Reprogramming of tumor cells: Signaling events and phenotypes

Dissertation

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Zusammenfassung

Krebs ist eine Erkrankung, die sich durch unkontrollierte Zellvermehrung und durch die Fähigkeit der Zellen in entfernte Organe zu disseminieren, auszeichnet. Diese Eigenschaften der Krebszellen gehen auf genetische und epigenetische Unterschiede zurück, die man entdeckt, wenn normale und Krebszellen miteinander verglichen werden. Genetische Mutationen, die in Onkogenen und Tumorsuppressorgenen auftreten, sind die ursprünglichen Ursachen der zellulären Transformation (Lengauer et al., 1998; Vogelstein and Kinzler, 2004). Zu den Genveränderungen, die auf der DNA Sequenzebene auftreten, gesellen sich epigenetische Veränderungen, die die Expression von Onkogenen und Tumorsuppressorgenen, unabhängig von Mutationen in der primären DNA Sequenz, beeinflussen können und so zur zellulären Transformation beitragen (Esteller and Herman, 2001; Sharma et al., 2010). Obwohl in Krebszellen eine grosse Zahl von Mutationen gefunden werden kann, trägt nur ein kleiner Prozentsatz dieser Mutationen zur zellulären Transformation bei. Insgesamt wurden bisher etwa 140 Gene als "Treiber" identifiziert, Gene, die die Tumorigenese begünstigen, wenn sie durch Mutationen in ihrer kodierenden Region verändert sind (Vogelstein et al., 2013). Individuelle Tumoren weisen aber immer nur eine kleine Anzahl mutierter "Treibergene" auf. Die grosse Mehrheit der Mutationen in Tumorzellen betrifft "bystander" Gene, das sind Gene, die nicht ursächlich an der Tumorigenese beteiligt sind. Die Funktion der "Treibergene" kann weiter eingegrenzt werden; diese Gene beeinflussen die Aktivitäten von 12 Signaltransduktionswegen, die wiederum das zelluläre Schicksal, ihr Überleben und die Genomintegrität regulieren (Vogelstein and Kinzler, 2004; Vogelstein et al., 2013).

Die Wirkstoff-Forschung hat sich diese Einsichten zunutze gemacht und sich auf Zielstrukturen fokussiert, die mit Funktionen von Proteinen interferieren, die bei der Tumorigenese eine zentrale Rolle spielen. Die Strategie der therapeutischen Forschung, hat sich hat sich von der Entwicklung unspezifisch, zytotoxischer Substanzen zur Entwicklung selektiver Wirkstoffe

hin gewandelt; Wirkstoffe, die in der Lage sind, einen definierten molekularen Prozess zu inhibieren, der für das Wachstum und das Überleben der Krebszellen unabdingbar ist. Eine Vorreiterrolle haben dabei die niedermolekularen Substanzen gespielt, die spezifische Proteinkinasen hemmen, und beachtliche Fortschritte bei der Behandlung von Krebspatienten begründet haben. Der klinische Benefit dieser Therapien ist jedoch selbst innerhalb definierter Patientenkollektive sehr unterschiedlich und meistens auch zeitlich begrenzt. Intrinsische und erworbene Resistenz gegenüber diesen Medikamenten ist dafür verantwortlich (Kubinyi, 2003). Trotz der beachtlichen Fortschritte im Verständnis der molekularen Ursachen der Krebsentstehung und der daraus resultierenden "gezielten" Wirkstoffe, sind weitere Durchbrüche unbedingt notwendig.

Die zelluläre Differenzierung und die zelluläre Transformation wird von epigenetischen Modifikationen determiniert. Der Vergleich von pluripotenten Zellen und differenzierten Zellen hat gezeigt, dass der Differenzierungsvorgang von einem globalen Chromatin "remodelling" begleitet ist, das sich in einem fortschreitenden Übergang von einer offenen Chromatinkonfiguration in eine kompaktere Konfiguration manifestiert. Histon modifizierende Enzyme und Chromatin "remodellers" spielen hierbei eine zentrale Rolle. Diese epigenetischen Modifikationen beeinflussen und stabilisieren die Genexpression über lange Intervalle und üben Einfluss auf die Zellteilung, die Apotoseregulation und die Empfindlichkeit der Zellen gegenüber extrazelluären Signalmolekülen aus. Es ist wahrscheinlich, dass die Signale, die von Onkogenen ausgehen, ein epigenetisches Muster diktieren, das als Grundlage der Stabilität und der transformierten Phänotypen von Tumorzellen angesehen werden kann. Da diese epigenetischen Stadien aber nicht absolut festgelegt sind, und Moleküle zur Verfügung stehen, die diese Stadien beeinflussen und verändern können, ist es denkbar, dass Regulatoren epigenetischer Stadien zur Krebstherapie herangezogen werden können.

Die Reprogrammierung ist ein zellulärer Prozess, der zunächst grundlegende Einsichten in die Reversibilität der Differenzierung somatischer Zellen verschaffte. Somatische Zellen eines erwachsenen Organismus konnten dazu

bewegt werden, wieder weniger differenzierte Eigenschaften anzunehmen und wurden schliesslich sogar in das Stadium pluripotenter Stammzellen zurückversetzt. Dieser Prozess der Dedifferenzierung von somatischen Zellen in das Stadium von induzierten pluripotenten Stammzellen, iPS, kann durch die Funktionen von vier definierten Transkriptionsfaktoren, Oct4, Sox2, Klf4 und Myc, eingeleitet werden, die eine Reihe von epigenetische Modifikationen in den somatischen Ausgangszellen bewirken (Anokye-Danso et al., 2011; Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Ganz ähnliche Auswirkungen auf differenzierte, somatische Zellen hat die Expression einer kleinen Familie von micro RNAs. Das miR302/367 cluster ist ebenfalls in der Lage, induzierte pluripotente Stammzellen, iPS, hervorzubringen. Die Reprogrammierung von somatischen Zellen durch das miR302/367 cluster resultiert aus ihrem Einfluss auf epigentische Modifikatoren, Zellzyklus regulierende Gene und der Inhibition des epithelialen zum mesenchymalen Übergangs (Lin and Ying, 2013).

Wir haben untersucht, ob der Prozess der Reprogrammierung auch das globale Genexpressionsmuster, und so die epigenetische Basis der Tumorigentät von Glioblastom Zellen, verändern kann. Er kann so möglicherweise zur Verringerung der Transformations-spezifischen Phänotypen dieser Zellen beitragen. Glioblastoma multiforme (GBM) ist eine häufige und aggressive Form von Hirntumoren des Menschen. Die durchschnittliche Überlebenszeit von GBM Patienten beträgt lediglich 15 Monate nach Stellung der Diagnose. Ähnlich wie in anderen Tumorentitäten, wurden in GBM Zellen genetische und epigenetische Veränderungen festgestellt. Genetische Mutationen beeinflussen das Wachstum dieser Zellen, verringern ihre Empfindlichkeit gegenüber apoptotischen Signalen, befähigen die Zellen pro-angiogene Faktoren auszuscheiden und invasive Eigenschaften zu entfalten. Epigenetische Veränderungen in GBM Zellen regulieren die Expression von Onkogenen und Tumorsuppressorgenen und verstärken die Malignität der Tumoren (Nagarajan and Costello, 2009).

Globale Expressionsanalysen haben ergeben, dass Stammzellen, induzierte pluripotente Stammzellen, iPS, differenzierte Zellen und Tumorzellen eines

Gewebes in ihren Genexpressionsmustern zum Teil überlappen (Ghosh et al., 2011). Es wurde gezeigt, dass die Tumorzellen im Muster der Genexpression den iPS Zellen ähnlicher sind, als den somatischen Zellen ihres Ursprungsgewebes (Ghosh et al., 2011). Weiterhin wurde die Expression embryonaler Stammzellmarker, einschliesslich der vier Reprogrammierungsfaktoren, in Tumorzellen gefunden, und ihre Expression kann direkt mit dem Tumorgrad und der Prognose korreliert werden (Ben-Porath et al., 2008; Schoenhals et al., 2009a).

In dieser Arbeit habe ich GBM und Brusttumor Zellen untersucht und die endogene Expression der Reprogrammierungsfaktoren gemessen. Meine Ergebnisse lassen vermuten, dass die de-differenzierten Phänotypen dieser Tumorzellen mit der Expression dieser Faktoren korreliert sind. Ich habe in diesen Zellen das miR 302/367 cluster zur Expression gebracht und den Einfluss des clusters auf die Signalwege und die transformierten Eigenschaften der Tumorzellen untersucht. Zu diesem Zweck wurde das miR 302/367 cluster in einen lentiviralen Gentransfervektor integriert und GBM Zellen mit den rekombinante Lentiviren infiziert. Die miR Expression beeinflusst die Eigenschaften der Tumorzellen drastisch und Veränderungen auf der Ebene der Genexpression, des zellulären Signalings und der transformierten Phänotypen der Zellen konnten beobachtet werden.

