paragraph 4.1.2, pages 49 and 50) were identified by comparing the microRNA expression of 6 osteosaroma cell lines and the reference lines (osteoblast and mesenchymal stem cell line).

Remarkably, five of these microRNA (miR-17-5p, miR-18a, miR-93, miR-106a and miR-106b) belong to the oncogenic microRNA-cluster 17/92 and its paralogue clusters miR-106a-363 and miR106b-25, respectively (Mogilyansky and Rigoutsos, 2013; Olive et al., 2010). The polycistronic cluster is located on the locus of the MIR17HG gene on chromosome 13 and encodes six microRNA (miR-17, 18a, 19a, 20a, 19b1 and 92a-1). Its two paralogues were identified on chromosome 7 (miR-106b, miR-93 and miR-25) and the X-chromosome (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363). Figure 24 is showing all the members organized by cluster and microRNA family and moreover illustrates what genes have been identified as potential targets in this study.

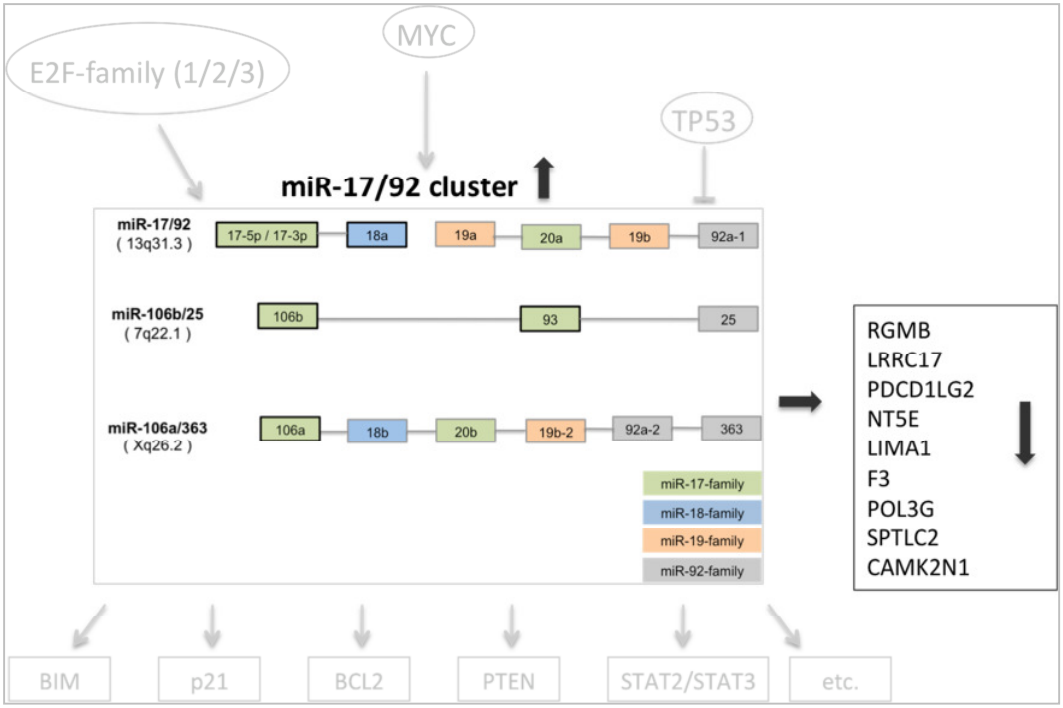



Figure 24: Schematic illustration of the miR-17-92-cluster, its main miR-17-92 cluster and the two paralogue members 106b/25 and 106a/363 (modified from: Mogilyansky et al, Cell Death and Differentiation, 2013) different family members and are displayed in green (miR-17-family), blue (miR-18-family), orange (miR-19-family) and grey (miR-92-family); around the middle section below and on the right side targeted genes are shown; above oncogenes targeting the cluster can be seen; results of this study are either written in bold letters or framed in black: i.e. the 5 members highlighted with a black frame (miR-17-5p/ miR-18a/ miR-106a/miR-106b and miR-93) are upregulated in our osteosarcoma cell lines versus progenitor/reference cell lines; targeted genes RGMB, LRRC217, PDCD1LG2, NT5E, LIMA1, F3, POL3G, SPTLC2 and CamK2N1) are known targets for the cluster and all downregulated in our osteosarcoma cell lines; most likely due to regulation by the upregulated miRNA; the genes below in grey are knowingly involved with regulation of miR-17-92, i.e. represent information derived from literature; arrow: activated by

In 2005 the oncogenic character of this cluster was first described in B-cell-lymphoma (He et al., 2005). Since then the miR-17/92 cluster, also referred to as “Oncomir-1” has been investigated thoroughly regarding its role in cancer development (Esquela-Kerscher and Slack, 2006b). The cluster, i.e. several of its member-microRNA, was shown to be responsible for an activation of proliferation or metastasis development (Hayashita et al., 2005; Huang et al., 2012). In recent studies examining tissue of Ewing sarcomas and osteosarcomas, it was found that expression levels of several miR-17/92-members help to distinguish between malignant and normal tissue (Dylla and Jedlicka, 2013).

This coincides with our study as we found the miR-17-5p, miR-18a, miR-93, miR-106a and miR-106b to be constantly up-regulated in the osteosarcoma cell lines vs. reference cell lines, supporting the idea that the miR-17/92 and its paraloguos clusters might have an oncogenic effect in osteosarcoma.

A study from Mogilansky et al. is reviewing the numerous works that have been published up to now and interestingly members of miR-17/92 or its paralogues have been shown overexpressed in all the solid tumors that have been examined (Mogilyansky and Rigoutsos, 2013).

As potential targets of the miR-17/92-cluster MYC is one of the most important to mention. This proto-oncogene, first described in 1982, is one of the key-players in the cell cycle. By regulating their transcription it enables genes, such as CDK4, to process from G1 to the S-phase; it stimulates proliferation, blocks differentiation of cells and triggers apoptosis (Dang, 2012; Hermeking et al., 2000; Vennstrom et al., 1982). In osteosarcoma in particular, MYC was shown to be amplified frequently.

It was demonstrated, not only by our group in 2010 but also by Gamberi et al. and Kuijjer, to have an unfavorable prognostic value (Gamberi et al., 1998; Kuijjer et al., 2012; Smida et al., 2010). On the other hand it was proven that MYC (c-myc) as well as MYCN (n-myc) and the EF2-family directly activate the transcription of miR-17/92 by binding to its promoter region (Olive et al., 2010).

The transcription of 17/92 clusters might be also regulated by TP53 (p53), as indicated by Yan et al. The group has detected that the expression of miR-17/92 is downregulated in hypoxic cells containing p53-wildtype (Yan et al., 2009).

Since the cluster and its paralogues consist of several miRNA, each can target large number of genes. It has been shown, for instance, that the cluster not only is activated by E2Fs-genes, but targets itself the E2F-family. This and the information earlier stated suggest that a feedback mechanism exists between miR-17/92, the E2Fs and MYC (Aguda et al., 2008; Sylvestre et al., 2007).

A recently published study examined the expression of the miR-17/92 cluster members in paraffin-samples of pre-therapeutic osteosarcomas. The group could demonstrate that upregulation of the cluster members correlates with unfavorable outcome (metastasis/survival). Furthermore they showed the relationship between

the expression of known regulating genes (MYC, TP53 or E2Fs), individual members of the miR-17-92-cluster and its identified targets, e.g. BIM, FAS (Arabi et al., 2014). An overview of the most important targets described in literature is given in figure 24 above.

*MicroRNA-34a potentially reflects differentiation state of cells*

In this study the miR-34a is underexpressed in 5/6 of the osteosarcoma cell lines when compared to the human osteoblast cell lines. On the other hand the OS cell lines show upregulation when compared to the mesenchymal stem cells. Figure 25 visualizes our findings concerning miR-34a- expression in the three different cell types.

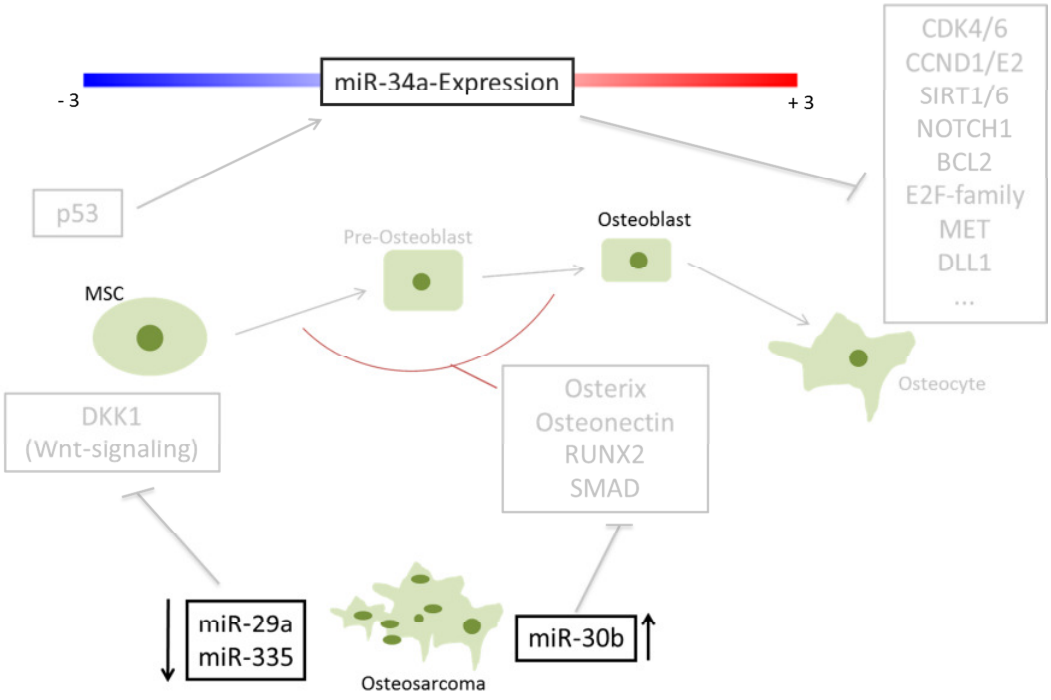



Figure 25: Potential role of miR-34a and miR-29a, miR-335 and miR-30b in osteoblast differentiation; MSC: mesenchymal stem cells, Pre-OB: pre-osteoblast, differentiation from mesenchymal stem cell to mature osteoblast/osteocyte is shown; coloured in grey: target genes or regulators extracted from literature, references see text; displayed in bold or black frame: results from this study; miR-34a expression has been found elevated (up to +3) in hFOB (human osteoblast cell line) and only slightly expressed or underexpressed in osteosarcoma cell lines (exception: U2OS); in MSC under expressed (-1,59 average); miR-29a and miR-335 were underexpressed in osteosarcoma vs. progenitor cell lines; miR-30b was found overexpressed in osteosarcoma cell lines when compared to osteoblasts and mesenchymal stem cells. (Data regarding targets and pathways mainly derived from: Harada and Rodan, 2003; Long, 2011)

The miRNA 34a has been already described in literature as being tumor suppressive. In neuroblastoma, for instance, its expression levels were low in tumor samples compared to normal tissue. A direct targeting of E2F2 by miR-34a led to a decrease of cell proliferation in this tumor. Another study examined the osteosarcoma cell lines U2OS and SaOS and found that the miR-34 family inhibits proliferation and metastasis and triggers apoptosis by directly targeting TP53 in these cell lines (Cole et al., 2008; Welch et al., 2007). On the other hand miR-34a is regulated by TP53, as well. He et al. did show that the miR-34 expression levels (miR-34a,b and c) were low in osteosarcoma samples versus normal tissue samples (He et al., 2009).

More recently several groups found miR-34a in particular responsible for inhibiting growth and metastasis in osteosarcoma (Yan et al., 2012; Zhao et al., 2013).

All these findings are in line with my results as I found 34a down-regulated in the osteosarcoma cell lines when compared to hFOB suggesting a tumor suppressive effect. The fact that, in our work, its expression in comparison to the stem cells (L-87/4) is upregulated cannot be explained so easily.

Certainly, the level of differentiation has impact on a microRNAs expression level. Bu et al. propose a “bimodal switch” mechanism for miR-34a. They found that, by targeting NOTCH, it regulates the degree of differentiation in colon cancer cells and their stem cells, respectively (Bu et al., 2013). Consistent with this and our results, recent publications described that the miR-34a expression level increases with the degree of cell differentiation in various tissues, among them osteosarcoma cells (Aranha et al., 2011; Lefort et al., 2013). Chen at al. identified miR-34a as a modulator of osteoblast differentiation. In their recently published work they propose a “dual regulatory effect” meaning that miR-34a affects both mesenchymal stem cell proliferation as well as the osteoblastic lineage (Chen et al., 2014).

The latter once again backs up our results and indicates that the miRNA-34a involvement in differentiation of osteoblasts and bone forming, respectively, might be crucial. Potential target genes of miR-34a are displayed in figure 25, as well.

*Other deregulated microRNA in osteosarcoma vs. progenitor cell lines*

MiR-29a is downregulated in the osteosarcoma cell lines in this study when compared to both progenitor lines.

In two, 2012 published articles, different groups compared microRNA epression profiles in osteosarcoma and normal tissue. Both showed a downregulation of the microRNA-expression levels in osteosarcoma tissue (Jones et al., 2012; Zhang et al., 2012).

This confirms our findings and the tumor suppressive function that miR-29a might have in osteosarcoma development. Its role in osteoblast differentiation has been examined by Kapinas et al. in 2010. The authors show an increase of miR-29a expression in the differentiation process of human osteoblasts (including our cell line hFOB 1.19) and propose a positive feedback mechanism for miR-29 and regulatory genes of the WNT-signaling pathway (Kapinas et al., 2010).

Another microRNA that has been connected to differentiation of osteoblasts and osteosarcoma pathogenesis is the miR-125b (Mizuno et al., 2008). It was characterized recently as a suppressor of both proliferation and migration in osteosarcoma. Liu et al. explained this effect by inhibition of STAT3 (Liu et al., 2011). Furthermore miR-125b has been linked to TP53 repeatedly, where it acts either indirectly by suppressing CDKN2A (p14arf) or in a direct manner (Amir et al., 2013; Le et al., 2011). The miR-125-family expression in general was examined in numerous cancer types and its properties vary immensely between the cancer entities, meaning that is seems to function as tumor suppressor and oncogene (Sun et al., 2013).