Zur Charakterisierung der Effekte der Expression des miR 302/367 cluster in U87MG Zellen habe ich zunächst eine globale Proteinanalyse durchgeführt, in der ich das gesamte Proteinexpressionsmuster der prentalen U87MG Zellen mit dem der miR exprimierenden Zellen verglich. Diese Analysen beruhten auf der "SILAC" Methodologie (stable isotope labeling by amino acids in cell culture). In diesen Experimenten werden U87MG Zellen in einem Medium mit den natürlich vorliegenden Aminosäuren kultiviert und die U87MG-miR 302/367 exprimierenden Zellen mit Arginin und Lysin, in die schwere Kohlenstoff und Stickstoff Isotope inkorporiert wurden. Proteinlysate der beiden Zell-Linien werden gemischt, auf ein-dimensionalen Polyacrylamidgelen werden die Proteine nach ihrer Grösse aufgetrennt, und einzelne Grössenfraktionen werden isoliert. Diese Proteine werden mit

Trypsin verdaut, und ihre Fragmente werden durch Massenspektometrie identifiziert. Quantitative Auswertungen zeigen dann die relative Menge individueller Proteine in den beiden Zellpopulationen an. Ich konnte feststellen, dass eine Vielzahl von Proteinen in ihren Expressionniveaus durch die miR stark beeinflusst ist. Mehr als 600 Proteine liegen in den miR 302/367 exprimierenden Zellen in verminderter Konzentration vor und mehr als 1600 Proteine sind in ihrer Konzentration erhöht. Dabei ist die verminderte Expression der Komponenten des PI3K/AKT Signalweges besonders auffällig. Die katalytische Untereinheit der PI3K und die AKT wurden auch in Western Blot Experimenten untersucht und die Verringerung ihrer Expression wurde bestätigt. Die Resultate dieser Protein Expressionanalysen sind nicht unerwartet, da in den mRNA Sequenzen der PI3K mehrere Bindungsstellen für die Mitglieder des miR 302/367 cluster gefunden wurden. Unter den Proteinen, die eine verstärkte Expression in miR 302/367 cluster exprimierenden Zellen aufweisen, sind eine Reihe von Tumorsuppressorgenen, z.B. CD9, MYBBP1A, PEA15 und UCHL1.

Eine zweite Kategorie von Proteinen, die durch die Expression von miR 302/367 in U87MG Zellen unterdrückt wird, sind die „CPM modules“ (core modules, PrC modules und myc modules), sowie die Reprogrammierungsfaktoren Oct3/4, Sox2 und Klf4. Diese Faktoren sind in ihrer Expression mit grosser Wahrscheinlichkeit von der Aktivität der AKT abhängig. Ich schliesse das aus der Tatsache, dass sowohl die Expression von miR 302/367, als auch die pharmakologische Inhibition von AKT durch LY294002 die Repression dieser Proteine bewirken können. Schliesslich habe ich die Expression und die Sekretion pro-inflammatorischer Zytokine in Abhängigkeit von der miR 302/367 gemessen. Ich konnte feststellen, dass die mRNA Expression von IL6, IL8 und MCP1 in den miR exprimierenden Zellen deutlich reduziert ist. Zytokin Array Experimente bestätigten diese Beobachtung auf der Ebene der sekretierten Proteine.

Auf der Ebene der Signaltransduktion bewirkte die Expression von miR 302/367 nicht nur die Inhibition des PI3K/AKT signalings, sondern auch die De-Aktivierung von Stat3. Diese Inhibition ist auf den Effekt der miR auf eine

Stat3 aktivierende Kinase zurückzuführen, da die Stat3 Proteinmenge in den Zellen von der miR nicht verändert wird.

Die weitgehenden Auswirkungen der miR Expression auf das globale Proteinexpressionsmuster und so zentrale Signalwege wie PI3K/AKT und Stat3, lassen erwarten, dass sich diese Veränderungen auch in phänotypischen Charakteristika der Zellen manifestieren. Aus diesem Grund habe ich zunächst die Wachstumsraten der U87MG Zellen mit den U87-miR 302/367 Zellen verglichen, konnte aber in zweidimensionaler Kultur keinen Unterschied feststellen. Ein deutlicher Unterschied trat jedoch zutage, als ich das "Substrat unabhängige Wachstum" der beiden Zellpopulationen miteinander verglich. Die parentalen U87 Zellen sind in der Lage, grosse Kolonien in Weichagar auszubilden, wärend die miR exprimierenden Zellen diese Fähigkeit fast vollkommen verloren haben. Die Ausbildung von Kolonien ist ein klassischer Transformationsparameter und die Ergebnisse dieser Experimente wiesen zum ersten Mal darauf hin, dass die miR Expression eine solche Eigenschaft unterdrücken kann. Diese Beobachtung wurde weiter verfolgt in Experimenten, in denen die Invasivität der Zellen untersucht wurde. Während U87MG Zellen eine hohe Invasivität im "Boyden chamber" Assay aufweisen, haben die U87-miR Zellen diese Fähigkeit eingebüsst. Diese Veränderung der Invasivität ist möglicherweise auf den Verlust der IL8 Sekretion zurückzuführen.

Der Übergang von einem epithelialen zu einem mesenchymalen Phänotyp hat in der Tumorforschung grosse Aufmerksamkeit gewonnen, da er mit der Progression lokaler, primärer Tumoren zu aggressiven metastasierenden Tumoren assoziiert wurde. EMT (epithelial to mesenchymal transition) beschreibt den Übergang von polarisierten, immobilen Epithelialzellen zu apolaren, beweglichen mesenchymalen Zellen(Gunasinghe et al., 2012; Wang and Shang, 2013; Yilmaz and Christofori, 2009). Diese mesenchymalen Zellen können Metastasen ausbilden, werden gegenüber Chemotherapeutika resistent und nehmen Eigenschaften an, die den Krebsstammzellen ähneln. Ich habe untersucht, ob die miR Expression Einfluss auf das Erscheinungsbild der U87MG Zellen hat, und ob der meschenchymale Charakter davon

beeinflusst werden kann. Ich fand, dass der mesenchmale Phänotyp der Zellen deutlich reduziert ist, und die Zellen epithelial erscheinen, also der der EMT umgekehrte Prozess, MET, induziert wird. Der Übergang von einer mesenchymalen zu einer epithelialen Zellmorphologie ist begleitet von verminderter Vimentin und verstärkter E-Cadherin Expression.

Auch in vivo wurden deutliche Veränderungen der Zelleigenschaften festgestellt. Die parentalen U87MG Zellen bilden nach Transplantation in Nacktmäuse schnell wachsende Tumoren an der Implantationsstelle aus, und metastasieren effizient in die Leber der Tiere. Kommen U87MG-miR Zellen zum Einsatz, ist das Tumorzellwachstum in den transplantierten Mäusen und die Ausbildung von Metastasen in der Leber dieser Tiere sind fast vollkommen verschwunden.

Meine Ergebnisse zeigen, dass die Expression des miR 302/367 clusters zu einer dramatischen Reprogrammierung der Tumorzellen führt. Diese Reprogrammierung übt zunächst Einfluss auf das gesamte Muster der Genexpression aus, inhibiert die onkogenen PI3K und Stat3 Signalwege und verändert dadurch die Eigenschaften der Zellen in einer äusserst günstigen Art und Weise. Die Transformationsphänotypen werden in vitro unterdrückt und die Tumorigenität in vivo ist fast vollkommen verschwunden. Die Reprogrammierung bietet sich daher als vielversprechende Option der Tumortherapie an.

Abstract

Cancer is a disease characterized by uncontrolled cell growth and the capacity to disseminate to distant organs. The properties of cancers are caused by genetic and epigenetic alterations when compared to their normal counterparts. Genetic mutations occur in oncogenes and tumor suppressor genes and are the initial drivers of cellular transformation (Lengauer et al., 1998; Vogelstein and Kinzler, 2004). In addition, epigenetic alterations, which influence the expression of oncogenes and tumor suppressor genes independently from sequence alterations, are also involved in the transformation process (Esteller and Herman, 2001; Sharma et al., 2010). Genetic alterations and epigenetic regulatory signals cooperate in tumor etiology.

Glioblastoma multiforme (GBM) is a frequent and aggressive malignant brain tumor in humans. The median survival of GBM patients is about 15 months after diagnosis. Like in other cancers, genetic and epigenetic alterations can be detected in GBM. Genetic alterations in GBM affect cell growth, apoptosis, angiogenesis, and invasion; however, epigenetic alterations in GBM also affect the expression of oncogenes or tumor suppresser genes that increase tumor malignancy (Nagarajan and Costello, 2009).

Reprogramming is a cellular process in which somatic cells can be induced to assume the properties of less differentiated stem cells. This process can be mediated through epigenetic modifications of the genome of somatic cells by the action of four defined transcription factors (Oct4, Sox2, Klf4 and Myc) or by the action of the miR 302/367 cluster (Anokye-Danso et al., 2011; Takahashi and Yamanaka, 2006; Takahashi et al., 2007) and result in the generation of induced pluripotent stem cells (iPS cells). Reprogramming of somatic cells by the miR 302/367 cluster can generate non-tumorigenic iPS cells through the inhibition of the epithelial to mesenchymal transition (EMT), cell cycle regulatory genes and epigenetic modifiers (Lin and Ying, 2013).

Global gene expression pattern analysis revealed that stem cells, iPS cells, differentiated cells and cancer cells share fractions of expressed genes (Ghosh et al., 2011). When cancer cells, stem cells including iPS cells, and their normal tissue cells were compared, relative distance measurements showed that the gene expression patterns of cancer cells were more similar to that of stem or iPS cells than to the pattern of their corresponding normal cells (Ghosh et al., 2011). Moreover, it has been reported that the expression of embryonic stem cell marker genes, including the four reprogramming factors, are associated with tumor grade and unfavorable

prognosis (Ben-Porath et al., 2008; Schoenhals et al., 2009a). I characterized GBM and breast cancer cell lines and measured the expression of reprogramming factors. The studies indicate that cancer cells assume de-differentiated phenotypes through the expression of reprogramming factors and epigenetic alterations.

I have also investigated the possibility to affect transformation parameters of tumor cells through the influence of mediators of reprogramming. The shift in gene expression from a pattern indicative of tumor cells to a pattern more closely related to stem cells could possibly be used to suppress transformation phenotypes and serve as a therapeutic strategy (Nishikawa et al., 2012). Recent results indicate that epigenetic modifications induced by reprogramming components are able to generate iPS cells from tumor cells and suppress their tumorigenicity (Carette et al., 2010; Liang et al., 2012; Lin et al., 2008; 2010; Miyoshi et al., 2010; Utikal et al., 2009).

I exposed tumor cells to the reprogramming effects of the miR 302/367 cluster and investigated the consequences of miR 302/367 cluster expression with respect to cellular signaling events and transformation phenotypes. Furthermore, I investigated the molecular mechanisms that confer the reprogramming potential of the miR 302/367 cluster in GBM cells and assessed the potential therapeutic benefit. As models for reprogramming of GBM cells and its effects on de-differentiation phenotypes and suppression of tumorigenicity, I used the GBM cell lines U87MG, SKGM3 and U373. I introduced the miR 302/367 cluster, encoded by a lentiviral gene transfer vector, into the GBM cell lines and expressed the miR 302/367 cluster in a conditional fashion. I monitored the effects of the miR 302/367 cluster on oncogenic signaling pathways and tumor phenotypes *in vitro* and *in vivo*. I found that the miR 302/367 cluster modified the GBM microenvironments and inhibited colony formation and metastasis formation upon suppression of PI3K/AKT and STAT3 signaling. It also suppressed epithelial to mesenchymal transition (EMT) *in vitro*. The expression of the miR 302/367 cluster inhibited U87MG tumor growth and liver metastasis in humanized, immunodeficient mice (NSG).

In conclusion, the reported role of the miR 302/367 cluster and our findings suggest that the functional effects of the miR 302/367 cluster expression can favorably influence the properties of tumor cells and can potentially become useful in cancer therapy.

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1. Introduction

1.1. Cancer cells are characterized by de-differentiated phenotypes

Hallmarks of cancer initially included uncontrolled cell growth and the potential to spread to distant organs. In the meantime, sustained proliferative signaling, evasion of growth suppressors, invasion and metastasis, replicative immortality, the induction of angiogenesis and resistance to cell death have been added (Hanahan and Weinberg, 2000). These hallmarks describe the acquired functional capabilities of cancer cells. Together with genomic instability of cancer cells and the intercellular communication of normal and transformed cells in the cancer tissue microenvironment, they provide explanations for most events observed in tumor initiation and progression (Hanahan and Weinberg, 2011).

Stem cells are non-differentiated cells, they are capable of self-renewal through successive mitoses and thus maintain the stem cell pool, and they are able to respond to particular stimuli to differentiate into specialized cell types. The differentiation process is accompanied by a loss of lineage potential and increasing functional restrictions. Adult stem cell e.g. divide into fully differentiated daughter cells during tissue repair and during normal cell turnover. Differentiation changes cell size, shape, membrane potential, metabolic activity and the cellular responsiveness to signals. These changes are largely due to highly controlled modifications in gene expression patterns mediated by epigenetic control mechanisms.

The discovery that the expression of four defined transcription factors, the reprogramming factors Oct4 / Sox2 / KLF4 / cMyc, are able to revert the state of fully differentiated cells into pluripotent stem cells (iPSCs), has been a major breakthrough in our appreciation of epigenetic biological processes (Takahashi et al., 2007). During reprogramming, imprinted epigenetic markers are erased and re-established in a way which promote pluripotent stem like cells (Lewitzky and Yamanaka, 2007; Yamanaka, 2008).

Cancer stem cells have been analogously defined. They have stem cell characteristics, such as self-renewal and a partial differentiation potential, and might be the basis for cancer relapse upon treatment, cancer heterogeneity and metastasis formation (Reya et al., 2001). The cancer stem-like cells also exhibit de-differentiated phenotypes (Reya et al., 2001).

1.1.1. Expression of stem cell genetic programs in cancers

Cancer cells share gene expression patterns with embryonic stem cells, iPS cells and differentiated cells (Ghosh et al., 2011). Global gene expression profiling showed that cancer cell gene expression overlaps with stem cell gene expression and with gene expression patterns found in differentiated cells. The gene expression patterns of cancer cells are more similar to stem cells than to patterns found in differentiated cells (Ghosh et al., 2011). Figure 1 shows the relative distance in the profiles between cancer cells and stem cells or cancer cells and differentiated cells. Cancer stem cells are thought be cells with stem cell properties in cancer tissue, all cancer cells seem to express stem cell related genes. This indicates that not only cancer stem cells, but the bulk of the cancer cells have de-differentiated phenotypes.

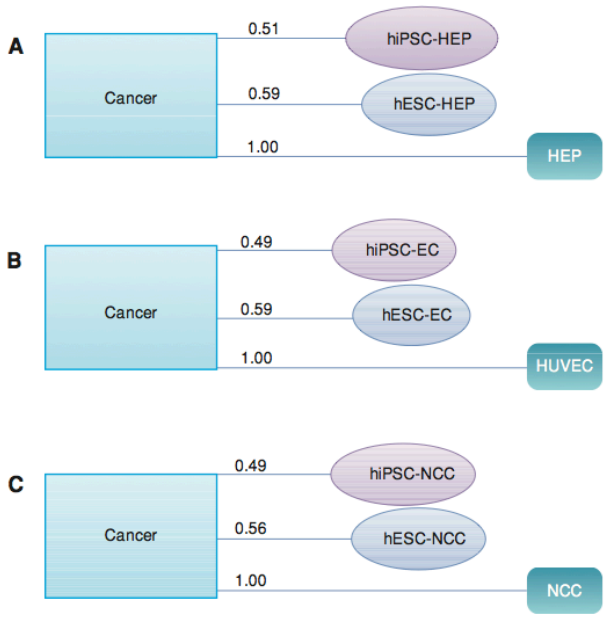


Figure 1. Relative distance measures between cancer cells versus hESC- and hiPSC- and primary tissues. Global gene expression analysis performed than Euclidean distance was measured by using the statistical software package SPSS (IBM). Relative distance measures between (A) cancer cells versus hESC- and hiPSC-derived hepatocytes and primary hepatocyte cells; (B) cancer cells versus hESC- and hiPSC-derived endothelial cells and HUVEC; and (C) cancer cells versus hESC- and hiPSC-derived neural crest cells (Ghosh et al., 2011).

1.1.2. Expression of embryonic stem cell markers in cancer cells

In 2009, Schoenhals et al. analyzed expression of embryonic stem cell marker genes in tumor tissues. They compared the expression of reprogramming factors, which are known to induce pluripotency in somatic cells, by using publicly available gene expression data, including the Oncomine Cancer Microarray database, Amazonia

database and RAGE database (Schoenhals et al., 2009a). In the study, they found that at least 1 out of the 4 reprogramming factors were over-expressed in 3/4 hematological cancers and in 15/40 solid tumors (Schoenhals et al., 2009a). Figure 2 indicates the over-expression of the reprogramming factors in human tumor types when compared to their normal tissues. These results corroborate the observation that tumor cells exhibit stem like properties and that endogenous expression of genes encoding the reprogramming could participate in the de-differentiation process of normal cells and the induction of tumorigenesis.

Tissue	Oct4	Sox2	KLF4	c-MYC
Lymphoma	no	no	no	yes
Leukemia	yes	no	yes	yes
Myeloma	no	no	yes	yes
Adrenal	no	no	no	no
Bladder	yes	yes	no	no
Blood	no	no	no	no
Brain	yes	yes	yes	yes
Breast	no	no	no	yes
Cervix	no	no	no	no
Chondrosarcoma	no	no	no	no
Colon	no	yes	no	yes
Endocrine	no	no	no	no
Endometrium	no	no	no	no
Esophagus	no	no	no	no
Gastric	no	no	no	no
Head-Neck	no	no	no	yes
Liver	no	yes	no	no
Lung	yes	yes	no	yes
Melanoma	no	no	no	no
Mesothelioma	no	no	no	no
Multi-cancer	no	yes	no	no
Muscle	no	no	no	no
Neuroblastoma	no	no	no	no
Oral	no	no	no	no
Others	no	no	no	no
Ovarian	yes	no	no	no
Pancreas	yes	no	no	yes
Parathyroid	no	no	no	no
Prostate	yes	yes	yes	yes
Rectum	no	no	no	no
Renal	yes	no	no	yes
Salivary-gland	no	no	no	yes
Sarcoma	no	no	no	no
Seminoma	yes	yes	no	yes
Skin	no	no	no	no
Testis	yes	yes	yes	no
Thyroid	no	no	no	no
Uterus	no	no	no	no

Figure 2. Over-expression of reprogramming factors in human tumor types. In 2009, Schoenhals et al. analyzed expression of reprogramming factors in 40 different human tumor types. It shows that most of tumors over-express at least 1 of 4 reprogramming factors endogenously when compared to their normal tissues (Schoenhals et al., 2009a).