Why in our study miR-125b is downregulated in all osteosarcoma cell lines except MG-63, where it was found up-regulated, cannot be explained explicitly.

The difference of this particular cell line is that, although highly proliferative, it does not show any migrative or invasive tendencies. Therefore MG-63 plays an exceptional role in our subset of cell lines. Pautke et al. underline this when stating that MG-63 has a very “heterogenous profile” in terms of immunocytochemistry labeling (Pautke et al., 2004).

The miR-30b, in our work up-regulated in 4/6 osteosarcoma cell lines, has been described in the context of bone differentiation as well.
Wu et al. characterized several miR-30 family members (among them miR-30b) as negative regulators of osteoblast differentiation as they target SMAD1 and RUNX2 (Wu et al., 2012). That the miR-30-family has a role in osteoblast differentiation was confirmed just recently by Eguchi et al. They published a biomarker panel that they named "ostemiRs" with the miR-30-family members as one of the most important. According to their expression patterns they were divided into groups: miR-30b/c and miR-30a/d/e (Eguchi et al., 2013).

The miR-193a-3p displays an up-regulation in our dataset (compared to both progenitor cell lines) again only in MG-63.
Few other data is available about this microRNA so far. One recent publication characterized miR-193a-3p as inducible by ionic radiation (different cell types) and in this context inducing apoptosis by directly targeting MCL-1 (Kwon et al., 2013).

Another microRNA that seems to have a special role is miR-193b being only downregulated in the osteosarcoma cell line MNNG.
This cell line, originating from HOS (ATCC CRL-1543), was transformed by treatment with cancerogenous nitrosamines. It has been examined only for a small number of cancer types, among them breast, prostate cancer and melanoma. Here miR-193b shows tumor suppressive effects (Chen et al., 2010; Li et al., 2009; Rauhala et al., 2010).

The miR-335 and miR-424 showed downregulation in 5/6 and 6/6 comparisons, respectively, in our osteosarcoma cell lines.
The assumable tumor suppressive effect of both microRNAs has been backed up by two studies from last year. They identified miR-335 lower expressed in osteosarcoma than in normal tissue and proposed a miR-424 tumor suppressive effect in osteosarcoma as well (Long et al., 2013; Wang et al., 2013). Furthermore,

both microRNA (miR-335 and miR-424) have been described as modulators of osteoblast differentiation (Gao et al., 2011; Zhang et al., 2011a).
The other miR-301a and miR-1275 demonstrated in our study as repeatedly up- or downregulated (the latter microRNA) have not yet been in focus in regard to osteosarcoma development. Therefore it might be worthwhile to further study their role in osteosarcoma.

To sum up, the 15 miR identified as deregulated in the osteosarcoma cell lines, especially the 17-92 cluster, miR-34a and miR-29a and 30b, seem to be essential components in osteoblast differentiation and bone homeostasis. An overview of these microRNA and their potential targets have been given in figure 24 and 25 (see pages 71 and 73). These facts might help to give new insights in bone differentiation and forming of malignant osteoid. Although the etiology of osteosarcoma has not been clarified yet, its high incidence during puberty indicates that pubertal hormonal changes and growth spurt are strongly associated. Therefore the understanding of how the described subset of microRNA and its expression changes can regulate the forming of new bone from stem cells is a crucial finding.

*Differential gene expression of osteosarcoma cell lines versus reference cell lines*

To predict potential targets for the 15 deregulated microRNAs I used the miRDB database. This online tool identified up to 207 target transcripts and up to 155 target genes per microRNA. The expression levels of these potential target genes were compared separately between the osteosarcoma and the reference cell lines. But only those with the appropriate regulation in the highest number of comparisons (≥4/12) were included in table 6 (consult results, page 47) and had to be evaluated further (n=16). Seven of the deregulated genes could be correlated again to the miR-17/92 cluster. Interestingly these genes were then only influenced by members of this cluster and not by other miR. RGMB, PDCD1LG2, LIMA1, F3, POLR3G, SPTLC2 and CAMK2N1 are the genes constantly down regulated by the oncogenic cluster but are not influenced by other microRNA of our subset.

Mainly the genes identified in this analysis take part in cell cycle processing, apoptosis and bone differentiation.

I found RGMB constantly downregulated in the osteosarcoma versus reference cell lines. Several members (miR-17, miR-93, miR-106a and miR-106b) have been predicted to target RGMB and show the proper regulation (all up) in at least 4/12 comparisons. The fact that RGMB is downregulated in the majority of the osteosarcoma cell lines utilized in this study suggests its relevance in the pathogenesis of this cancer type.

RGMB (also referred to as DRAGON) acts as a co-receptor for BMP (bone morphogenic protein) and in this role is responsible for differentiation processes in bone (Halbrooks et al., 2007; Samad et al., 2005).

Other genes that seem to be regulated by the 17/92 cluster members include the oncogenic LIMA1 (also referred to as EPLIN) and CAMK2N1, both so far described as tumor-suppressive in prostate and breast cancer (Maul and Chang, 1999; Wang et al.; Zhang et al., 2011b). Additionally, Ma et al. found that CAMK2 induced apoptosis and cell cycle arrest in ovarian cancer cells (Ma et al., 2009).

Furthermore PDCD1LG2 (programmed cell death 1 ligand 2) is down-regulated by 17/92 in a high number of comparisons. This second ligand of the programmed cell death protein has been first described in 2001 and seems mostly to be involved in T-cell-regulation (Latchman et al., 2001).

Another gene from our list that is known to be involved in differentiation of bone is LRRC17. In this study it was specifically targeted and down-regulated only by miR-30b. Consistent with this, it was described as a negative regulator, by targeting RANKL, of osteoclast function (Kim et al., 2009).

In this work, CCNE1 (Cyclin E1) is upregulated supposedly by the miR-424a only.

It is a member of the Cyclin family, which members are known for their regulatory role for CDK kinases. Cyclin E1 in particular forms a complex with CDK2 and in this function supports progression from G1 to S-phase in cell cycle. It has not only been linked to several different cancer types but also to osteosarcoma genesis (Honda et al., 2005; Lockwood et al., 2011).

The identified target genes NT5E, MGAT4A (also referred to as GnT-Iva) and MYBL1 have been described as potential biomarkers in breast or pancreatic cancer as well as in pediatric brain tumors (Ide et al., 2006; Lo Nigro et al., 2012; Ramkissoon et al., 2013). In this analysis I could identify these 3 genes as deregulated in 4 of 12 and 6 of 12 comparisons, respectively.

Another gene important in cancer pathogenesis in the list is the gene FLI 1, whose expression has been described as helpful to distinguish between small cell osteosarcoma, chondrosarcoma and Ewing sarcoma (Lee et al., 2011).

Before, it was described extensively as an important player in the pathogenesis of Ewing's sarcoma, certain types of leukemia or breast cancer (Kauer et al., 2009; Sakurai et al., 2007; Truong and Ben-David, 2000).

Whether F3, a gene encoding for Thromboplastin, POLR3G (Polymerase III polypeptide G) or SPTLC2 (serine palmitoyltransferase, long chain base unit 2) take part in the pathogenesis of cancer has not been described yet.

Therefore the results of our study, describing these genes as potential targets of 17-92, give interesting novel information about their function.

According to several studies nearly all the genes found to be targeted by ths list of microRNAs are involved in the transformation to malignancy. This fact underlines the biological relevance of the identified microRNA. Especially the influence of the miRNA-17-family (17-92 cluster or its paralogous clusters miR-106a-92 and 106b-25) might be crucial. All these connections to genes involved in bone forming/bone differentiation imply that the comparison of gene expression between osteosarcoma tissue and precursor tissue shows a real "biologic path" that leads from mesenchymal stem cells to bone cancer cells.

## 5.4. Correlation of microRNA-mRNA data with phenotype

*In vitro assays - results in comparison with literature*

A cancer's growth properties and its tendency to infiltrate foreign tissue distinguish an aggressive from a non-aggressive tumor. That's why in this study I assessed, in addition to the genotype, the proliferational, migrative and invasive potential of the

osteosarcoma cell lines. I characterized HOS-58 and SaOS as slow proliferating cell lines based on their doubling time (>30 hours, see figure 14, page 48).

As already mentioned, I could verify that the alleged cell lines HOS-58, ZK-58 and HOS originate from the same cell line. Only HOS-58 was kept for further analysis and will be discussed here. In literature HOS-58 is poorly described. The data about this cell line range in doubling time from 20-36 hours in a state of pre-confluence and up to 80 hours when the cells are completely confluent (Siggelkow et al., 1998).

SaOS has been shown to be slow proliferating in this study (51 hours doubling time), a similar observation was found by others (Evdokiou et al., 2003; Jia et al., 2000).

As the cell lines with the highest growth potential MG-63 with an average of 26 h doubling time, MNNG with 23h, U2OS with 27 h and SJSA-01 with 19 h have been identfied. The literature backs up this findings, demonstrating doubling times for these 4 cell lines ranging from 18 hours for SJSA-01 up to 30 hours for MG-63 (Evdokiou et al., 2003; Luu et al., 2005a; Manara et al., 2000; Pautke et al., 2004).

Generally, migration is the process helping multicellular organisms to move from one place to the other, a complex sequence of extension, formation of adhesive networks, translocation and re-integration. Invasion, based on an initial ability of the cell to migrate is the ability to penetrate into other cell membranes (Friedl and Wolf, 2003).

In this work I demonstrated that the cell lines SJSA, MNNG and U2OS are osteosarcoma cell lines with a high migrative and invasive potential. The cell lines SaOS, HOS58 and MG-63 could be classified as little or not migrative and invasive, respectively (see figure 15+16 on pages 50 and 51).

For supporting my findings in regard to migration and invasion potential few data was available. Several groups performed the same procedures, i.e. Boyden chamber assay with (invasion assay) or without Matrigel® covered (migration assay) cell culture inserts. Another method to use for migration analysis was the so-called “scratch assay”, which I initially performed with a lab colleague to decide for an appropriate method. The scratch assay holds a high risk of cell membrane damage and additionally lacks reliable quantification of the result (Kramer et al., 2013; Valster et al., 2005). Due to its better reproducibility, the possibility of testing with a

chemogradient and a matrigel coating (to mimic the cell membrane) I selected the transmembrane method.

Analogue to my findings, MG-63 is described to be a non-migrating and non-invading cell line. U2OS is characterized as being highly invasive and migrative. Other groups underpinned my work as well, their findings just differ in the number of cells migrating/invading through the Matrigel® membrane. Others simply display microscopic pictures with the invaded/migrated cells and show a grading without any quantification (Cheng et al., 2004; Fromigué et al., 2008; Luu et al., 2005b; Mills et al., 2009).

In summary, literature supports the findings of the in-vitro assays conducted in this work. This is an indication that this approach to further characterize and categorize the osteosarcoma cell lines is valid and reproducible.

*MiRNA-181-family and miR-186 may have an anti-proliferative effect in osteosarcoma cell lines*

Table 8 on page 53 displays that the miR-181a, b, d and miR-181* as well as miR-186 are significantly downregulated in the highly proliferative group of cell lines.

The miR-181a,b, d and miR-181* (miR-181a-2-3p) belong to the human 181-family and their genes are located on chromosomes 1, 9 and 19. The family of miR was first described in homo sapiens in 2007 (Landgraf et al., 2007).

miR-186-5p (previously referred to as solo miR-186) was first mentioned in the context of human microRNA research some years later (Lagos-Quintana et al., 2003). Its stem-loop-sequence (hsa-miR-186) is located on chromosome 1.

The role of the miRNA-181-family in cancer pathogenesis has been previously investigated by different studies. Only recently, miR-181a has been described to have a tumor suppressive effect by targeting K-RAS in squamos cell carcinoma (Shin et al., 2011). Furthermore miR-181a and miR-181b have been outlined as one of the relevant regulators in Cisplatin-induced apoptotic processes in NSCLC (Galluzzi et al., 2010; Zhu et al., 2010). Shi et al. examined a tumor suppressive effect of miR-181a and miR-181b in glioma cells, showing that these microRNA inhibit the cancer cells' proliferation in vitro (Shi et al., 2008). Another study showed miR-181b as a

potential response marker in colorectal carcinomas, i.e. showing higher expression levels in carcinoma vs. normal tissue and altered levels in surveillance (Nakajima et al., 2006).

These results are in line with the antiproliferative effects that all the miR-181-family-members seem to have in the osteosarcoma cell lines. Only recently, a collaborating group has shown, based on the proliferation assay results of this study but using a different analytical approach, that the miR-181a, b and d are downregulated in osteosarcoma cell lines with high proliferative potential (Poos et al., 2013). Two other studies described an upregulation of the miR-181a and b in osteosarcoma (obtained from open biopsies) versus control bone samples (Jones et al., 2012; Namløs et al., 2012).

The importance of miR-186 in cancerogenesis has been described in only a few studies. It was shown to be overexpressed in epithelial cancer cells (via targeting PTX7) for example (Zhou et al., 2008) . Moreover, Cai et al. found that downregulation of miR-186 is associated to poor prognosis in adenocarcinoma of the lung (Cai et al., 2013).

Its role in malignancies and osteosarcoma in particular has to be investigated further. Nevertheless, my results show that miR-186 in addition to the miR-181-family could be a set of biomarkers distinguishing between highly proliferative cells and slowly growing osteosarcoma cells providing that these microRNA-set shows the same potential in primary tumor samples.

*Transfection of miR-181a inhibitor does not influence growth of the cell lines*

The active role of mir-181a in osteosarcoma biology could not be validated.