1.1.3. Association of stem cell phenotypes with tumor grade and prognosis

It has been reported that over-expression of stem cell related genes are highly associated with tumor grade and prognosis (Ben-Porath et al., 2008; Hassan et al., 2009; Schoenhals et al., 2009a). Global gene expression analysis in tumor tissues of different grades showed that high-grade tumors over-expresses stem cell related genes (Ben-Porath et al., 2008; Hassan et al., 2009). Especially, high-grade brain tumors strongly expressed reprogramming factors and their target genes; in addition the expression of reprogramming factors in brain tumors is associated with an unfavorable prognosis for the brain tumor patients (Ben-Porath et al., 2008; Schoenhals et al., 2009a).

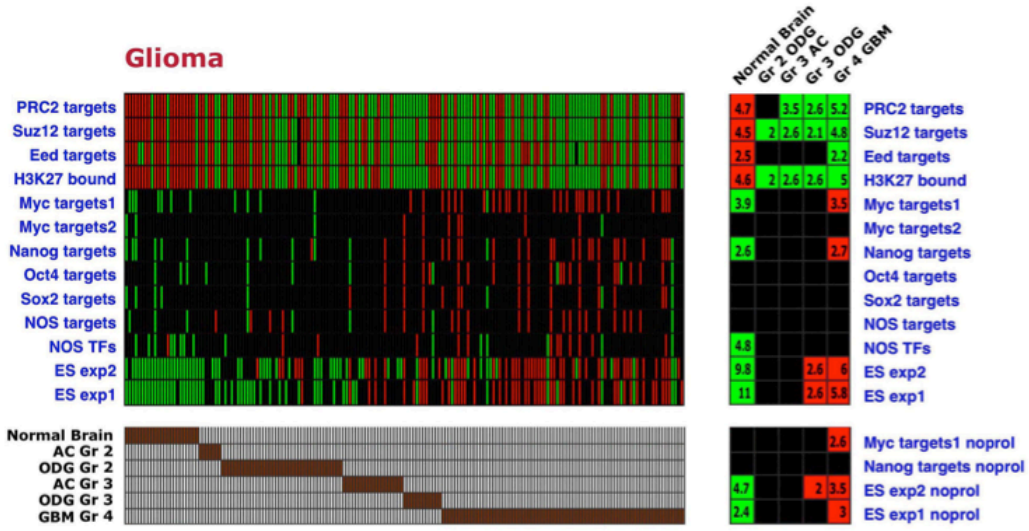


Figure 3. Expression of reprogramming factor target genes and ES cell expressed genes in glioma patient samples. Ben-Porath and collaborators performed gene expression analysis with different grades of glioma patient samples and normal brain tissues. In this study, they categorized different gene sets that are targeted by each or combined reprogramming factors or ES expressed gene sets. The global gene analysis data shows that reprogramming factor target genes and ES cell expressed genes are over-expressed in high-grade glioma patient samples when compared to low-grade glioma patient samples and nomal brain tissues (Ben-Porath et al., 2008).

Figure 3 shows that the reprogramming target genes (Oct4, Myc, Nanog, Sox2 and NOS; Nanog, Oct4 and Sox2 targets) and ES cell expressed genes are highly over-expressed in grade 4 glioma (glioblastoma; GBM) patient samples when compared to low-grade glioma patient samples. NANOG and Myc target genes and ES cell expressed genes are strongly over-expressed in glioblastoma patient samples when compared to normal brain tissues (Figure 3). Interestingly, markers of epigenetic regulators such as PRC2, Suz12 and H3K27 bound genes are also over-expressed in glioma patients. This indicates that glioma formation is not only due to genetic

aberrations, but also caused by epigenetic changes (Figure 3). Clinical data show that the high expression of cMyc in brain tumor patients is correlated with short survival intervals of brain tumor patients (Schoenhals et al., 2009a; Shai et al., 2003). These data suggest that the expression of reprogramming factors and genes indicative for stem cells are strongly associated with brain tumor aggressiveness.

1.2. Epigenetic and cancer therapy

Although cells of tissues and organs have the same genetic information, there is a big difference in function and appearance. This is due to different expression of gene sets in different tissue cells. The specialized gene set expressions are developed during stem cell or progenitor cell differentiation. Epigenetics is a compound word with Epi- , which means above, and genetics. Epigenetics is the study of heritable changes in gene activity, which are not caused by changes in the DNA sequence. Even cellular differentiation is raised without changing of DNA sequence, obtained phenotypic trait through the cellular differentiation is inherited to daughter cells via cell division. Epigenetic is the inheritance process of the obtained phenotypic trait to daughter cells regardless of the gene sequences.

1.2.1. The relationship between epigenetic regulation of gene expression and cancer

An epigenetic gene regulatory system should be heritable, self-perpetuating and reversible (Bonasio et al., 2010). Epigenetic modifications mainly occur through the interplay of three factors: DNA methylation, histone modification and non-coding RNAs (Figure 4). The factors have various molecular mechanisms to regulate epigenetic alterations in the genome through chromatin remodeling or direct alterations on genomic DNA. The factors do not function in isolation, but in a highly coordinated fashion. In chromatin remodeling, DNA methylation and histone modifications are the main contributors. In DNA methylation, it has been reported that non-coding RNA, RNA not translated into proteins, is regulating DNA methylation, DNA imprinting and chromatin alterations (Bird, 2002).

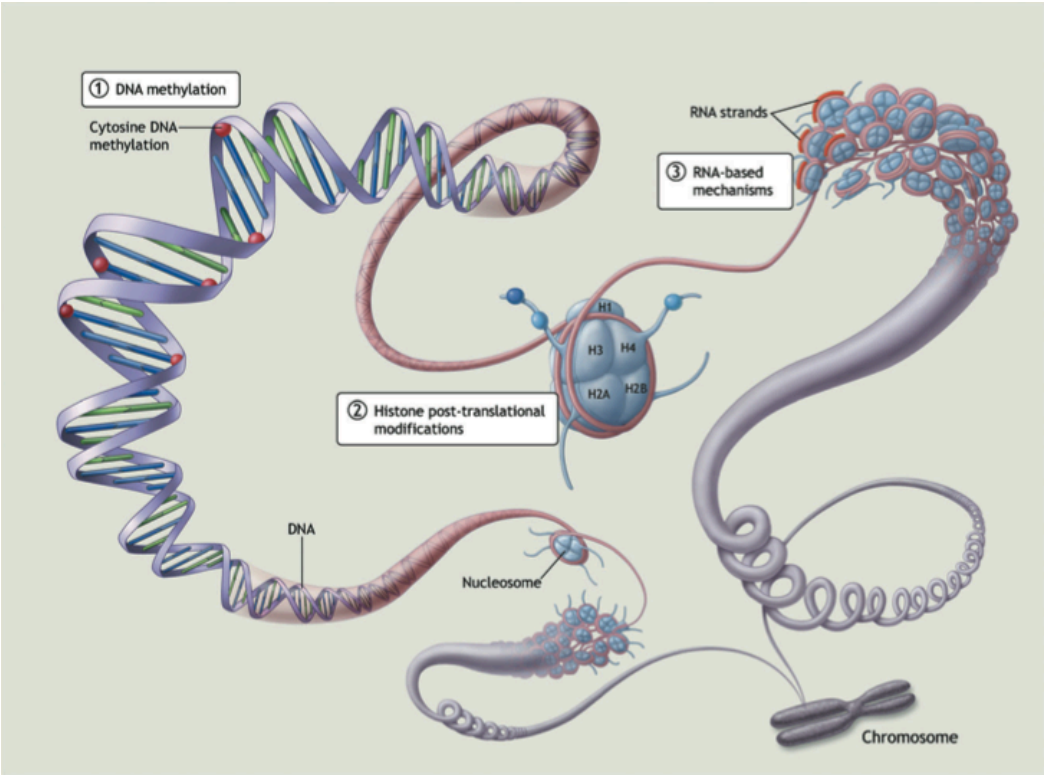


Figure 4. Epigenetic mechanisms of gene regulation. Epigenetic mechanisms are regulated by three main factors: DNA methylation, Histone modifications and RNA-based mechanisms. (Matouk and Marsden, 2008).

1.2.1.1. Promoter DNA methylation

A most important factor of epigenetic alteration is DNA methylation. DNA methylation has been studied in developmental processes and controls gene activity and the architecture of the nucleus of the cell. Because DNA methylation is inherited over multiple cell generations and persistently regulates gene expression, it was suspected early on to also participate in tumorigenesis.

The main function of DNA methylation in normal cells is concerned with gene repression, control of cellular differentiation and development, maintenance of the conformation and integrity of the chromosome, imprinted genes, inactivation of X chromosomes and genome defense against surreptitious mobile genetic elements. In mammalians, DNA methylation occurs in cytosines that precede guanines, also called CpG dinucleotides.

Each nucleotide can be combined with several chemical bonds. The CpG dinucleotide is a combination of cytosine and guanine via a phosphodiester bond. In humans, CpGs are rare in 98% of the genome. However, in 1 ~ 2% of the human genome, specific regions are present, which are CpG-rich. They are called CpG islands. CpG islands are often located around the promoter regions of housekeeping genes or other

gene frequently expressed in a cell. These islands are usually not methylated in normal cells, and results in the expression of genes that are related with normal cell functions. In these cases, the CpG islands are protected from methylation and the associated promoter regions are active. Near by genes are not expressed when the promoter is methylated. However, there are two exceptions concerning the non-methylation of CpG islands in normal cells: genes on X chromosomes and imprinted genes. In these cases, most of the CpGs in the CpG islands are methylated and the methylation is strongly associated with translational suppression. These processes occur by the action of maintenance methyltransferase.

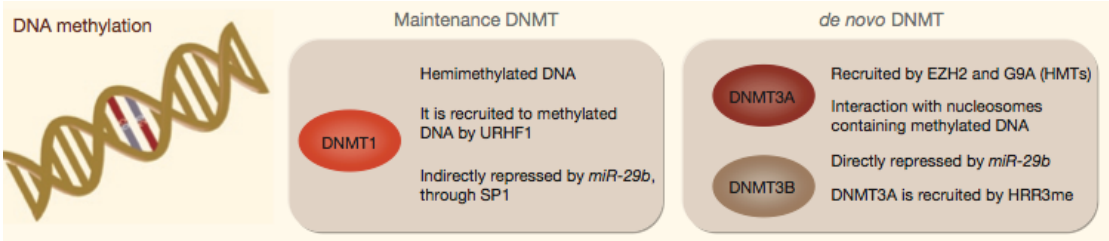


Figure 5. DNA methyltransferases and their main functions. The DNMT family of enzymes that catalyze the transfer of a methyl group from SAM to DNA regulates DNA methylation. Generally, DNMT classified into maintenance DNMTs (DNMT1) and de novo DNMTs (DNMT3A and DNMT3B) (Portela and Esteller, 2010).

There are three main enzymes, which regulate DNA methylation: DNA methyl transferase (DNMT) -1, DNMT-3A and DNMT-3B (Chen and Riggs, 2011). DNMT catalyze the transfer of a methyl group to DNA by using S-adenosyl methionine (SAM) as the methyl donor (Figure 5). DNMT1 is the most abundant DNA methyl-transferase in mammalian cells. The main function of DNMT1 is the maintenance of DNA methylation in mammalian cells. DNMT1 methylates hemimethylated CpG dinucleotides in the mammalian genome. DNMT-3A and 3B mainly regulate *de novo* DNA methylation in dividing cells (Jones and Liang, 2009). These enzymes methylate hemimethylated and unmethylated CpG at the same rate.

DNMTs catalyze the transfer of methyl groups onto DNA. *De novo* methyltransferases introduce methyl group (CH_3) onto CG sites, which were not previously methylated on the parental template strands of DNA. Figure 6 shows the role of DNMTs in *de novo* DNA methylation during cell division. All DNMTs are shown to possess *de novo* methylation activity, but DNMT1 is very inefficient in *de novo* methylation. Demethylases remove methyl groups to create unmethylated CG sites. Once a DNA methylation pattern is carved by *de novo* methyltransferases and demethylases, it is maintained during DNA replication by maintenance DNA methyltransferase (DNMT1). The enzyme copies the DNA methylation pattern from

the template strand to the unmethylated daughter nascent strand (Chen and Riggs, 2011; Day and Sweatt, 2010; Okano et al., 1999; Szyf, 2005) (Figure 6).

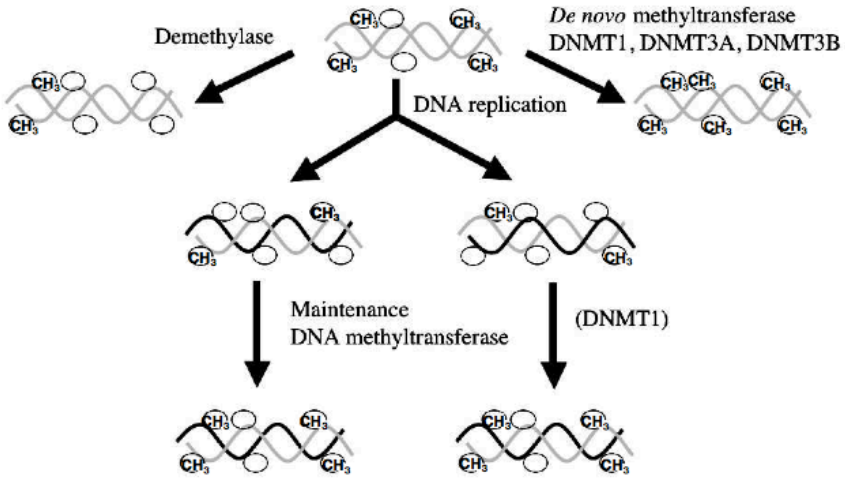


Figure 6. *de novo* DNA methylation by DNMTs during cell division. DNMTs regulate *de novo* DNA methylation during cell division (Szyf, 2005).

1.2.1.2. Histone modifications

Histones have been known as highly positively charged proteins in eukaryotes that package and order the DNA into structural units called nucleosomes. Nucleosomes, the basic unit of chromatin, are composed of an octamer of the four core histones: H2A, H2B, H3, H4 (Luger et al., 1997). These core histones consist of globular C-terminal and unstructured N-terminal tails (Figure 7) (Gräff and Mansuy, 2008). Most of histone modifications occur on the histone N-terminal tails.

Histone modifications have been reported that effect on DNA transcription, repair, replication, and chromatin condensation (Bannister and Kouzarides, 2011; Fischle et al., 2003). Until now, at least 8 histone modifications have been identified, namely acetylation, methylation, demethylation, phosphorylation, ubiquitination, sumoylation, ADP-ribosylation, deamination, tail clipping and proline isomerization (Bannister and Kouzarides, 2011). Histone modification research is rapidly progressing and it is likely that these modifications are acting in a combinatorial manner.

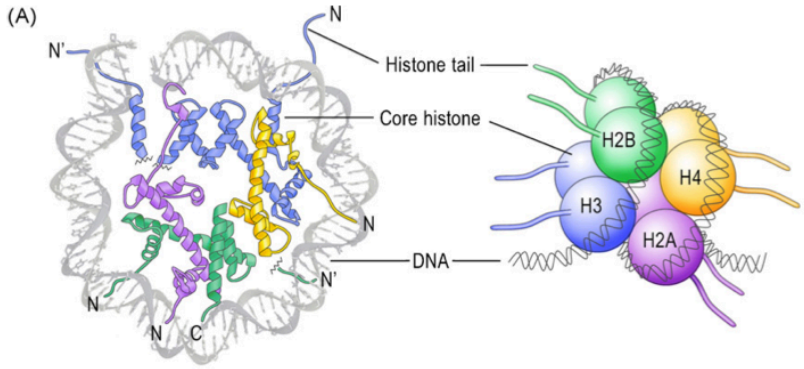


Figure 7. Structure of nucleosomes and histones. Nucleosomes (left) are consisted with 4 pairs of histones (H2A, H2B, H3, H4) (right) and DNA. These histones have unstructured N-terminal tails and histone modifications occur on these N-terminal tails (Gräff and Mansuy, 2008).

Histones are directly concerned with chromatin remodeling, condensed and loosened chromatin structure. The structural dynamics of chromatin by histone modifications influence gene expression because chromatin remodeling regulates the accessibility of transcription factors to the genes. When the chromatin has a condensed chromatin structure (inaccessible chromatin), the recognition sequence of transcription factors is shielded and results in silencing of gene expression or binding of transcriptional repressor on the methylated DNA. However, if the chromatin has a slackened chromatin structure (accessible chromatin), the recognition sequence of transcription factors is opened and results in gene induction due to binding of transcription factors to the promoter of target genes (Figure 8).

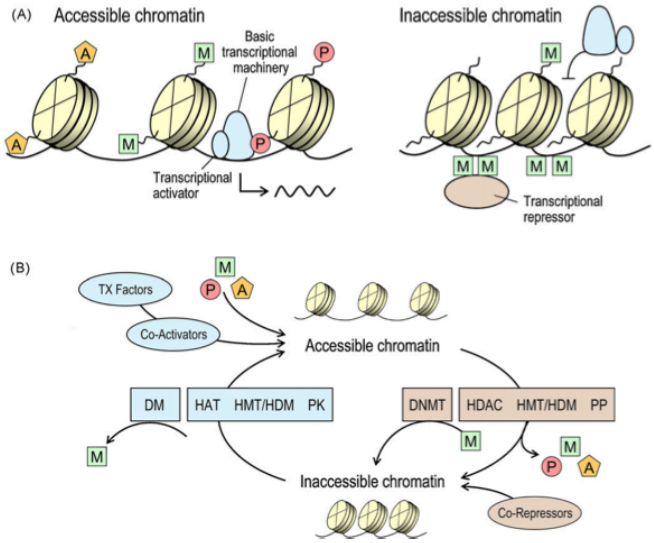


Figure 8. Different chromatin structures by modification of histones with specific enzymes regulate gene expressions. (A) In the condensed chromatin structure (right), gene expression is suppressed by interruption of target gene promoter binding with transcriptional activator or by binding of methylated target genes with transcriptional repressor. In the slackened chromatin structure (left), transcriptional activator can bind with its target gene promoter results in increasing of target gene expression. (B) Chromatin can be remodeling through histone methylation(M), acetylation(A), and phosphorylation(P) by the action of specific enzymes. The

chromatin remodeling occurs by the combination action of histone modifications (Gräff and Mansuy, 2008).