Transfection of the miR-181a inhibitor did not change the doubling times of the osteosarcoma cell lines significantly in comparison to the controls. A number of reasons, methodically and biologically, have to be taken into consideration to explain these results:

The methodology for the transfection holds several sources of errors. It is not yet well-established for osteosarcoma cells. Therefore optimal conditions for transfecting this cell type (although certain studies including cell line transfection were consulted) were not described. A number of questions remain: Which amount

of transfection reagent is the adequate? Is it advisable to use stable instead of transient transfection? In transient transfection the genetic material brought into the cell is not integrated in the genome, it has a limited life span due to cell division or other factors. Stable transfection is usually virus-mediated and integration in the genome takes place. Although the implication of a virus in the cells of interest might be recently done with high efficiency and reproducibility, the risk of cytotoxicity or (due to its random integration in the genome) the alteration or even damage of oncogenes of interest is high (Kim and Eberwine, 2010).

Additionally, from a biological point of view, one microRNA may not act as an isolated regulator of proliferation. So even if this study proofs an involvement of miR-181a, b, d in the proliferation process they would be only components in the complex growth mechanism of this cancer.

*Genes constantly deregulated in highly proliferative versus slowly proliferating cell lines*

Table 10, on pages 56 and 57 exhibits the genes that show differential expression when the fast growing cell lines and the slow ones were compared.

A high number of genes (n=60) was identified as deregulated, i.e. were found to be significantly distinguishing (p-value< 0,05) between osteosarcoma cells with high and low growth potential. I conducted a search in the DAVID Annotation tool to put this gene list into a biological context. A significant (p-value 0,0011) clustering of 4 genes (CDK6, CDKN2A, CCND1 and Serpine1) involved in the p53 pathway was found. Furthermore, a functional enrichment analysis (GOTerm BP; demonstrated in table 11 on pages 63/64) was performed. It showed that 10 of the 60 genes (CCND1, CDKN2A, DLX5, SCIN, SERPINE1, ADRA1A, CDK6, MAB21L2, ADRA1D, FOXP2) are known to be involved in regulation of cell proliferation (p-value: 0,0017). The following genes mainly take part in regulation of cell differentiation (CCND1, CDKN2A, DLX5, SCIN, CDK6, ROBO2, CNTN4) and growth (SERPINE1, TIMP3, EMP1, FOXP2).

These results indicate that several genes that are differentially expressed among the fast and slow proliferating groups in our analysis really have a biological role in proliferation and growth processes.

It is beyond the scope of this work to discuss every gene in detail. Therefore only the earlier mentioned relevant genes involved in proliferation or growth will be reviewed further.

The cell-cycle regulators (CDK6, CDKN2A, CCND1) and their significance in the pathogenesis of osteosarcoma have been emphasized when molecular genetics was described in the introduction.

In my analysis, CDK6 and CCND1 are upregulated in the comparison of slow and fast proliferating cell lines. CDKN2A, in contrast, we found down regulated in this comparison.

CDKN2A in particular, has been intensively characterized by Ottaviano et al. regarding its relevance in osteosarcoma cell lines or osteosarcoma in general, respectively (Ottaviano et al., 2010).

Cai et al. characterized CCND1, as a target of miR-15a and 16-1, as a contributor to proliferation processes in osteosarcoma (Cai et al., 2012). In numerous other cancer types (e.g. breast, endometrial, pancreatic, prostate cancer) it has been found to be deregulated and therefore marking a focus of research for targeted therapy in these malignomas. CDK6, as well as CDK4, participates in the phosphorylation of the RB gene and thus plays an important role not only in osteosarcoma but in various cancers (Kovar et al., 1997; Musgrove et al., 2011).

Interestingly, CCND1, CDK6 (upregulated) and DLX5, MAB21L2, ADRA1D, FOXP2 (all downregulated in the highly proliferative cell lines) have been linked specifically to the Go-term positive cell proliferation in my secondary analysis indicating that they stimulate proliferation.

DLX5 (distal-less homeobox 5) has been mentioned in the context of osteoblast differentiation (in vitro) repeatedly. Its expression has been found to be increasing during the bone forming process. Furthermore it was shown that RUNX2 is a direct target (Holleville et al., 2007; Ryoo et al., 1997).

In a comprehensive study of the genetic and epigenetic changes in osteosarcoma cell lines and tumor samples by Kresse et al. DLX5 was found deregulated. Although a different approach was used, the mRNA expression in the cell line SaOS was upregulated as well. This confirms the findings of my work, with SaOS being a slow proliferating cell line. However, as far as the the osteosarcoma tumor samples are

concerned, the group of Kresse et al. showed an overexpression of DLX5 in all the (five) examined tumors (Kresse et al., 2012). This fact cannot be explained unequivocally as we found downregulation of DLX5 in highly proliferative cell lines. Clearly, its role in proliferation and osteosarcoma pathogenesis has to be studied further.

MAB21L2 (mab-21 like-2) has been described in growth factor-beta and BMP signaling and it is assumingly involved in neural development (Baldessari et al., 2004).

As far as the ADRA1D (adrenoreceptor alpha 1D) and other members of this gene family are concerned, there is evidence that they induce apoptosis of prostate cancer cells (Partin et al., 2003). In breast cancer, adrenoreceptors have been discussed as a target for therapy but the rather paradox effects when antagonizing the individual adrenoceptors obviated further attempts to introduce this clinically. (Pérez Piñero et al., 2012).

The Forkhead-Box-Protein-2 (FOXP2) has been found expressed in the healthy brain but neurologic diseases additionally (Enard et al., 2002).

The three latter genes have not been linked to bone or osteosarcoma development so far. Thus, this study's findings might help understanding the complex mechanisms of osteosarcoma cell proliferation.

*Let-7 d and let-7f are down regulated in migrative and invasive osteosarcoma cell lines*

Comparing the microRNA expression levels of the migrative/invasive with the group of osteosarcoma cell lines showing obverse behavior I found the miR-let-7d and let-7f to be down regulated in the osteosarcoma cell lines with high invasive and migrative potential (to be seen in table 9 and figure 18 on page 55).

The let-7 (lethal-7) family of microRNA was not only one of the first microRNAs identified but also the first human microRNA that has been described in scientific literature. In 2000 it was found in *C. elegans* and a bit later the same year its existence in homo sapiens was proven (Pasquinelli et al., 2000; Reinhart et al., 2000). By now 11 mature let-7-family sequences are known (Roush and Slack, 2008). The role of let-7 in cancer pathogenesis seems to be manifold (Boyerinas et

al., 2010). The regulation of cell differentiation, cell migration and invasion appears to be of great relevance among its various functions. Liang et al., for example, outlined that let-7f reduces the potential to invade and migrate in gastric cancer cells (Liang et al., 2011). Let-7d, the other microRNA we found downregulated in the osteosarcoma cells in our study, showed the same tendency in advanced-grade renal cell carcinoma (Su et al., 2014). A study published in 2010, using immunochemistry, characterized retinoblastoma samples with lower let-7 expression levels in the tumor samples with high mobility compared to normal tissue or those samples with lower mobility (Mu et al., 2010).This is very interesting since it is a well-known fact that people with RB1-germline mutation have a significantly higher risk of developing osteosarcoma.

Though it has been indicated recently it is still not clear what role the let-7-family members might play in osteosarcoma development.

*Genes differentially regulated in migrative and invasive cell lines*

Only one gene, TMEM119, showed significant (p=0,00295) deregulation in this comparison. Table 12 on page 61 shows an overview of 10 genes differentially expressed among the groups with high and low invasive/migrative potential. I included 9 genes that presented with p-values <0,1 because of their apparent relevance after conducting a GoTerm enrichment analysis. TP53, KRT8, UNC5B, ARHGEF2 were significantly (p=0,00082 and 0,00086, respectively) associated to apoptosis and programmed cell death. Furthermore, ARHGEF2, TP53 and TRPS1 are known to be involved in the biological processes of protein transport and intracellular transport.

TMEM119 is a known promoter of osteoblast differentiation is directly targeting RUNX2. By interaction with the different bone morphogenetic proteins it participates in the differentiation from myoblasts to osteoblastic cells (Hisa et al., 2011; Tanaka et al., 2012).

The probably best-characterized tumor suppressor gene TP53, though not reaching a significant level, is upregulated in the highly migrative versus non-migrative osteosarcoma cell lines in this study. Introducing this work, I already described that TP53 has been found altered in osteosarcoma repeatedly (see section molecular

genetics). Its influence on migration, invasion and metastasis forming in cancer in general has been reported to be significant as well (Muller et al., 2011).

## 5.5. Comprehensive microRNA-mRNA network analysis based on phenotype

By using the integrative approach, based on the Peng et al. publication, two large networks have been identified for each group (Peng et al., 2009b). These were merged for both the migration/invasion and the proliferation group because they comprised overlapping molecules each. The original analytical method was modified. The expression matrices of all the cell lines were used for testing mRNA and microRNA for negative correlation. The pre-selection of only differentially expressed microRNA, as it has been done by the Peng group, was not done in my work. It was assumed that microRNAs disable the expression of their target mRNA. The miRDB prediction database was used to identify likely biologic relationships. Using this approach described by Peng et al. a number of bipartite networks were identified and fed into the Ingenuity Pathway Analysis tool for illustrating the biological context of the respective microRNA-mRNA-coupling.
In the following section the two largest proliferation and migration networks (as demonstrated in figures 19 and 22) will be discussed further.

*Proliferation network*

The top-rated networks resulting from the IPA query for proliferation were connective tissue disorders, inflammatory disease and response (score 37) and cellular development, cellular growth and proliferation and cancer (score 19). These two networks were merged and displayed in figure 19 on page 64.

One of the central nodes in this figure is the so called “Guardian of the Genome” TP53 (Lane, 1992). This is line with its known role as tumor suppressor gene regulating cell divison and proliferation in all kinds of cancer. I found it, though not reaching significant level, differentially expressed in migrative versus non-migrative cell lines.

Furthermore, in the earlier analyses, I identified numerous microRNA (e.g. miR-34a) deregulated that are knowingly targeted by or target TP53. In this network the miR-21, miR-30c-5p and the miR-130 (=miR-130a) are frequently upregulated and directly targeted by TP53. Underexpressed target molecules of TP53 are the miR-19 and miR-30* (comprising the miR-30b and d). Considering the fact that TP53 is known as a protector from a cell's development to cancer it is remarkable to see that its targeting of microRNA in my study leads to deregulation in both ways. Not only is TP53 a target of hundreds of microRNA but it reciprocally regulates the processing/transcription of microRNA (Boominathan, 2010).

The deregulation of (onco)mir-21 has been shown in nearly all sorts of cancer. Its knock-down or suppression demonstrably results in inhibition of cell proliferation and its overexpression on the other hand to cancer progression and metastasis (Esquela-Kerscher and Slack, 2006b).

This is in line with this work's findings as I see the miR-21 upregulated in the proliferation network and therefore show its relevance in osteosarcoma cell lines.

AHR is one of the molecules upregulated in our proliferation network.

The AHR, the Aryl hydrocarbon receptor, a ligand-activated transcription factor and its heterodimeric counterpart, the aryl hydrocarbon receptor nuclear translocator (ARNT), build together the so called aryl hydrocarbon receptor complex (AhRC) (Beischlag et al., 2008). AHR has been described to regulate the expression of numerous genes. Thereby it is an interesting fact that many cancerogenous pollutants and other environmental factors (e.g. EBV) seem to be activating the receptor (Chopra and Schrenk, 2011; Inoue et al., 2012). Moreover, it has been found to inhibit the transcription of MYC in breast tumors (Yang et al., 2005).

The Estrogen receptor 1 (ESR1) is one of the central nodes of the created IPA network. It is known for its involvement mainly in breast cancer and other gynecological cancers (Chen et al., 2008). Prior studies have examined and proven the correlation of osteosarcoma and the Estrogen pathway Musselman et al., 2012; Stossi et al., 2004). Though not deregulated in the osteosarcoma cell lines, the Estrogen Receptor Gene (ESR) shows direct and indirect connections to various

molecules in the Proliferation network (TP53, miR-21, miR-181a etc.) This is consistent with the assumption that hormonal (especially estrogenic) changes, extensive skeletal growth and the underlying genetic and epigenetic alterations are highly related to the incidence peek of osteosarcoma at age 10-19 (Savage and Mirabello, 2011).

I was able to show miR-181a and miR-186 differentially expressed when proliferative and non-proliferative osteosarcoma cell lines were compared. That these two microRNAs appear in the proliferation network, though based on a different approach, backs up the solidity of my analysis.

CAMK2N1 has been shown downregulated when comparing the osteosarcoma cell lines with the references in the very beginning. This seems to be in contradiction with the upregulation in the proliferation network. But one has to take into consideration that the analysis here was different from the other. In a one-by-one comparison of osteosarcoma cell lines and precursor cells the CAMK2N1 showed lower expression. However, the integrative analysis resulting in the genes' overexpression in the network focused on the expression in osteosarcoma cell lines with high growth potential only.

The miR-17, miR-20a and miR-106a belong to the well-described oncogenic cluster 17-92 which turned up with 5 members upregulated in the same analysis. Its representation in this proliferation network with three members shows once again its relevance in osteosarcoma pathogenesis or osteosarcoma cell proliferation, respectively.

*Migration/Invasion network*

The two top-rated networks cellular development, reproductive system disease, cellular growth and proliferation (score 28) and reproductive system disease, cellular development, genetic disorder (score 22) were merged and displayed by means of the IPA software (see figure 22).

MYC is, as well as TP53, a central molecule in this network. Although not differentially expressed between the two groups in this study, it seems to have a central role in the migration/invasion of osteosarcoma cells, as some of the

downstream targets and interacting proteins are differentially expressed. The fact that amplification of MYC (c-myc previously) has been observed repeatedly in osteosarcoma has already been discussed in earlier.

I have outlined its connection to the miR-17/92-cluster, as I found 5 of its members (miR-17, miR-18a, miR-93, miR-106a and b) to be differentially expressed when compared to reference non-cancer cell lines. Quite recently, Arabi et al. demonstrated the regulative effect that MYC has in these miR-17/92 members in analogy to the proliferation network discussed before (Arabi et al., 2014). This adds up since the one of the top rated networks includes cellular growth and proliferation.

As depicted in the network, MYC directly acts on GJA1 (Gap junction protein alpha 1), which is downregulated frequently in proliferative versus non-proliferative osteosarcoma cell lines.

Gap junction proteins (connexins) are integral proteins in the cell membrane and responsible for cell-to-cell communication and transfer of ions and metabolites. Gap junction protein alpha 1 (GJA1), that is also known as Connexin 43, is probably the best-described family member. Interestingly, it has been shown to be a suppressor of proliferation in the osteosarcoma cell line U2OS (Zhang et al., 2001). Only recently, another study, examining potential biomarkers for osteosarcoma surveillance, reported GJA1 to be differentially expressed between osteosarcoma and normal tissue samples (Wu et al., 2014).

This supports the findings in this study, as GJA1 is differentially expressed, e.g. upregulated in the non-proliferative cell lines. Furthermore, one has to note its connectivity to three microRNAs that are frequently upregulated in the network: miR-17*(=miR-106a, miR-17 and miR-20a), miR-23 and miR-101. Hence, GJA1 seems to have a distinct effect in this regulatory module for osteosarcoma migration/invasion and proliferation.

It was beyond the scope of this work to discuss all the molecules represented in the networks. In fact, I intended to elucidate the miR-mRNA-interactions in osteosarcoma cell proliferation and migration/invasion. These basic features hypothetically define the aggressiveness of a cancer. So I focused more on the

connectivity of the deregulated microRNAs and genes with known tumor suppressors and oncogenes (TP53, MYC, MET etc.) to give further insight into the complex mechanism of osteosarcoma development.

Finding several microRNAs (miR-181a, miR-186, the miR-17-92-cluster) and genes (CAMK2N1, TP53) repeatedly, though using different analytical approaches, underlines the solidity and reliability of this work.

# 6. Summary and conclusion

The history of treatment in childhood cancer is a story of success; this is especially true for lymphoblastic leukemia or lymphoma. In contrast to these malignancies osteosarcoma is a cancer type that is still characterized by a high mortality. Even with the actual multimodal treatment regime, a 5-year-survival rate of ~70% could not be improved. In recurrent disease this number is even considerably lower (30%) The main reason may lie in its pattern of molecular and chromosomal changes: they are highly complex and not yet fully understood.

The search for novel therapy strategies and biomarkers for surveillance/earlier diagnosis of osteosarcoma relapse is ongoing but could not yet provide a clinically applicable result. The importance of microRNA in regulating not only physiological cell function but also progression to cancer has been described extensively over the past years. It has been indicated that microRNA dysregulation may play a major role in osteosarcoma development. Whether microRNA are useful biomarkers in this bone tumor has not been finally proven yet.

Therefore the goal of this study was to provide a comprehensive analysis of microRNA and their targets in osteosarcoma. Hereby, eight established osteosarcoma cell lines have been used as study material. Human osteoblasts and mesenchymal stem cells have served as references.

First I compared the expression of osteosarcoma cells and the reference cell lines for both microRNA and mRNA separately. By this means, I discovered a subset of microRNA distinguishing between osteosarcoma and normal tissue (e.g. 17-92-cluster). Among this subset, the miR-34a might serve as differentiation marker for osteoblasts.

Moreover, using conventional association testing, I identified deregulated microRNA (e.g.181-family, let-7-family) and their potential target genes (among them CDKN2A, TMEM19) significantly correlating with the osteosarcoma cell lines' potential to proliferate, migrate and invade, respectively.

In a more sophisticated analytical approach the expression matrices of both microRNA and mRNA have been utilized to find gene regulatory networks. To identify real biologically active mRNA-microRNA-systems (for proliferation and

migration/invasion of osteosarcoma) I validated the microR-mRNA-couples that are deregulated between the groups in the prediction database miRdb. Subsequently, networks for proliferation and migration/invasion were created against the background of canonical pathways in cancer and disease. I demonstrated several well-described microRNA (e.g. miR-21, miR-181-family) not only to be deregulated in osteosarcoma but additionally involved in a network of well-characterized oncogenes (MYC) and tumor suppressor (TP53) genes.

A first validation of these results has been initialized by transfection of the miRNA-181a-inhibitor into the osteosarcoma cells. A solid proof of biological relevance though has not been accomplished yet and is subject of further research. To validate my results in primary tumor material and to correlate this with patient data has to be the next step.

# 7. Perspective

Further experiments to further elucidate the role of microRNA in osteosarcoma have to be done. This includes comprehensive transfection experiments of candidate microRNAs combined in primary osteosarcoma samples and follow up measurements of expression changes after transfection (quantification by RT-PCR, western blot etc.) for both microRNA and gene expression. The use of stable transfection systems has to be considered.

A sequencing of microRNA and gene expression in patient material is necessary to compare differences in expression profiles and to correlate this with patient data (different disease states, histology type, gender, age and localization to name only some of the important data). When a number of miRNA has been identified as biologically active in osteosarcoma the establishment of blood testing for microRNA expression is desirable. Thus, the goal would be to take blood routinely at examination points outlined in treatment protocol.

It is rather unlikely that a malignoma with such a bandwidth of genetic changes as already found in Osteosarcoma can be attributed to deregulation of a single microRNA. The key might be to use a panel of interesting miRNA for stratification of, for example, the risk for developing metastases in osteosarcoma. A molecular stratification in addition to traditional histopathology characteristics, as it is clinical routine in leukemia or breast cancer, should be the objective.

The frequently growing amounts of data about osteosarcoma pathogenesis (genetic and epigenetic alterations, copy number changes etc.) should be collected and to be made accessible. The osteosarcoma database that was mentioned, introducing this work, is the first one realizing this in a comprehensive manner. A further development of such databases and osteosarcoma data collection in general has to be the goal in order to bundle knowledge in this area and to facilitate the development of new therapeutic options.

# 8. Bibliography

Aguda, B.D., Kim, Y., Piper-Hunter, M.G., Friedman, A., and Marsh, C.B. (2008). MicroRNA regulation of a cancer network: consequences of the feedback loops involving miR-17-92, E2F, and Myc. Proc. Natl. Acad. Sci. U. S. A. *105*, 19678–19683.

Al-Romaih, K., Bayani, J., Vorobyova, J., Karaskova, J., Park, P.., Zielenska, M., and Squire, J.. (2003). Chromosomal instability in osteosarcoma and its association with centrosome abnormalities. Cancer Genet. Cytogenet. *144*, 91–99.

Ambros, V. (2004). The functions of animal microRNAs. Nature *431*, 350–355.

Amir, S., Ma, A.H., Shi, X.B., Xue, L., Kung, H.J., and deVere White, R.W. (2013). Oncomir miR-125b Suppresses p14ARF to Modulate p53-Dependent and p53-Independent Apoptosis in Prostate Cancer. PLoS One *8,* e61064.

Arabi, L., Gsponer, J.R., Smida, J., Nathrath, M., Perrina, V., Jundt, G., Ruiz, C., Quagliata, L., and Baumhoer, D. (2014). Upregulation of the miR-17-92 cluster and its two paraloga in osteosarcoma – reasons and consequences. Gene and Cancer, Vol 5

Araki, N Uchida, A. (1991). Involvement of the retinoblastoma gene in primary osteosarcomas and other bone and soft-tissue tumors. Clin. Orthop. Relat. Res. *270*, 271–277.

Aranha, M.M., Santos, D.M., Solá, S., Steer, C.J., Rodrigues, C.M.P. (2011). miR-34a regulates mouse neural stem cell differentiation. PLoS One *6*, e21396.

Asangani, I.A., Rasheed, S.A.K., Nikolova, D.A., Leupold, J.H., Colburn, N.H., Post, S., and Allgayer, H. (2008). MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene *27*, 2128–2136.

Baldessari, D., Badaloni, A., Longhi, R., Zappavigna, V., and Consalez, G.G. (2004). MAB21L2, a vertebrate member of the Male-abnormal 21 family, modulates BMP signaling and interacts with SMAD1. BMC Cell Biol. *5*, 48.

Baranwal, S., and Alahari, S. (2009). miRNA control of tumor cell invasion and metastasis. Int. J. Cancer J. Int. Du Cancer *126*, 1283–1290.

Bartel, D.P. (2004). MicroRNAs : Genomics , Biogenesis , Mechanism , and Function. *116*, 281–297.

Bartel, D.P., and Chen, C.-Z. (2004). Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat. Rev. Genet. *5*, 396–400.

Baumhoer, D., Smida, J., Specht, K., Bink, K., Quintanilla-Martinez, L., Rosemann, M., Siggelkow, H., Nathrath, W.B., Atkinson, M.J., Bielack, S., et al. (2011). Aberrant expression of the human epidermal growth factor receptor 2 oncogene is not a common feature in osteosarcoma. Hum Pathol *42*, 859–866.

Bayani, J., Zielenska, M., Pandita, A., Al-Romaih, K., Karaskova, J., Harrison, K., Bridge, J. a, Sorensen, P., Thorner, P., and Squire, J. a (2003). Spectral karyotyping identifies recurrent complex rearrangements of chromosomes 8, 17, and 20 in osteosarcomas. Genes. Chromosomes Cancer *36*, 7–16.

Beischlag, T. V, Luis Morales, J., Hollingshead, B.D., and Perdew, G.H. (2008). The aryl hydrocarbon receptor complex and the control of gene expression. Crit. Rev. Eukaryot. Gene Expr. *18*, 207–250.

Benassi, M., and Molendini, L. (1999). Alteration of pRb/p16/cdk4 regulation in human osteosarcoma. Int. J. Cancer *493*, 489–493.

Benassi, M.S., Molendini, L., Gamberi, G., Magagnoli, G., Ragazzini, P., Gobbi, G.A., Sangiorgi, L., Pazzaglia, L., Asp, J., Brantsing, C., et al. (2001). Involvement of INK4A gene products in the pathogenesis and development of human osteosarcoma. Cancer *92*, 3062–3067.

Bielack, S., Jürgens, H., Jundt, G., Kevric, M., Kühne, T., Reichardt, P., Zoubek, A., Werner, M., Winkelmann, W., and Kotz, R. (2009). Osteosarcoma: The COSS experience. In Cancer Treatment and Research, pp. 289–308.

Bielack, S.S., Kempf-Bielack, B., Delling, G., Exner, G.U., Flege, S., Helmke, K., Kotz, R., Salzer-Kuntschik, M., Werner, M., Winkelmann, W., et al. (2002). Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J. Clin. Oncol. *20*, 776–790.

Billiau, A., and Edy, V. (1977). Human interferon: mass production in a newly established cell line, MG-63. Antimicrob. Agentsand Chemotherapy *12*, 11–15.

Boehm, A., and Neff J.R., Squire J.A., Bayani J., N.M.B.J.A. (2000). Literature, Cytogenetic Findings in 36 Osteosarcoma specimens and a review of the. Pediatr. Pathol. Mol. Med. *19*, 359–376.

Boominathan, L. (2010). The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. PLoS One *5*, e10615.

Boyerinas, B., Park, S.-M., Hau, A., Murmann, A.E., and Peter, M.E. (2010). The role of let-7 in cell differentiation and cancer. Endocr. Relat. Cancer *17*, F19–F36.

Bridge, J. a., Nelson, M., McComb, E., McGuire, M.H., Rosenthal, H., Vergara, G., Maale, G.E., Spanier, S., and Neff, J.R. (1997). Cytogenetic findings in 73 osteosarcoma specimens and a review of the literature. Cancer Genet. Cytogenet. *95*, 74–87.

Bu, P., Chen, K., Chen, J., Wang, L., and Walters, J. (2013). A microRNA miR-34a-regulated bimodal switch targets notch in colon cancer stem cells. Cell Stem Cell *12*, 602–615.

Cai, C.K., Zhao, G.Y., Tian, L.Y., Liu, L., Yan, K., Ma, Y.L., Ji, Z.W., Li, X.X., Han, K., Gao, J., et al. (2012). miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma. Oncol. Rep. *28*, 1764–1770.

Cai, J., Wu, J., Zhang, H., Fang, L., Huang, Y., Yang, Y., Zhu, X., Li, R., and Li, M. (2013). MiR-186 downregulation correlates with poor survival in lung adenocarcinoma, where it interferes with cell-cycle regulation. Cancer Res. *73*, 756–766.

Calin, G.A., and Croce, C.M. (2006a). MicroRNA-cancer connection: the beginning of a new tale. Cancer Res. *66*, 7390–7394.

Calin, G.A., and Croce, C.M. (2006b). MicroRNA signatures in human cancers. Nat. Rev. Cancer *6*, 857–866.

Calin, G.A., Dumitru, C.D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., et al. (2002). Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. U. S. A. *99*, 15524–15529.

Calin, G.A., Sevignani, C., Dumitru, C.D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc. Natl. Acad. Sci. U. S. A. *101*, 2999–3004.

Carrle D, Bielack, S. (2007). Diagnostik und multimodales Therapiekonzept des Osteosarkoms. Ärztliches J. Onkol. 34–38.

Chen, G.G., Zeng, Q., and Tse, G.M.K. (2008). Estrogen and Its Receptors in Cancer.Medicinal Research Reviews *28*, 954–974.

Chen, J., Feilotter, H.E., Paré, G.C., Zhang, X., Pemberton, J.G.W., Garady, C., Lai, D., Yang, X., and Tron, V.A. (2010). MicroRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma. Am. J. Pathol. *176*, 2520–2529.

Chen, L., Holmstrøm, K., Qiu, W., and Ditzel, N. (2014). MicroRNA-34a Inhibits Osteoblast Differentiation and In Vivo Bone Formation of Human Stromal Stem Cells. Stem Cells *32*, 902–912.

Chen, X., Guo, X., Zhang, H., Xiang, Y., Chen, J., Yin, Y., Cai, X., Wang, K., Wang, G., Ba, Y., et al. (2009). Role of miR-143 targeting KRAS in colorectal tumorigenesis. Oncogene *28*, 1385–1392.

Cheng, Y.Y., Huang, L., Lee, K.M., Li, K., and Kumta, S.M. (2004). Alendronate regulates cell invasion and MMP-2 secretion in human osteosarcoma cell lines. Pediatr. Blood Cancer *42*, 410–415.

Chopra, M., and Schrenk, D. (2011). Dioxin toxicity, aryl hydrocarbon receptor signaling, and apoptosis-persistent pollutants affect programmed cell death. Crit. Rev. Toxicol. *41*, 292–320.