The majority of the histone modifications are contributed by acetylation, methylation, and phosphorylation. Figure 9 shows the enzymes that regulate each of these histone modifications (Figure 9). Histone acetylation of lysines is regulated by the action of two main enzymes, histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Bannister and Kouzarides, 2011; Luger et al., 1997). The HATs neutralize the lysine's positive charge and results in the weakening the interactions between histones and DNA. HDACs oppose the effects of HATs and reverse the lysine acetylation. Histone phosphorylations are controlled by kinases and phosphatases. They take place on serines, threonines and tyrosines in the N-terminal histone tails. Histone methylation occurs on the side chains of lysines and arginines. Unlike acetylation and phosphorylation, histone methylation does not alter the charge of the histone protein. It has been reported that histone methyl transferases (HMTs) and histone de-methyl transferases (HDMs) regulate histone methylation (Bannister and Kouzarides, 2011; Fischle et al., 2003; Kouzarides, 2007).

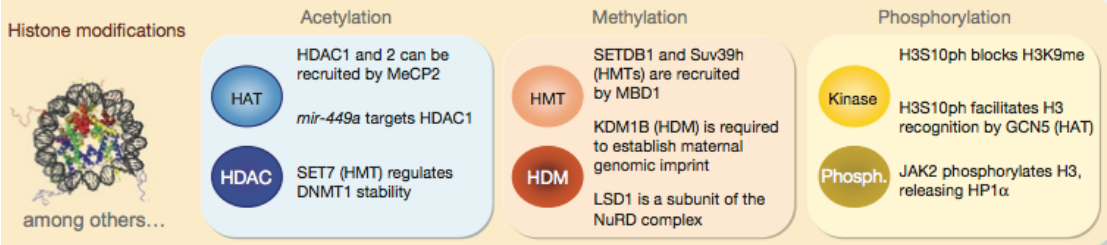


Figure 9. Specific enzymes for histone modifications. HAT and HDAC regulate histone acetylation, HMT and HDM regulate histone methylation, and Kinase and Phosphatase regulate histone phosphorylation. Chromatin structure is regulated through these histone modifications by the specific enzymes (Portela and Esteller, 2010).

1.2.1.3. Epigenetic patterns in normal and in cancer cells

In 1983, researchers found that the DNA of colorectal cancer cells was less methylated (hypomethylated) than the DNA of normal tissues from colorectal cancer patients (Feinberg and Vogelstein, 1983). Because methylated genes are generally less actively transcribed, DNA hypomethlyation causes abnormally high gene activation by altering the arrangement of chromatin. Conversely, too much methylation (hypermethylation) can block the functions of protective tumor suppressor genes. The finding that DNA methylation regulates the expression of oncogenes and tumor suppressor genes in cancer cells, has led to the conclusion that cancer is not only a genetic disease, but also determined by epigenetic factors

(Esteller and Herman, 2001; Feinberg and Tycko, 2004; Momparler, 2003; Sharma et al., 2010). Abnormal control of gene expression through epigenetic modifications, DNA methylation and histone modification, causes tumor progression and contributes to tumor aggressiveness (Figure 10). As previously mentioned, DNA methylation occurs at CpG sites. CpG islands are mostly free of methylation in normal cells. However, the CpG islands can become excessively methylated in cancer cells, and CpG island methylation can result in the silencing of tumor suppresser gene expression (Esteller, 2007; Ron-Bigger et al., 2010). Figure 10 shows a model how the epigenetic alterations can cause tumor progression in skin. It has been reported that during the progression of a neoplasm, the degree of CpG island methylation and altered histone modification patterns are increased, and that these changes increase tumor proliferation and invasiveness (Esteller, 2008; Fraga et al., 2004).

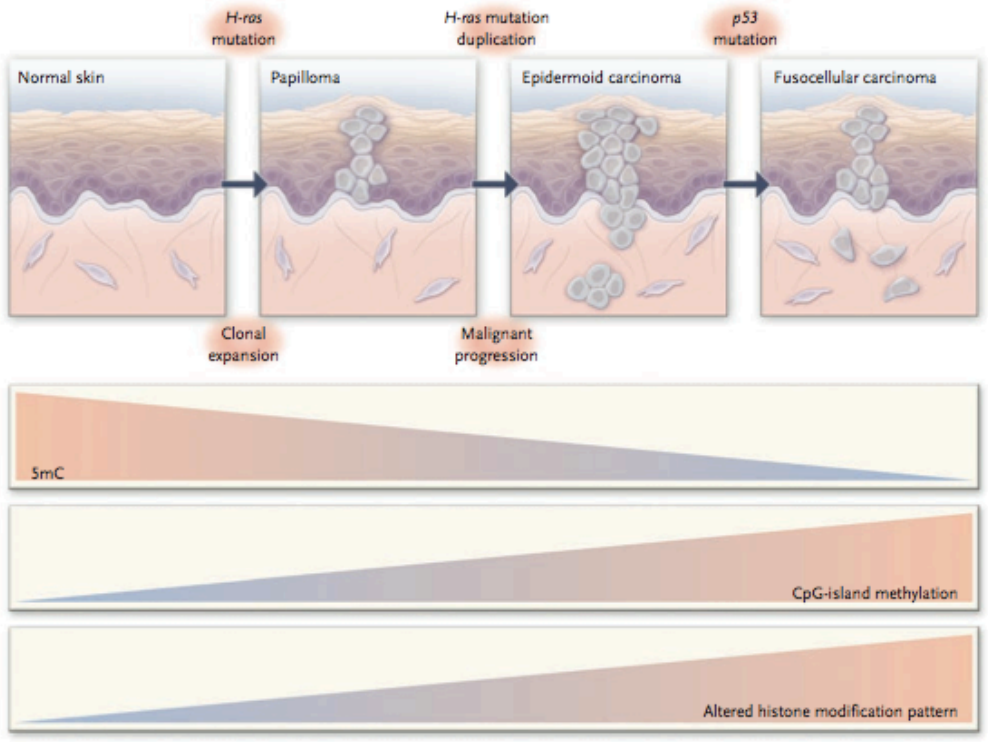


Figure 10. A model of epigenetic alterations in skin tumor progression. H-ras Genetic mutation causes papilloma in skin, then the papilloma progress into malignant tumors upon increasing of methylation of CpG-island and altered histone modification pattern (Esteller, 2008).

Epigenetic modifications in cancer are not only due to DNA methylation, but also to histone modifications and nucleosome positioning (Portela and Esteller, 2010). CpG island hypermethylation in the promoter regions of tumor suppressor genes is a major epigenetic alteration in many cancers. These hypermethylations affect genes, which regulate the cell cycle, DNA repair, the metabolism of carcinogenesis, cell-to-

cell interaction, apoptosis and angiogenesis and are involved in the tumor progressions (Esteller, 2007; 2008; 2011a).

However, the inactivation of tumor suppressor genes is also associated with histone modifications. Deacetylation of histone H3 and H4, loss of H3K4 trimethylation, and gain of H3K9 methylation and H3K27 trimethylation have been reported as a histone modification associated with the inactivation of tumor suppressor genes in cancers (Momparler, 2003; Sharma et al., 2010). The hypoacetylation and hypermethylation of histone H3 and H4 inactivate certain genes which are known as tumor suppressor like genes, such as $p21^{WAF1}$, despite the absence of hypermethylation of the CpG island (Richon et al., 2000). It has been reported that histone H4 exhibits a loss of acetylation at lys 16 and trimethylation at lys 20 in most cancers (Fraga et al., 2005). Interestingly, these changes appear early and accumulated during the tumorigenic process.

Figure 11 and 12 show that epigenetic changes, especially, DNA methylation (Figure 11) and histone modifications (Figure 12) regulate gene expression patterns of oncogenes and tumor suppressor genes in cancers, and these epigenetic modifications can cause and accelerate tumorigenesis (Esteller, 2007).

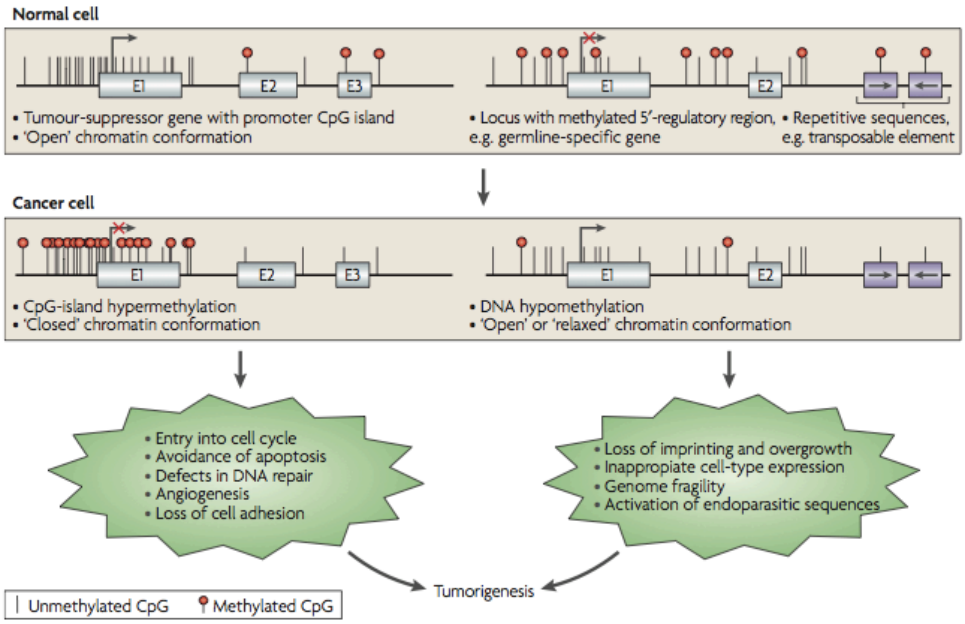


Figure 11. Altered DNA-methylation patterns in tumorigenesis. CpG islands in cancer have altered DNA methylation patterns when compared to normal cells CpG islands. In cancer cells, the promoter region of tumor suppressor genes is highly methylated results in inactivation of tumor suppressor genes. When compared to normal cells, the promoter region of oncogenes has less methylation patterns in cancer cells results in increasing of tumorigenesis (Esteller, 2007).