Cole, K. a, Attiyeh, E.F., Mosse, Y.P., Laquaglia, M.J., Diskin, S.J., Brodeur, G.M., and Maris, J.M. (2008). A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene. Mol. Cancer Res. *6*, 735–742.

Croce, C.M. (2009). Causes and consequences of microRNA dysregulation in cancer. Nat. Rev. Genet. *10*, 704–714.

Dang, C. V. (2012). MYC on the path to cancer. Cell *149*, 22–35.

Van Dartel, M., and Hulsebos, T.J.M. (2004). Amplification and overexpression of genes in 17p11.2 ~ p12 in osteosarcoma. Cancer Genet. Cytogenet. *153*, 77–80.

Dylla, L., and Jedlicka, P. (2013). Growth-promoting role of the miR-106a~363 cluster in Ewing sarcoma. PLoS One *8*, e63032.

Eguchi, T., Watanabe, K., Hara, E.S., Ono, M., Kuboki, T., and Calderwood, S.K. (2013). OstemiR: a novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesencymal stem cells. PLoS One *8*, e58796.

Enard, W., Przeworski, M., Fisher, S.E., Lai, C.S.L., Wiebe, V., Kitano, T., Monaco, A.P., and Pääbo, S. (2002). Molecular evolution of FOXP2, a gene involved in speech and language. Nature *418*, 869–872.

Esquela-Kerscher, A., and Slack, F.J. (2006a and b). Oncomirs - microRNAs with a role in cancer. Nat. Rev. Cancer *6*, 259–269.

Evdokiou, a, Labrinidis, a, Bouralexis, S., Hay, S., and Findlay, D.. (2003). Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis. Bone *33*, 216–228.

Fakharzadeh, S.S., Trusko, S.P., and George, D.L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J. *10*, 1565–1569.

Fletcher, J.A., Gebhardt, M.C., and Kozakewich, H.P. (1994). Cytogenetic aberrations in osteosarcomas. Nonrandom deletions, rings, and double-minute chromosomes. Cancer Genet. Cytogenet. *77*, 81–88.

Fogh, J., Fogh, J.M., and Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst. *59*, 221–226.

Frankel, L.B., Christoffersen, N.R., Jacobsen, A., Lindow, M., Krogh, A., and Lund, A.H. (2008). Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J. Biol. Chem. *283*, 1026–1033.

Friedl, P., and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nat. Rev. Cancer *3*, 362–374.

Friend, S., Weinberg, R., Rapaport, J., and Albert, D. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature. *323*, 643-646

Fromigué, O., Hamidouche, Z., and Marie, P.J. (2008). Blockade of the RhoA-JNK-c-Jun-MMP2 cascade by atorvastatin reduces osteosarcoma cell invasion. J. Biol. Chem. *283*, 30549–30556.

Galluzzi, L., Morselli, E., Vitale, I., Kepp, O., Senovilla, L., Criollo, A., Servant, N., Paccard, C., Hupé, P., Robert, T., et al. (2010). miR-181a and miR-630 regulate cisplatin-induced cancer cell death. Cancer Res. *70*, 1793–1803.

Gamberi, G., Benassi, M.S., Bohling, T., Ragazzini, P., Molendini, L., Sollazzo, M.R., Pompetti, F., Merli, M., Magagnoli, G., Balladelli, A., et al. (1998). C-myc and c-fos in human osteosarcoma: Prognostic value of mRNA and protein expression. Oncology *55*, 556–563.

Gao, J., Yang, T., Han, J., Yan, K., Qiu, X., Zhou, Y., Fan, Q., and Ma, B. (2011). MicroRNA expression during osteogenic differentiation of human multipotent mesenchymal stromal cells from Bone Marrow. J. Cell. Biochem. *112*, 1844–1856.

Gokgoz, N., Wunder S., et al. (2001). Comparison of p53 Mutations in Patients with Localized Osteosarcoma and metastatic osteosarcoma. Cancer *416*, 2181-2189

Gorlick, B.R., Huvos, A.G., Heller, G., Aledo, A., Beardsley, G.P., Healey, J.H., and Meyers, P.A. (1999). Expression of HER2/erbB-2 correlates with survival in Osteosarcoma. Journal of Clinical Oncology *17*, 2781–2788.

Gougelet, A., Pissaloux, D., Besse, A., Perez, J., Duc, A., Dutour, A., Blay, J.-Y., and Alberti, L. (2011). Micro-RNA profiles in osteosarcoma as a predictive tool for ifosfamide response. Int. J. Cancer *129*, 680–690.

Greshock, J., Nathanson, K., Martin, A.-M., Zhang, L., Coukos, G., Weber, B.L., and Zaks, T.Z. (2007). Cancer cell lines as genetic models of their parent histology: analyses based on array comparative genomic hybridization. Cancer Res. *67*, 3594–3600.

Griffiths-Jones, S. (2004). The microRNA Registry. Nucleic Acids Res. *32*, D109–11.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., and Enright, A.J. (2008). miRBase: tools for microRNA genomics. Nucleic Acids Res. *36*, D154–8.

Grosshans, H., and Filipowicz, W. (2008). Molecular biology: the expanding world of small RNAs. Nature *451*, 414–416.

Halbrooks, P.J., Ding, R., Wozney, J.M., and Bain, G. (2007). Role of RGM coreceptors in bone morphogenetic protein signaling. J. Mol. Signal. *2*, 4.

Harada, S., and Rodan, G.A. (2003). Control of osteoblast function and regulation of bone mass. Nature *423*, 349–355.

Hayashita, Y., Osada, H., Tatematsu, Y., Yamada, H., Yanagisawa, K., Tomida, S., Yatabe, Y., Kawahara, K., Sekido, Y., and Takahashi, T. (2005). A polycistronic MicroRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. *65*, 9628–9632.

He, L., and Hannon, G.J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. *5*, 522–531.

He, C., Xiong, J., Xu, X., Lu, W., Liu, L., Xiao, D., and Wang, D. (2009). Functional elucidation of MiR-34 in osteosarcoma cells and primary tumor samples. Biochem. Biophys. Res. Commun. *388*, 35–40.

He, L., Thomson, J.M., Hemann, M.T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S.W., Hannon, G.J., et al. (2005). A microRNA polycistron as a potential human oncogene. Nature *435*, 828–833.

Heldin, C., Johnsson, A., and Wennergren, S. (1986). A human osteosarcoma cell line secretes a growth factor structurally related to a homodimer of PDGF A-chains. Letters to Nature, *319,* 511-514.

Heremans, H., Billiau, A., Cassiman, J.J., Mulier, J.C., and de Somer, P. (1978). In vitro cultivation of human tumor tissues. II. Morphological and virological characterization of three cell lines. Oncology *35*, 246–252.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., et al. (2000). Identification of CDK4 as a target of c-MYC. Proc. Natl. Acad. Sci. U. S. A. *97*, 2229–2234.

Hisa, I., Inoue, Y., Hendy, G.N., Canaff, L., Kitazawa, R., Kitazawa, S., Komori, T., Sugimoto, T., Seino, S., and Kaji, H. (2011). Parathyroid hormone-responsive Smad3-related factor, Tmem119, promotes osteoblast differentiation and interacts with the bone morphogenetic protein-Runx2 pathway. J. Biol. Chem. *286*, 9787–9796.

Holleville, N., Matéos, S., Bontoux, M., Bollerot, K., and Monsoro-Burq, A. (2007). Dlx5 drives Runx2 expression and osteogenic differentiation in developing cranial suture mesenchyme. Dev. Biol. *304*, 860–874.

Honda, R., Lowe, E.D., Dubinina, E., Skamnaki, V., Cook, A., Brown, N.R., and Johnson, L.N. (2005). The structure of cyclin E1/CDK2: implications for CDK2 activation and CDK2-independent roles. EMBO J. *24*, 452–463.

Huang, D.W., Sherman, B.T., and Lempicki, R. a (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. *4*, 44–57.

Huang, G., Nishimoto, K., Zhou, Z., Hughes, D., and Kleinerman, E.S. (2012). miR-20a encoded by the miR-17-92 cluster increases the metastatic potential of osteosarcoma cells by regulating fas expression. Cancer Res. *72*, 908–916.

Ide, Y., Miyoshi, E., Nakagawa, T., Gu, J., Tanemura, M., Nishida, T., Ito, T., Yamamoto, H., Kozutsumi, Y., and Taniguchi, N. (2006). Aberrant expression of N-acetylglucosaminyltransferase-IVa and IVb (GnT-IVa and b) in pancreatic cancer. Biochem. Biophys. Res. Commun. *341*, 478–482.

Inoue, H., Mishima, K., Yamamoto-Yoshida, S., Ushikoshi-Nakayama, R., Nakagawa, Y., Yamamoto, K., Ryo, K., Ide, F., and Saito, I. (2012). Aryl hydrocarbon receptor-mediated induction of EBV reactivation as a risk factor for Sjögren's syndrome. J. Immunol. *188*, 4654–4662.

Iorio, M. V., Ferracin, M., Liu, C.G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Campiglio, M., et al. (2005). MicroRNA gene expression deregulation in human breast cancer. Cancer Res. *65*, 7065–7070.

Isakoff, Harris, M.J., Gebhardt, M.C., and Grier, H.E. (2007). Bone Sarcomas. In Cancer in Adolescents and Young Adults, pp. 203–218.

Jia, S., Worth, L.L., and Kleinerman, E.S. (2000). A nude mouse model of human osteosarcoma lung metastases for evaluating new therapeutic strategies. Clinical & Experimental Metastasis, *17*, 501–506.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D., and Slack, F.J. (2005). RAS is regulated by the let-7 microRNA family. Cell *120*, 635–647.

Jones, K.B., Salah, Z., Del Mare, S., Galasso, M., Gaudio, E., Nuovo, G.J., Lovat, F., LeBlanc, K., Palatini, J., Randall, R.L., et al. (2012). miRNA signatures associate with pathogenesis and progression of osteosarcoma. Cancer Res. *72*, 1865–1877.

Kager, L., Zoubek, A., Pötschger, U., Kastner, U., Flege, S., Kempf-Bielack, B., Branscheid, D., Kotz, R., Salzer-Kuntschik, M., Winkelmann, W., et al. (2003). Primary metastatic osteosarcoma: Presentation and outcome of patients treated on neoadjuvant Cooperative Osteosarcoma Study Group protocols. J. Clin. Oncol. *21*, 2011–2018.

Kapinas, K., Kessler, C., Ricks, T., Gronowicz, G., and Delany, A.M. (2010). miR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. J. Biol. Chem. *285*, 25221–25231.

Kauer, M., Ban, J., Kofler, R., Walker, B., Davis, S., Meltzer, P., and Kovar, H. (2009). A molecular function map of Ewing's sarcoma. PLoS One *4,* e5415.

Kim, V.N. (2005). MicroRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. Cell Biol. *6*, 376–385.

Kim, T.K., and Eberwine, J.H. (2010). Mammalian cell transfection: the present and the future. Anal. Bioanal. Chem. *397*, 3173–3178.

Kim, T., Kim, K., Lee, S.H., So, H.-S., Lee, J., Kim, N., and Choi, Y. (2009). Identification of LRRc17 as a negative regulator of receptor activator of NF-kappaB ligand (RANKL)-induced osteoclast differentiation. J. Biol. Chem. *284*, 15308–15316.

Klein, M.J., and Siegal, G.P. (2006). Osteosarcoma. Am. J. Clin. Pathol. *125*, 555–581.

Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W., and Martin, G.R. (1986). Basement membrane complexes with biological activity. Biochemistry *25*, 312–318.

Kong, Y.W., Cannell, I.G., de Moor, C.H., Hill, K., Garside, P.G., Hamilton, T.L., Meijer, H.A., Dobbyn, H.C., Stoneley, M., Spriggs, K.A., et al. (2008). The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. Proc. Natl. Acad. Sci. U. S. A. *105*, 8866–8871.

Kovar, H., Jug, G., Aryee, D.N., Zoubek, a, Ambros, P., Gruber, B., Windhager, R., and Gadner, H. (1997). Among genes involved in the RB dependent cell cycle regulatory cascade, the p16 tumor suppressor gene is frequently lost in the Ewing family of tumors. Oncogene *15*, 2225–2232.

Kramer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschläger, M., and Dolznig, H. (2013). In vitro cell migration and invasion assays. Mutat. Res. *752*, 10–24.

Kresse, S.H., Rydbeck, H., Skarn, M., Namlos, H.M., Barragan-Polania, A.H., Cleton-Jansen, A.M., Serra, M., Liestol, K., Hogendoorn, P.C., Hovig, E., et al. (2012). Integrative analysis reveals relationships of genetic and epigenetic alterations in osteosarcoma. PLoS One *7*, e48262.

Krichevsky, A.M., and Gabriely, G. (2009). miR-21: A small multi-faceted RNA. J. Cell. Mol. Med. *13*, 39–53.

Kuijjer, M.L., Rydbeck, H., Kresse, S.H., Buddingh, E.P., Lid, A.B., Roelofs, H., Bürger, H., Myklebost, O., Hogendoorn, P.C.W., Meza-Zepeda, L.A., et al. (2012). Identification of osteosarcoma driver genes by integrative analysis of copy number and gene expression data. Genes Chromosom. Cancer *51*, 696–706.

Kwon, J.E., Kim, B.Y., Kwak, S.Y., Bae, I.H., and Han, Y.H. (2013). Ionizing radiation-inducible microRNA miR-193a-3p induces apoptosis by directly targeting Mcl-1. Apoptosis *18*, 896–909.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., and Tuschl, T. (2003). New microRNAs from mouse and human. RNA *9*, 175–179.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A.O., Landthaler, M., et al. (2007). A Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing. Cell *129*, 1401–1414.

Lane, D. (1992). p53, guardian of the genome. Nature *358*, 15–16.

Latchman, Y., Wood, C.R., Chernova, T., Chaudhary, D., Chernova, I., Iwai, Y., Long, A.J., Brown, J.A., Nunes, R., Greenfield, E.A., et al. (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *2*, 261–268.

Le, M.T.N., Shyh-Chang, N., Khaw, S.L., Chin, L., Teh, C., Tay, J., O'Day, E., Korzh, V., Yang, H., Lal, A., et al. (2011). Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs. PLoS Genet. *7,* e1002242.

Lee, A.F., Hayes, M.M., Lebrun, D., Espinosa, I., Nielsen, G.P., Rosenberg, A.E., and Lee, C.-H. (2011). FLI-1 distinguishes Ewing sarcoma from small cell osteosarcoma and mesenchymal chondrosarcoma. Appl. Immunohistochem. Mol. Morphol. *19*, 233–238.

Lee, R.C., Feinbaum, R.L., and Ambrost, V. (1993). The C . elegans Heterochronic Gene lin-4 Encodes Small RNAs with Antisense Complementarity to & II-14. *75*, 843–854.

Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: Stepwise processing and subcellular localization. EMBO J. *21*, 4663–4670.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Lefort, K., Brooks, Y., Ostano, P., Cario-André, M., Calpini, V., Guinea-Viniegra, J., Albinger-Hegyi, A., Hoetzenecker, W., Kolfschoten, I., Wagner, E.F., et al. (2013). A miR-34a-SIRT6 axis in the squamous cell differentiation network. EMBO J. *32*, 2248–2263.

Li, X.-F., Yan, P.-J., and Shao, Z.-M. (2009). Downregulation of miR-193b contributes to enhance urokinase-type plasminogen activator (uPA) expression and tumor progression and invasion in human breast cancer. Oncogene *28*, 3937–3948.

Liang, S., He, L., Zhao, X., Miao, Y., Gu, Y., Guo, C., Xue, Z., Dou, W., Hu, F., Wu, K., et al. (2011). MicroRNA let-7f inhibits tumor invasion and metastasis by targeting MYH9 in human gastric cancer. PLoS One *6*, e18409.

Liu, L. hong, Li, H., Li, J. ping, Zhong, H., Zhang, H. chon, Chen, J., and Xiao, T. (2011). miR-125b suppresses the proliferation and migration of osteosarcoma cells through down-regulation of STAT3. Biochem. Biophys. Res. Commun. *416*, 31–38.

Lockwood, W.W., Stack, D., Morris, T., Grehan, D., O'Keane, C., Stewart, G.L., Cumiskey, J., Lam, W.L., Squire, J.A., Thomas, D.M., et al. (2011). Cyclin E1 is amplified and overexpressed in osteosarcoma. J. Mol. Diagnostics *13*, 289–296.

Lonardo, F., Ueda, T., Huvos, A.G., Healey, J., and Ladanyi, M. (1997). p53 and MDM2 alterations in osteosarcomas: Correlation with clinicopathologic features and proliferative rate. Cancer *79*, 1541–1547.

Long, F. (2011). Building strong bones: molecular regulation of the osteoblast lineage. Nat. Rev. Mol. Cell Biol. *13*, 27–38.

Long, X.H., Mao, J.H., Peng, A.F., Zhou, Y., Huang, S.H., and Liu, Z.L. (2013). Tumor suppressive microRNA-424 inhibits osteosarcoma cell migration and invasion via targeting fatty acid synthase. Exp. Ther. Med. *5*, 1048–1052.

Longhi, A., and Pasini, A. (2005). Height as a risk factor for osteosarcoma. J. Pediatr. Hematology/Oncology *27*, 314-318.

Lu, J., Getz, G., Miska, E. a, Alvarez-Saavedra, E., Lamb, J., Peck, D., Sweet-Cordero, A., Ebert, B.L., Mak, R.H., Ferrando, A. a, et al. (2005). MicroRNA expression profiles classify human cancers. Nature *435*, 834–838.

Luu, H.H., Kang, Q., Jong, K.P., Si, W., Luo, Q., Jiang, W., Yin, H., Montag, A.G., Simon, M.A., Peabody, T.D., et al. (2005a). An orthotopic model of human osteosarcoma growth and spontaneous pulmonary metastasis. Clin. Exp. Metastasis *22*, 319–329.

Luu, H.H., Kang, Q., Park, J.K., Si, W., Luo, Q., Jiang, W., Yin, H., Montag, A.G., Simon, M. a, Peabody, T.D., et al. (2005b). An orthotopic model of human osteosarcoma growth and spontaneous pulmonary metastasis. Clin. Exp. Metastasis *22*, 319–329.

Ma, Q., Zhou, Y., Ma, B., Chen, X., Wen, Y., and Liu, Y. (2012). The clinical value of CXCR4, HER2 and CD44 in human osteosarcoma: A pilot study. Oncol.letters *3*, 797–801.

Ma, S., Yang, Y., Wang, C., Hui, N., Gu, L., Zhong, H., Cai, Z., Wang, Q., Zhang, Q., Li, N., et al. (2009). Endogenous human CaMKII inhibitory protein suppresses tumor growth by inducing cell cycle arrest and apoptosis through down-regulation of the phosphatidylinositide 3-kinase/Akt/HDM2 pathway. J. Biol. Chem. *284*, 24773–24782.

Maire, G., Martin, J.W., Yoshimoto, M., Chilton-MacNeill, S., Zielenska, M., and Squire, J. a (2011). Analysis of miRNA-gene expression-genomic profiles reveals complex mechanisms of microRNA deregulation in osteosarcoma. Cancer Genet. *204*, 138–146.

Maitra, A., Wanzer, D., Weinberg, A.G., and Ashfaq, R. (2001). Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. Cancer *92*, 677–683.

Man, T.-K., Lu, X.-Y., Jaeweon, K., Perlaky, L., Harris, C.P., Shah, S., Ladanyi, M., Gorlick, R., Lau, C.C., and Rao, P.H. (2004). Genome-wide array comparative genomic hybridization analysis reveals distinct amplifications in osteosarcoma. BMC Cancer *4*, 45.

Manara, M.C., Baldini, N., Serra, M., Lollini, P., Giovanni, C. De, Vaccari, M., Argnani, A., Benini, S., Maurici, D., Picci, P., et al. (2000). Reversal of Malignant Phenotype in Human Osteosarcoma Cells Transduced With the Alkaline Phosphatase Gene. *26*, 215–220.

Martin, J.W., Squire, J. a, and Zielenska, M. (2012). The genetics of osteosarcoma. Sarcoma *2012*, 627254.

Maul, R.S., and Chang, D.D. (1999). EPLIN, epithelial protein lost in neoplasm. Oncogene *18*, 7838–7841.

McAllister, R., Gardner, M., and Greene, A. (1971). Cultivation in vitro of cells derived from a human osteosarcoma. Cancer *27*, 397-402

Mertens, F., and Mandahl, N. (1993). Cytogenetic findings in 33 osteosarcomas. Int J. Cancer *50*, 44–50.

Michael, M.Z., O' Connor, S.M., van Holst Pellekaan, N.G., Young, G.P., and James, R.J. (2003). Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol. Cancer Res. *1*, 882–891.

Miller, C., Aslo, A., Won, A., and Tan, M. (1996). Alterations of thep53, Rb andMDM2 genes in osteosarcoms. J. Cancer Research and Clinical Oncology *122,* 559–565.

Mills, J., Matos, T., Charytonowicz, E., Hricik, T., Castillo-Martin, M., Remotti, F., Lee, F.Y., and Matushansky, I. (2009). Characterization and comparison of the properties of sarcoma cell lines in vitro and in vivo. Hum. Cell *22*, 85–93.

Mizuno, Y., Yagi, K., Tokuzawa, Y., Kanesaki-Yatsuka, Y., Suda, T., Katagiri, T., Fukuda, T., Maruyama, M., Okuda, A., Amemiya, T., et al. (2008). miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. Biochem. Biophys. Res. Commun. *368*, 267–272.

Mogilyansky, E., and Rigoutsos, I. (2013). The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. Cell Death Differ. *20*, 1603–1614.

Mohseny, A.B., Machado, I., Cai, Y., Schaefer, K.-L., Serra, M., Hogendoorn, P.C.W., Llombart-Bosch, A., and Cleton-Jansen, A.-M. (2011). Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Lab. Invest. *91*, 1195–1205.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237–1245.

Momand, J., Jung, D., Wilczynski, S., and Niland, J. (1998). The MDM2 gene amplification database. Nucl Acids Research *26*, 3453–3459.

Mu, G., Liu, H., Zhou, F., Xu, X., Jiang, H., Wang, Y., and Qu, Y. (2010). Correlation of overexpression of HMGA1 and HMGA2 with poor tumor differentiation, invasion, and proliferation associated with let-7 down-regulation in retinoblastomas. Hum. Pathol. *41*, 493–502.

Muller, P. a J., Vousden, K.H., and Norman, J.C. (2011). P53 and Its Mutants in Tumor Cell Migration and Invasion. J. Cell Biol. *192*, 209–218.

Musgrove, E. a, Caldon, C.E., Barraclough, J., Stone, A., and Sutherland, R.L. (2011). Cyclin D as a therapeutic target in cancer. Nat. Rev. Cancer *11*, 558–572.

Musselman, J.R.B., Bergemann, T.L., Ross, J.A., Sklar, C., Silverstein, K.A.T., and Erica, K. (2012). Case-parent analysis of variation in pubertal hormone genes and pediatric osteosarcoma : a Children ' s Oncology Group ( COG ) study. International Journal of Molecular Epidemiology and Genetics *3*, 286–293.

Nakajima, G., Hayashi, K., and Xi, Y. (2006). Non-coding MicroRNAs hsa-let-7g and hsa-miR-181b are associated with Chemoresponse to S-1 in colon cancer. Cancer Genomics and Proteomics *3*, 317–324.

Namløs, H.M., Meza-Zepeda, L. a, Barøy, T., Østensen, I.H.G., Kresse, S.H., Kuijjer, M.L., Serra, M., Bürger, H., Cleton-Jansen, A.-M., and Myklebost, O. (2012). Modulation of the osteosarcoma expression phenotype by microRNAs. PLoS One *7*, e48086.

Nevins, J.R. (2001). The Rb / E2F pathway and cancer. Human Mol Genetics *10*, 699–704.

Nielsen, G.P., Burns, K.L., Rosenberg, A.E., and Louis, D.N. (1998). CDKN2A Gene Deletions and Loss of p16 Expression Occur in Osteosarcomas That Lack RB Alterations. The Am J Path *153*, 159–163.

Lo Nigro, C., Monteverde, M., Lee, S., Lattanzio, L., Vivenza, D., Comino, a, Syed, N., McHugh, a, Wang, H., Proby, C., et al. (2012). NT5E CpG island methylation is a favourable breast cancer biomarker. Br. J. Cancer *107*, 75–83.

Oliner, J., Kinzler, K., and Meltzer, P. (1992). Amplification of a gene encoding p53-associated protein in human sarcomas. Letters to Nature *358*, 80-83

Olive, V., Jiang, I., and He, L. (2010). Mir-17-92, a cluster of miRNAs in the midst of the cancer network. Int. J. Biochem. Cell Biol. *42*, 1348–1354.

Ottaviani, G., and Jaffe, N. (2010). The epidemiology of osteosarcoma. Pediatr. Adolesc. Osteosarcoma, Cancer Treatment and Research book chapter, *152*, 3-13

Ottaviano, L., Schaefer, K., Gajewski, M., Huckenbeck, W., Baldus, S., Rogel, U., Mackintosh, C., Alava, E. De, Myklebost, O., Kresse, S.H., et al. (2010). Molecular Characterization of Commonly Used Cell Lines for Bone Tumor Research : A Trans-European EuroBoNet Effort. Genes, Chromos and Cancer *49*, 40–51.

Overholtzer, M., Rao, P.H., Favis, R., Lu, X.-Y., Elowitz, M.B., Barany, F., Ladanyi, M., Gorlick, R., and Levine, A.J. (2003). The presence of p53 mutations in human osteosarcomas correlates with high levels of genomic instability. Proc. Natl. Acad. Sci. U. S. A. *100*, 11547–11552.

Ozaki, T., Neumann, T., Wai, D., Schäfer, K., Valen, F. Van, Dockhorn-dworniczak, B., Horst, J., and Poremba, C. (2003). Chromosomal alterations in osteosarcoma cell lines revealed by comparative genomic hybridization and multicolor karyotyping. Cancer Genetics and Cytogenetics *140*, 145–152.

Partin, J. V, Anglin, I.E., and Kyprianou, N. (2003). Quinazoline-based alpha 1-adrenoceptor antagonists induce prostate cancer cell apoptosis via TGF-beta signalling and I kappa B alpha induction. Br. J. Cancer *88*, 1615–1621.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Srinivasan, A., Fishman, M., Finnerty, J., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 1998–2001.

Pautke, C., Schieker, M., Tischer, T., Kolk, A., Neth, P., Mutschler, W., and Milz, S. (2004). Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res. *24*, 3743–3748.

Peng, X., Li, Y., Walters, K.-A., Rosenzweig, E.R., Lederer, S.L., Aicher, L.D., Proll, S., and Katze, M.G. (2009a). Computational identification of hepatitis C virus associated microRNA-mRNA regulatory modules in human livers. BMC Genomics *10*, 373.

Peng, X., Li, Y., Walters, K.-A., Rosenzweig, E.R., Lederer, S.L., Aicher, L.D., Proll, S., and Katze, M.G. (2009b). Computational identification of hepatitis C virus associated microRNA-mRNA regulatory modules in human livers. BMC Genomics *10*, 373.

Pérez Piñero, C., Bruzzone, A., Sarappa, M.G., Castillo, L.F., and Lüthy, I.A. (2012). Involvement of α2- and β2-adrenoceptors on breast cancer cell proliferation and tumour growth regulation. Br. J. Pharmacol. *166*, 721–736.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573–1576.

Ponten, J., and Saksela, E. (1967). Two established in vitro cell lines from human mesenchymal tumours. Int. J. Cancer *447*, 434–447.

Poos, K., Smida, J., Nathrath, M., Maugg, D., Baumhoer, D., and Korsching, E. (2013). How MicroRNA and Transcription Factor Co-regulatory Networks Affect Osteosarcoma Cell Proliferation. PLoS Comput. Biol. *9*, e1003210.