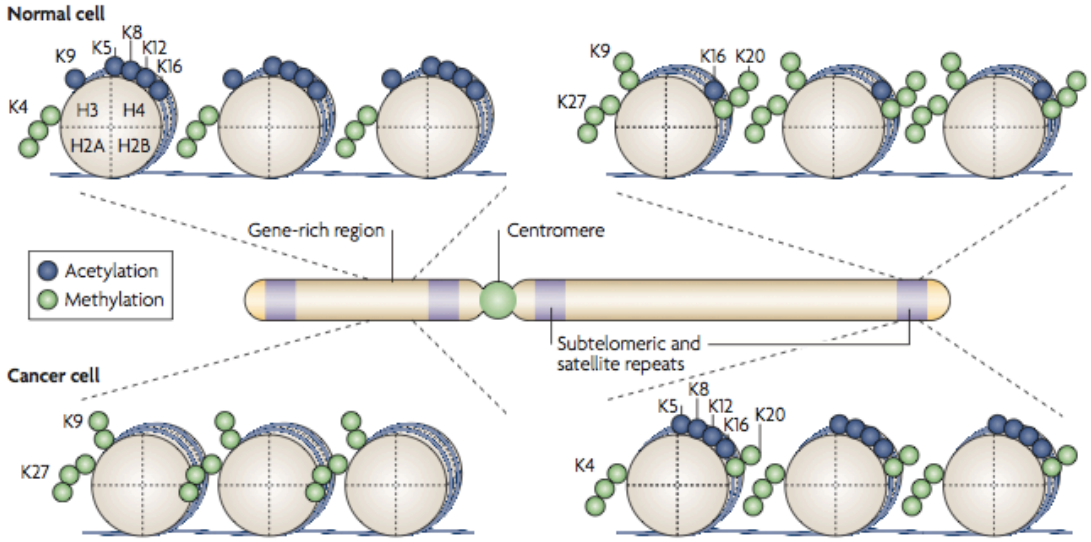


Figure 12. Histone modification maps for a typical chromosome in normal and cancer cells. Histone H3 and H4 have been known that include the promoters of tumor suppressor genes. It has been shown that histone H4 has strong acetylation form in normal cells but not in cancer cells by the Nucleosomal arrays. Moreover, histone has more methylated in cancer cells when compared to normal cells. These histone H4 modification with de-acetylation and trymethylation inactivate tumor suppressor genes (Esteller, 2007).

Additional molecular components participate in the epigenetic regulation of gene expression. Recently it has been shown that small non-coding RNAs, especially microRNAs, also regulate epigenetic modifications of cells (Kala et al., 2013). microRNA expression profiling shows that most sub-groups of microRNAs are down-regulated in cancer when compared to normal cells. The down-regulation increases the expression of onco-proteins in cancer cells and results in tumor progression (Calin and Croce, 2006; Dalmay and Edwards, 2006; Kala et al., 2013). However, there are also up-regulated microRNAs in cancer cells. The up-regulation of the microRNA suppresses tumor suppressor genes and thus also participates in tumor progression (Cho, 2007; Esquela-Kerscher and Slack, 2006; Manikandan et al., 2008). The functions of individual microRNAs in cancer will be described in detail in chapter 1.5, microRNAs.

1.2.1.4. The epigenetic stem cell signature of cancer cells

The origin of cancer cells is still a controversial question in cancer research. It is conceivable that cancer cells originates from mutations in stem cells or it is possible that differentiated cells reacquire stem cell characteristics through mutational and epigenetic mechanisms and a process of de-differentiation. However, both hypotheses imply that cancer cells exhibit de-differentiated phenotypes when compared to normal differentiated cells.

Three gene sets, the CPM modules, are the main regulatory factors for stem cell phenotypes such as pluripotency and self-renew (Kim and Orkin, 2011). They comprise the Core module, the PrC (polycomb related factors) module and Myc module. Recently, it has been shown that these CPM modules are highly expressed in the cells upon the induction of reprogramming factors and differentially expressed at different stages of the reprogramming process (Nagata et al., 2012). The Core module comprises transcription factors with numerous chromosomal targets, and mainly regulates stem cell pluripotency. The Core module is only up-regulated in the reprogramming cells and iPS cells, but it is down-regulated in differentiated cells and cancer cells (Nagata et al., 2012). The PrC module encodes stem cell epigenetic modulators that regulate chromatin structure through modulation of histone methylation (Surface et al., 2010). The PrC module also directly regulates *de novo* DNA methylation of target genes (Widschwendter et al., 2007). Polycomb group proteins were initially reported in *Drosophila* and found to regulate *Drosophila* embryonic development (Sparmann and van Lohuizen, 2006). Although it is unclear how Polycomb proteins are recruited to specific chromatin sites in mammalian cells, the main function of polycomb repressive complexes in eukaryotes is silencing of genes by epigenetic control (Morey and Helin, 2010). As previous mentioned, histone H3 contains gene suppressing marks (Momparler, 2003; Sharma et al., 2010). Because of their repression function, the PrC module is only expressed in differentiated cells that have more specialized phenotypes than stem cells. However, because the function of PrC module is highly related with histone modifications causing chromatin remodeling, expression of PrC module is increased in the reprogramming cells. Global gene expression analysis showed that the PrC module is also expressed in cancer stem cells and results in suppression of tumor suppressor genes (Nagata et al., 2012). The Myc module includes Myc related proteins (Kim and Orkin, 2011; Kim et al., 2010). In stem cells, it regulates stem cell self-renew and proliferation. Most notably, it is reported that Myc also influences global histone modifications. It can maintain normal histone methylation patterns and restore altered histone acetylation (Knoepfler et al., 2006). Global gene expression analysis shows that the Myc module is down-regulated in differentiated cells, but it is highly expressed in the reprogramming cells, iPS cells and in cancer stem cells (Nagata et al., 2012).

The epigenetic stem cell markers are highly expressed in cancer cells, with the exception of the core modules, when compared to differentiated cells. These reports suggest that cancer cells have de-differentiated phenotypes through the expression of epigenetic stem cell markers.

1.2.2. Therapeutic approaches targeting epigenetic determinants

Cancer as previous mentioned is not only characterized by genetic lesions, but also by epigenetic modifications. Epigenetic alterations are strongly associated with de-differentiation phenotypes of cancer cells and cancer progression. Epigenetic alterations of cancer cells are manifested in DNA methylation, histone de-acetylation and histone methylation. These result in an increased inactivation of gene expression and e.g. the suppression of tumor suppressor gene activities. In contrast to genetic alterations which are difficult to reverse, epigenetic alterations are potentially reversible. This might allow to reprogram the malignant phenotypes of cancer cells to a more normal state. For this reason, drugs that target specific enzymes which regulate epigenetic modifications, are being developed. Most of the "epigenetic drugs" target DNA methyltransferase (DNMT) and histone de-acetylase (HDAC) (Yoo and Jones, 2006).

1.2.2.1. Inhibition of DNA methyltransferase (DNMTs)

DNMT, as previous mentioned, is an enzyme that methylates CpG dinucleotide rich regions of genomic DNA. DNMT1 has been reported as a maintenance methyltransferase that causes the inheritance of DNA methylation patterns from the template strand to the nascent daughter strand. DNMT-3A and DNMT-3B have been reported as *de novo* methyltransferases (Figure 6) (Szyf, 2005). Activation of DNMT increases the methylation of tumor suppressor genes in cancer cells, and the methylation results in the inhibition of tumor suppressor gene expressions. For these reasons, several potentially useful DNAMT inhibitors are undergoing preclinical and clinical drug trials. DNMT inhibitors can be classified into two different types of small molecules, nucleoside analogues (Table 1), and non-nucleoside analogues (Table 2). Nucleoside analogues have a modified cytosine ring that is attached to either a ribose or deoxyribose group (Yoo and Jones, 2006). These nucleoside analogue drugs are methylated by DNMTs and protect genomic DNA from hypermethylation. Although these nucleoside analogues were the first class of compounds used as DNMT inhibitors, they have shown side effects in clinical trials, they are myelotoxic and induce cytopenia (Peedicayil, 2006). In contrast, non-nucleoside analogues directly inhibit DNMTs through targeting the catalytic region of DNMTs. These non-nucleoside analogues do not have myelotoxic side effects and are the preferred compounds in preclinical and clinical trials (Peedicayil, 2006; Yoo and Jones, 2006).