Poos, K., Smida, J., Nathrath, M., Maugg, D., Baumhoer, D., Neumann, A., and Korsching, E. (2014). Structuring osteosarcoma knowledge: an osteosarcoma-gene association

database based on literature mining and manual annotation. Database : the journal of biological databases and curation (Oxford). *2014*, 1-9

Potratz, J., Zoubek, a., and Bielack, S. (2006). Maligne Knochentumoren bei Kindern und Jugendlichen. Monatsschrift Kinderheilkd. *154*, 10–19.

Price, C.H.G. (1958). Primary bone-forming tumours and their relationship to skeletal growth. J. Bone Jt. Surgery, Br. Vol. *40 B*, 574–593.

Ramkissoon, L. a, Horowitz, P.M., Craig, J.M., Ramkissoon, S.H., Rich, B.E., Schumacher, S.E., McKenna, A., Lawrence, M.S., Bergthold, G., Brastianos, P.K., et al. (2013). Genomic analysis of diffuse pediatric low-grade gliomas identifies recurrent oncogenic truncating rearrangements in the transcription factor MYBL1. Proc. Natl. Acad. Sci. U. S. A. *110*, 8188–8193.

Rauhala, H.E., Jalava, S.E., Isotalo, J., Bracken, H., Lehmusvaara, S., Tammela, T.L.J., Oja, H., and Visakorpi, T. (2010). miR-193b is an epigenetically regulated putative tumor suppressor in prostate cancer. Int. J. Cancer *127*, 1363–1372.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901–906.

Rhim, J., Park, D., and Arnstein, P. (1975a). Transformation of human cells in culture by N-methyl-N'-nitro-N-nitrosoguanidine.Nature *256*, 751-753.

Rhim, J.S., Cho, H.Y., and Huebner, R.J. (1975b). Non-producer human cells induced by murine sarcoma virus. Int. J. Cancer *15*, 23–29.

Rhim, J.S., Cho, H.Y., Vernon, M.L., Arnstein, P., Huebner, R.J., and Gilden, R. V (1975c). Characterization of non-producer human cells induced by Kirsten sarcoma virus. Int. J. Cancer *16*, 840–849.

Ritchie, M.E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A., and Smyth, G.K. (2007). A comparison of background correction methods for two-colour microarrays. Bioinformatics *23*, 2700–2707.

Roberts, W.M., Douglass, E.C., Peiper, S.C., Houghton, P.J., and Look, A.T. (1989). Amplification of the gli Gene in Childhood Sarcomas Amplification of the gli Gene in Childhood Sarcomas. Cancer Research *49*, 5407–5413.

Rosemann, M., Kuosaite, V., and Nathrath, M. The genetics of radiation-induced and sporadic osteosarcoma : a unifying theory ? Journal of radiological protection *22*, *113-116*.

Ross, J., and Fletcher, J. (1998). The HER-2/neu Oncogene in Breast Cancer: Prognostic Factor, Predictive Factor, and Target for Therapy. Oncologist *2*, 237–252.

Roush, S., and Slack, F.J. (2008). The let-7 family of microRNAs. Trends Cell Biol. *18*, 505–516.

Ryoo, H.M., Hoffmann, H.M., Beumer, T., Frenkel, B., Towler, D.A., Stein, G.S., Stein, J.L., van Wijnen, A.J., and Lian, J.B. (1997). Stage-specific expression of Dlx-5 during osteoblast differentiation: involvement in regulation of osteocalcin gene expression. Mol. Endocrinol. *11*, 1681–1694.

Sakurai, T., Kondoh, N., Arai, M., Hamada, J. ichi, Yamada, T., Kihara-negishi, F., Izawa, T., Ohno, H., Yamamoto, M., and Oikawa, T. (2007). Functional roles of Fli-1, a member of the Ets family of transcription factors, in human breast malignancy. Cancer Sci. *98*, 1775–1784.

Salzer-Kuntschik, M., and Delling, G. (1983). Morphological grades of regression in osteosarcoma after polychemotherapy. J. Cancer Res Clin Oncol *166*, 21–24.

Samad, T.A., Rebbapragada, A., Bell, E., Zhang, Y., Sidis, Y., Jeong, S.J., Campagna, J.A., Perusini, S., Fabrizio, D.A., Schneyer, A.L., et al. (2005). DRAGON, a bone morphogenetic protein co-receptor. J. Biol. Chem. *280*, 14122–14129.

Sandberg, A. a, and Bridge, J. a (2003). Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: osteosarcoma and related tumors. Cancer Genet. Cytogenet. *145*, 1–30.

Savage, S. a, and Mirabello, L. (2011). Using epidemiology and genomics to understand osteosarcoma etiology. Sarcoma *2011*, 548151.

Schauwecker, J., Weirich, G., Gradinger, R., and Rechl, H. (2006). Das Osteosarkom. Orthopädie und Rheuma, *2002,* 36-42.

Scotlandi, K., Manara, M.C., Hattinger, C.M., Benini, S., Perdichizzi, S., Pasello, M., Bacci, G., Zanella, L., Bertoni, F., Picci, P., et al. (2005). Prognostic and therapeutic relevance of HER2 expression in osteosarcoma and Ewing's sarcoma. Eur. J. Cancer *41*, 1349–1361.

Shi, L., Cheng, Z., Zhang, J., Li, R., Zhao, P., Fu, Z., and You, Y. (2008). hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. Brain Res. *1236*, 185–193.

Shin, K.-H., Bae, S.D., Hong, H.S., Kim, R.H., Kang, M.K., and Park, N.-H. (2011). miR-181a shows tumor suppressive effect against oral squamous cell carcinoma cells by downregulating K-ras. Biochem. Biophys. Res. Commun. *404*, 896–902.

Siggelkow, H., Niedhart, C., and Kurre, W. (1998). In vitro differentiation potential of a new human osteosarcoma cell line (HOS 58). Differentiation research in biological diversity *63*, 81–91.

Smida, J., Baumhoer, D., Rosemann, M., Walch, A., Bielack, S., Poremba, C., Remberger, K., Korsching, E., Scheurlen, W., Dierkes, C., et al. (2010). Genomic alterations and allelic

imbalances are strong prognostic predictors in osteosarcoma. Clin. Cancer Res. *16*, 4256–4267.

Song, B., Wang, Y., Titmus, M.A., Botchkina, G., Formentini, A., Kornmann, M., and Ju, J. (2010). Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. Mol. Cancer *9*, 96.

Stephens, P.J., Greenman, C.D., Fu, B., Yang, F., Bignell, G.R., Mudie, L.J., Pleasance, E.D., Lau, K.W., Beare, D., Stebbings, L. a, et al. (2011). Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell *144*, 27–40.

Stiller, C. (2002). Epidemiology of cancer in adolescents. Med. Pediatr. Oncol. *39*, 149–155.

Stossi, F., Barnett, D.H., Frasor, J., Komm, B., Lyttle, C.R., and Katzenellenbogen, B.S. (2004). Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors. Endocrinology *145*, 3473–3486.

Stratton, M. R. , Campbell, P. J., Futreal, P. A. (2009). The cancer genome. Nature *458*, 719-724.

Su, B., Zhao, W., Shi, B., Zhang, Z., Yu, X., Xie, F., Guo, Z., Zhang, X., Liu, J., Shen, Q., et al. (2014). Let-7d suppresses growth, metastasis, and tumor macrophage infiltration in renal cell carcinoma by targeting COL3A1 and CCL7. Mol. Cancer *13*, 206.

Subramaniam, M., Jalal, S.M., Rickard, D.J., Harris, S.A., Bolander, M.E., and Spelsberg, T.C. (2002). Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ER alpha cells: bone formation in vivo and karyotype analysis using multicolor fluorescent in situ hybridization. J. Cell. Biochem. *87*, 9–15.

Sun, Y.-M., Lin, K.-Y., and Chen, Y.-Q. (2013). Diverse functions of miR-125 family in different cell contexts. J. Hematol. Oncol. *6*, 6.

Sylvestre, Y., De Guire, V., Querido, E., Mukhopadhyay, U.K., Bourdeau, V., Major, F., Ferbeyre, G., and Chartrand, P. (2007). An E2F/miR-20a autoregulatory feedback loop. J. Biol. Chem. *282*, 2135–2143.

Tanaka, K.I., Inoue, Y., Hendy, G.N., Canaff, L., Katagiri, T., Kitazawa, R., Komori, T., Sugimoto, T., Seino, S., and Kaji, H. (2012). Interaction of Tmem119 and the bone morphogenetic protein pathway in the commitment of myoblastic into osteoblastic cells. Bone *51*, 158–167.

Thalmeier, K., and Meissner, P. (1994). Establishment of two permanent human bone marrow stromal cell lines with long-term post irradiation feeder capacity.Blood *83*, 1799–1807.

Troisi, R., Masters, M.N., Joshipura, K., Douglass, C., Cole, B.F., and Hoover, R.N. (2006). Perinatal factors, growth and development, and osteosarcoma risk. Br. J. Cancer *95*, 1603–1607.

Truong, A.H., and Ben-David, Y. (2000). The role of Fli-1 in normal cell function and malignant transformation. Oncogene *19*, 6482–6489.

Tucker, M. a, D'Angio, G.J., Boice, J.D., Strong, L.C., Li, F.P., Stovall, M., Stone, B.J., Green, D.M., Lombardi, F., and Newton, W. (1987). Bone sarcomas linked to radiotherapy and chemotherapy in children. N. Engl. J. Med. *317*, 588–593.

Valster, A., Tran, N.L., Nakada, M., Berens, M.E., Chan, A.Y., and Symons, M. (2005). Cell migration and invasion assays. Methods *37*, 208–215.

Vennstrom, B., Sheiness, D., Zabielski, J., and Bishop, J.M. (1982). Isolation and characterization of c-myc, a cellular homolog of the oncogene (v-myc) of avian myelocytomatosis virus strain 29. J. Virol. *42*, 773–779.

Wang, X., and El Naqa, I.M. (2008). Prediction of both conserved and nonconserved microRNA targets in animals. Bioinformatics *24*, 325–332.

Wang, T., Guo, S., Liu, Z., Wu, L., and Li, M. (2014) CAMK2N1 inhibits prostate cancer progression androgen receptor-dependent signaling through.Oncotarget *5,* 10293-10306.

Wang, Y., Zhao, W., and Fu, Q. (2013). MiR-335 suppresses migration and invasion by targeting ROCK1 in osteosarcoma cells. Mol. Cell. Biochem. *384*, 105–111.

Welch, C., Chen, Y., and Stallings, R.L. (2007). MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells. Oncogene *26*, 5017–5022.

Wu, D., Chen, K., and Bai, Y. (2014). Screening of diagnostic markers for osteosarcoma. Mol. Med Reports *10*, 2415-2420.

Wu, T., Zhou, H., Hong, Y., Li, J., Jiang, X., and Huang, H. (2012). miR-30 family members negatively regulate osteoblast differentiation. J. Biol. Chem. *287*, 7503–7511.

Yan, H., Xue, G., Mei, Q., Wang, Y., Ding, F., Liu, M.-F., Lu, M.-H., Tang, Y., Yu, H., and Sun, S. (2009). Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis. EMBO J. *28*, 2719–2732.

Yan, K., Gao, J., Yang, T., Ma, Q., Qiu, X., Fan, Q., and Ma, B. (2012). MicroRNA-34a inhibits the proliferation and metastasis of osteosarcoma cells both in vitro and in vivo. PLoS One *7*, e33778.

Yang, X., Liu, D., Murray, T.J., Mitchell, G.C., Hesterman, E. V, Karchner, S.I., Merson, R.R., Hahn, M.E., and Sherr, D.H. (2005). The aryl hydrocarbon receptor constitutively represses c-myc transcription in human mammary tumor cells. Oncogene *24*, 7869–7881.

Yekta, S., Shih, I.-H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. Science *304*, 594–596.

Zhang, J., Tu, Q., Bonewald, L.F., He, X., Stein, G., Lian, J., and Chen, J. (2011a). Effects of miR-335-5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1. J. Bone Miner. Res. *26*, 1953–1963.

Zhang, S., Wang, X., Osunkoya, A.O., Iqbal, S., Wang, Y., Chen, Z., Müller, S., Josson, S., Coleman, I.M., Nelson, P.S., et al. (2011b). EPLIN downregulation promotes epithelial–mesenchymal transition in prostate cancer cells and correlates with clinical lymph node metastasis. Oncogene *30*, 4941–4952.

Zhang, W., Qian, J.-X., Yi, H.-L., Yang, Z.-D., Wang, C.-F., Chen, J.-Y., Wei, X.-Z., Fu, Q., and Ma, H. (2012). The microRNA-29 plays a central role in osteosarcoma pathogenesis and progression. Mol. Biol. *46*, 557–562.

Zhang, Y.W., Morita, I., Ikeda, M., Ma, K.W., and Murota, S. (2001). Connexin43 suppresses proliferation of osteosarcoma U2OS cells through post-transcriptional regulation of p27. Oncogene *20*, 4138–4149.

Zhao, H., Ma, B., Wang, Y., Han, T., Zheng, L., Sun, C., Liu, T., Zhang, Y., Qiu, X., and Fan, Q. (2013). miR-34a inhibits the metastasis of osteosarcoma cells by repressing the expression of CD44. Oncol. Rep. *29*, 1027–1036.

Zhou, H., Randall, R.L., Brothman, A.R., Ph, D., Maxwell, T., Coffin, C.M., and Goldsby, R.E. (2003). Her-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. J Pedr Hem/Oncol. *25,* 27-32.

Zhou, L., Qi, X., Potashkin, J. a, Abdul-Karim, F.W., and Gorodeski, G.I. (2008). MicroRNAs miR-186 and miR-150 down-regulate expression of the pro-apoptotic purinergic P2X7 receptor by activation of instability sites at the 3'-untranslated region of the gene that decrease steady-state levels of the transcript. J. Biol. Chem. *283*, 28274–28286.

Zhu, W., Shan, X., Wang, T., Shu, Y., and Liu, P. (2010). miR-181b modulates multidrug resistance by targeting BCL2 in human cancer cell lines. Int. J. Cancer *127*, 2520–2529.

Ziyan, W., Shuhua, Y., Xiufang, W., and Xiaoyun, L. (2011). MicroRNA-21 is involved in osteosarcoma cell invasion and migration. Med. Oncol. *28*, 1469–1474.

Zoubek, A., Windhager, R., and Bielack, S. (2006). Osteosarkome. Pädiatrische Hämatologie und Onkologie, book chapter pp. 882–893.

# 9. List of figures

# 10. List of tables

# 11. List of Abbreviations

| Abbreviation | Explanation |
|---|---|
| OS | Osteosarcoma |
| ATCC | American Type Culture Collection |
| N/A | Not Available |
| FCS | Fetal Calf Serum |
| CG | Chemogradient |
| CI | Cell Index |
| Lowess-Algorithm | Locally Weighted Scatterplot Smoothing |
| miRNA | MicroRNA |
| mRNA | Messenger RNA |
| PBS | Phosphate Buffered Saline |
| RPMI | Roswell Park Memorial Institute |
| RMA | Robust Multichip Average |
| QC | Quality Control |
| WT | Whole Transcript |
| OGS | Official Gene Symbol |
| SEER | Surveillance, Epidemiology and End Results Program |
| Dt | Doubling time |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| EURAMOS | European and American Osteosarcoma Study group |
| CTOS | Connective Tissue Oncology Society |
| Go-Term | Gene Ontology Term |
| FISH | Flourescence In Situ Hybridization |
| SKY | Spectral Karyotyping |
| CGH | Comparative Genomic Hybridization |
| DAVID | Database for Annotation, Visualization and Integrated Discovery |
| CLL | Chronic Lymphocytic Leukemia |
| MAP | Methotrexate, Adriamycin, Cisplatin |
| MAPIE | Methotrexate, Adriamycin, Cisplatin, Ifosfamide, Etoposide |

# 12. Acknowledgements

I would like to express my thanks to:

- Prof. Dr. med. Michaela Nathrath, who had confidence in me and gave me the opportunity to do this thesis. Throughout all this time you have been animating me and you have been always supportive. With your apparently infinite motivation and honest commitment to medicine, in clinic or in research, you are an inspiring example.

- The Holzhauer Stiftung, in person Peter Vaupel, who kindly provided the funding for this work, especially for my stay in Munich. Without your generous support this thesis would not have been possible.

- Dr. rer. nat. Jan Smida, who was an extraordinary supervisor throughout the whole time of this thesis. You have answered all my questions patiently and you helped with your comprehensive knowledge not only in molecular biology. Besides, thanks a lot for being such an enjoyable and wise office partner during my stay in Munich.

- My particluar thanks to all the other (past and actual) KKG-members for the help with laboratory work and plenty of fruitful and amusing discussions about science and life in general: PD Dr. med. Daniel Baumhoer, Laura Dajka, Dr. rer. nat. Doris Maugg, Dr. rer. nat. Ela Karshovska, Dr. Michael Rosemann.

- Furthermore, I am very grateful for the warm welcome in his institute and the insights into softball that I happily received by Prof. Michael Atkinson.
  To the members of the Radiation Biology unit and the Department of Analytic Pathology, Mareike, Klaudia, Isi, Anne, Fred, Natasha, Ines, Simone, Omid, Bahar, Solvejg und Steffi I give my sincere thanks for their constant scientific advice and support as well as regular diverting lunch breaks and discussions.

- Moreover, I thankfully acknowledge the special support that I received from my former colleagues and supervisors of the Children's Hospital Kassel.
  You all created a very pleasant ambience at work that was exemplarily and even during the time of my leave you were always supportive.

- Special thanks to our cooperation partners from the Helmholtz Centre Munich and Institute of Bioinformatics, WWU Münster:
- Martin Irmler and Johannes Beckers, Intitute for Experimental Genetics (Munich)
- Kristian Unger, Research Unit of Radiation Cytogenetics (Munich)
- PD Dr. Eberhard Korsching and Dr. rer. nat. Kathrin Poos (Münster)

# 13. Publications

## 13.1. Congress contributions

miRNA im Osteosarkom – von der Zelllinie zum primären Tumormaterial“
Annual Meeting of the German Society for Pediatrics and Adolescents (DGKJ) and Society for Pediatric Oncology and Hematology (GPOH) , Hamburg 12th – 16th September 2012 (oral presentation)

miRNA deregulation as a critical milestone on the road to osteosarcoma development and metastasis?“ ; Annual Meeting of the European Musculo-Skeletal Oncology Society (EMSOS), Bologna, Italy; 15th – 16th Mai 2012 (oral presentation)

## 13.2. Original articles

Baumhoer D, Elsner M, Smida J, **Zillmer S**, Rauser S, Schoene C, Balluff B, Bielack S, Jundt G, Walch A, Nathrath M.: CRIP1 expression is correlated with a favorable outcome and less metastases in osteosarcoma patients. Oncotarget. 2011 Dec;2(12):970-5.

Baumhoer D, Smida J, **Zillmer S**, Rosemann M, Atkinson MJ, Nelson PJ, Jundt G, Luettichau IV, Nathrath M.: Strong expression of CXCL12 is associated with a favorable outcome in osteosarcoma. Mod Pathol. 2011 Dec 16.; doi: 10.1038/modpathol.2011.193

**Parts of this thesis have been published in this original article:**
Baumhoer D*, **Zillmer S***, Unger K, Rosemann M, Atkinson MJ, Irmler M, Siggelkow H., von Luettichau I, Jundt G, Smida J, Nathrath M:
MicroRNA profiling with correlation to gene expression revealed the oncogenic miR17-92 cluster to be up-regulated in osteosarcoma Cancer Genetics 2012 May; 205 (5): 212-9 (*both authors contributed equally to this work)

# APPENDIX

## 1. Materials, Kits and Reagents

### A. Cell culture (Material and Media)

- PBS, manufacturer PAA
- RPMI 1640 + L-Glutamine, GIBCO
- Trypsin 0,05% + EDTA 0,02%
- FBS, manufacturer PAA
- Ethanol 80%
- Culture flasks (T25, T75), manufacturer Greiner bio-one
- Cell counter Z1 , manufacturer Beckman
- Incubator
- Water bath, manufacturer Memmert
- Glass-Pipettes ( 5, 10, 25 ml), manufacturer Greiner bio-one
- Aspiration pipette (2ml), manufacturer Sarstedt
- Pipette tips (1-10; 1-100, 101-1000µl), TipOne Starlab
- Pipettes (1-10, 1-100, 101-1000µl), manufacturer Eppendorf
- Pipette controller (Accu jet pro)
- Microscope 1 (Axiovert 25), manufacturer Zeiss
- Microscope 2 (LH 50 A), manufacturer Olympus
- Multiwell-tissue culture plates (6-well; 24-well), manufacturer Falcon
- Conical Tubes (15 ml, 50 ml), manufacturer Greiner bio-one
- Aspiration vacuum pump (BVC 21), manufacturer Vacuubrand
- Laminar flow hood, manufacturer BDK Luft- und Reinraumtechnik
- Precision hot plate, manufacturer Harry Gestigheit GmbH
- Tissue culture flasks (25cm2 , 75cm2), Greiner bio-one

B. Migration and Invasion Assays

- BD Biocoat Invasion Chamber (Nr.: 354480), BD Biosciences
- Touludine blue 1%
- Methanole
- Ampuwa Water
- Canula/needle

## 2. Protocols

C. A. RNA-Extraction

- see protocol of mirVana miRNA Isolation Kit

D. Proliferation Assay

- Seed 1x$10^5$ cells in 25$cm^2$ cell culture flasks (for every cell line in duplicate)
- Count cells every 24 hours for 7 days using an automated cell counter (Beckman Coulter), for this harvest cells after 24h, 48h, 72h, 96h und 168h hours by trypsinization (for trypsinisation process, consult cell cultivation protocol)
- Calculate mean cell numbers for every cell line plot into a growth curve with logarithmic scaling
- Calculate doubling time

E. Matrigel® Invasion Assay

- Remove the package from -20°C storage and allow to come to room temperature
- Add around 500µl of warm (37°C) culture medium (RPMI, no FCS) to the interior of the inserts and bottom of wells. Allow to rehydrate for 2 hours in humidified tissue culture incubator, 37°C, 5% CO2 atmosphere.
- In the meantime: Harvest the cells (washing procedure with 5-6ml PBS and 1/10 of Trypsin 2-3 min, watch under microscope, neutralization with 5ml FCS 0,2%)
- Cell counting and re-suspension of 2,5x$10^4$ cells of each cell line and fill with medium to reach an amount of 350µl
- After rehydration, carefully remove the medium without disturbing the layer of Matrigel™™ Matrix on the membrane.

- Add chemoattractant (750µl 10% FCS) to the wells of a new BD Falcon 24-well-plate
- Transfer the inserts to these wells and add the cell suspension (350µl)
- Use sterile forceps to transfer the chambers. Be sure that no air bubbles are trapped beneath the membranes. This can be avoided by tipping the insert or chamber at a slight angle as it is lowered into the liquid.
- Incubate the BD BioCoat Matrigel Invasion Chambers for 48 hours in a humidified tissue culture incubator, at 37°C, 5% CO2 atmosphere.
- Observe under light microscope after 24hours and take pictures
- After incubation time aspirate the medium very carefully
- Transfer the inserts to a new 24-well-plate (wells filled with PBS) and swab the insert with a PBS-humidified cotton swab 2-3 times to remove the non-invading cells on the upper part of the membrane
- Fix the lower part with 600µl of Methanol for 2 minutes
- Transfer inserts to wells with Ampuwa water and wash for 2 minutes
- Transfer the inserts to well containing Touluidin blue 2% and stain for ca. 10min
- After rinse the inserts gently under water and remove remaining colour
- Allow the inserts to dry at 37°C for at least 1 hour
- Remove the membrane from the insert housing by inverting the insert and inserting the tip of a sharp scalpel blade through the membrane at the edge adjacent to the housing wall. Do not fully release the membrane from the housing but leave a very small point of attachment
- Use forceps to peel the membrane from the remaining point of attachment and place it bottom side down on a microscope slide on which a small drop of immersion oil has been placed. Place a second very small drop of immersion oil on top of the membrane
- Place a second slide or cover slip on top of the membrane and apply gentle pressure to expel any air bubbles
- Observe and photograph the invading cells under the microscope at approximately 40 - 200X magnifications depending on cell density. Count cells in several fields

F. Migration assay

- Harvest cells at approx. 80-85% confluence and count
- Resuspend the cells in 0,2% FCS
- Add chemoattractant (900µl of 10% FCS RPMI) to bottom of the wells
- Transfer inserts to the wells and add cells ($2,5x10^4$ )
- Incubate the chambers for 24 and 48 hours each (37°C and 5% $CO^2$)
- After each time point: aspirate medium
- Transfer chambers to a clean 24-well-plate with 600µl PBS in each well
- Use a PBS-humidified cotton swab to remove the non-migrating cells in the upper part of the inserts (2-times)
- Last part of procedure analogue to invasion assay (protocol above)

G. Fast-Forward Transfection of HOS cells (or similar) with miRNA/miRNAinhibitor (using HiPerFect Transfection Reagent)

- Shortly before transfection, seed 1.2 x $10_5$ cells per well of a 12-well plate in 1100µl of an appropriate culture medium containing serum and antibiotics. For the short time until transfection, incubate the cells under normal growth conditions (typically 37°C and 5% CO2).
- Cells may alternatively be seeded after step 3 of this protocol. Dilute 75 ng miRNA (or 10 times this amount of miRNA inhibitor) in 100µl culture medium without serum (this will give a final miRNA concentration of 5 nM after adding complexes to cells in step 5). Add 6µl of HiPerFect Transfection Reagent to the diluted miRNA/miRNA inhibitor and mix by vortexing. IMPORTANT: The amount of transfection reagent and miRNA/miRNA inhibitor required for optimal performance may vary, depending on the cell line and gene target. Incubate the samples for 5−10 min at room temperature (15−25°C) to allow the formation of transfection complexes. Add the complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes. Incubate the cells with the transfection complexes under their normal growth conditions and monitor gene silencing after an appropriate time (e.g., 6−72 h after transfection, depending on experimental setup). Change the medium as required. Note: The optimal incubation time for gene silencing analysis depends on the cell

type, the gene targeted, and the method of analysis. This can be determined by performing a time-course experiment. If working with fluorescently labeled miRNA/miRNA inhibitor, microscopic analysis should be performed 4−24 h after transfection.

- Optimizing miRNA/miRNA inhibitor and HiPerFect Transfection Reagent amounts
- As a general rule, optimization of transfection can be performed by varying miRNA/miRNA inhibitor and HiPerFect Reagent amount as follows:
- Use the reagent and nucleic acid amounts listed in the protocol. Use 0.2x lower and 2x higher miRNA/miRNA inhibitor amounts. Use 0.5x lower and 1.5x higher reagent amounts.
- The table below shows a pipetting scheme with 5 different conditions that is recommended to test when optimizing transfection (i.e., when determining the conditions that provide highest transfection efficiency and/or lowest cytotoxicity).

| | | | |
|---|---|---|---|
| Optimizing miRNA/miRNA inhibitor | 15 ng miRNA/miRNA (1 nM*)<br>6 µl HiPerFect | 75 ng miRNA/miRNA inhibitor (5 nM*)<br>6 µl HiPerFect | 150 ng miRNA/miRNA inhibitor (10 nM*)<br>6 µl HiPerFect |
| Optimizing reagent | 75 ng miRNA/miRNA inhibitor (5 nM*)<br>3 µl HiPerFect | 75 ng miRNA/miRNA inhibitor (5 nM*)<br>6 µl HiPerFect | 75 ng miRNA/miRNA inhibitor (5 nM*)<br>9 µl HiPerFect |

- As a starting point, it is recommened to use 75 ng miRNA/miRNA inhibitor and 6 µl reagent as stated in the protocol. The effect of using greater or lesser amounts of miRNA/miRNA inhibitor and HiPerFect Transfection Reagent can be observed using these combinations.

  Approximate values for a double-stranded, 21 nt miRNA/miRNA inhibitor molecule: ~20 µM miRNA/miRNA inhibitor is equivalent to approximately 0.25 µg/µl.