Table 1. DNA methylation inhibitors: nucleoside analogues (Yoo and Jones, 2006)

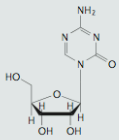
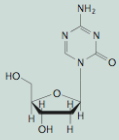
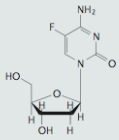
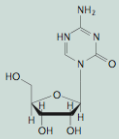
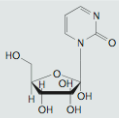
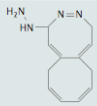
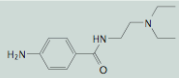
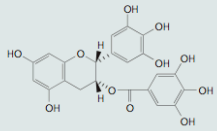
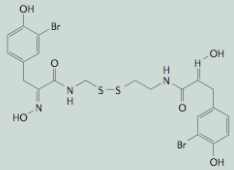
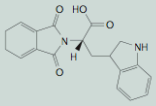
Inhibitor	Structure	Dose range	Clinical trials
5-Azacytidine		μM	Phase I, II, III: haematological malignancies
5-Aza-2′-deoxycytidine		μM	Phase I, II, III: haematological malignancies; cervical, non-small-cell lung cancer
5-Fluoro-2′-deoxycytidine		μM	Phase I
5,6-Dihydro-5-azacytidine		μM	Phase I, II: ovarian cancer and lymphomas
Zebularine		μM–mM	Preclinical

Table 2. DNA methylation inhibitors: non-nucleoside analogues (Yoo and Jones, 2006)

Inhibitor	Structure	Dose range	Clinical trials
Hydralazine		μM	Phase I: cervical cancer
Procainamide		μM	Preclinical
EGCG		μM	Preclinical
Psammaplin A		nM–μM	Preclinical
MG98	N/A	N/A	Phase I: advanced/ metastatic solid tumour
RG108		μM	Preclinical

1.2.2.2. Inhibition of histone deacetylases (HDACs)

Secondary histone modifications are important determinants of chromatin structure and gene activity. The loss of acetylation and trimethylation in histone H3 and H4 are commonly found in various cancers. These patterns cause disruption of the constitutive heterochromatin structure and the loss of histone acetylation has been identified as a first step in gene silencing (Mutskov and Felsenfeld, 2004). For these reasons, HDACs, which are able to remove acetyl groups from histones, have been recognized as drug targets in epigenetic therapies. HDAC inhibitors have been found to have antitumor effects; however, the inhibitors are not only causing the inhibition of HDACs, but also deacetylate other, unidentified targets. The mechanisms of their action are still not unequivocally defined. HDAC inhibitors can be classified into four different groups: short-chain fatty acids, hydroxamic acids (Table 3), cyclic tetrapeptides and benzamides (Table 4). These inhibitors directly inhibit HDACs, and the inhibition results in an increase in histone acetylation. (Mund and Lyko, 2010; Peedicayil, 2006; Yoo and Jones, 2006). Short-chain fatty acids (SCFAs) were the first drugs used as HDAC inhibitors. Butyrate and valproic effect cell growth and induce apoptosis *in vitro* and *in vivo* (Candido, 1978; Gottlicher, 2001; Yoo and Jones, 2006). However, these compounds have pleiotropic-effects and also affect other enzymes. Because of their unspecific actions and low bioavailability, SCFAs have not been considered as desirable drugs (Perrine et al., 1994). Nevertheless, SCFAs have shown promising results in the treatment of leukaemia (Raffoux et al., 2005; Yang et al., 2005).

Hydroxamic acids are potent inhibitors of HDACs. Trichostatin A, a *Streptomyces* product, was first shown as an inducer of differentiation and cell cycle arrest, and later reported as a HDAC inhibitor (Yoshida et al., 1990). Suberoylanilide hydroxamic acid (SAHA) also inhibits the catalytic domain of HDAC. It also has been shown to have beneficial therapeutic effects on advanced cancers (Garcia-Manero et al., 2007). Cyclic tetrapeptides inhibit HDACs at very low concentrations, through the epoxyketone group which alkylates the catalytic pocket of HDACs (Yoo and Jones, 2006). These inhibitors are also undergoing preclinical evaluation. Depsipeptide, the best known HDAC inhibitor in this group has shown antitumor activity against leukaemias and T-cell lymphomas (Byrd et al., 2005; Marshall et al., 2002; Sandor et al., 2002). Benzamide groups inhibit HDACs by binding to the catalytic zinc ion (Yoo and Jones, 2006). MS-275 and CI-994 are the most well-known synthetically derived inhibitors of HDACs in this group. MS-275 has anti-tumor effects on renal cell carcinoma and prostate cancer cells (Wang et al., 2005). CI-994 has been used in

several Phase I studies alone or in combination with other chemotherapeutic agents (Pauer et al., 2004; Undevia et al., 2004).

HDAC inhibitors prevent hypomethylation of histones, promote transcriptional activity and modify malignant cells in the direction of more normal cell states. Moreover, recently it has been reported that HDAC inhibitors also effect angiogenesis, cell differentiation, cell migration and promote apoptosis (New et al., 2012).

Table 3. Histone-deacetylase inhibitors: shor-chain fatty acids and hydroxamic acids (Yoo and Jones, 2006)

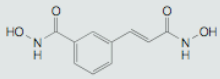
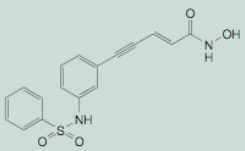
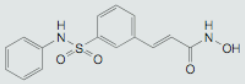
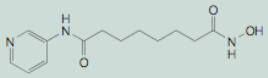
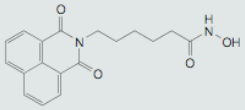
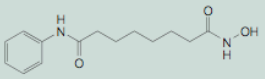
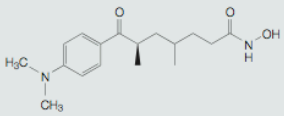
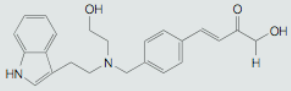
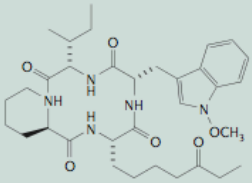
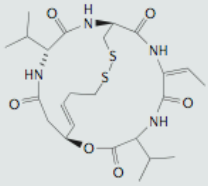
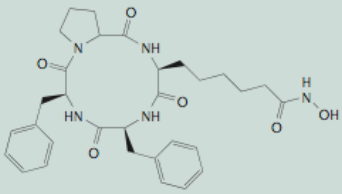
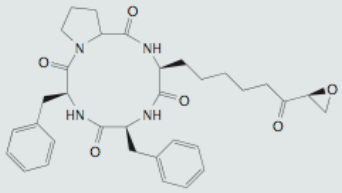
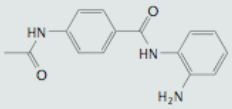
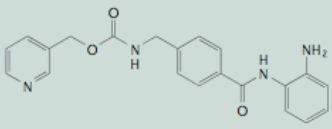
Inhibitor	Structure	Dose range	Clinical trials
Short-chain fatty acids			
Butyrate		mM	Phase I, II: colorectal
Valproic acid		mM	Phase I: AML, leukaemias
Hydroxamic acids			
m-Carboxy cinnamic acid bishydroxamic acid (CBHA)		μM	Preclinical
Oxamflatin		μM	Preclinical
PDX 101		μM	Phase I
Pyroxamide		nM	Preclinical
Scriptaid		μM	Preclinical
Suberoylanilide hydroxamic acid (SAHA)		μM	Phase I, II: haematological and solid tumours
Trichostatin A (TSA)		nM	Preclinical
LBH589	N/A	nM	Phase I
NVP-LAQ824		nM	Phase I

Table 4. Histone-deacetylase inhibitors: cyclic tetrapeptides and benzamides (Yoo and Jones, 2006)

Inhibitor	Structure	Dose range	Clinical trials
Cyclic tetrapeptides			
Apicidin		nM	Preclinical
Depsipeptide (FK-228, FR901228)		μM	Phase I, II: CLL, AML, T-cell lymphoma
TPX-HA analogue (CHAP)		nM	Preclinical
Trapoxin		nM	Preclinical
Benzamides			
CI-994 (N-acetyl dinaline)		μM	Phase I, II: solid tumours
MS-275		μM	Phase I, II: solid tumours and lymphoma

1.3. Reprogramming of somatic cells into pluripotent stem cells

In 1962, *John Gurdon* found that adult frogs could be derived from the nuclei of single somatic cells (Gurdon, 1962). It was the first report that the epigenetic memory of somatic cell nuclei could be erased and reprogrammed in oocytes. The cloning of a lamb showed for the first time, that animal cloning from somatic cell nuclei was possible. In 1997, *Ian Wilmut* cloned a lamb, named *Dolly*, from adult sheep mammary epithelial cell nuclei (Wilmut et al., 1997). To clone *Dolly*, *Wilmut* and *colleagues* used the nuclear transfer of a single nucleus of differentiated cells to an enucleated unfertilized egg. These findings showed that differentiated cells can be

reprogrammed into stem like cells by epigenetic control exerted the oocyte microenvironment.

In 2005, *Taranger* and *colleagues* found that extracts from embryonic stem cells are able to induce cell dedifferentiation and epigenetic reprogramming (Taranger et al., 2005). Finally in 2006, *Shinya Yamanaka* and *colleagues* found four defined transcription factors are sufficient to induce somatic cells to become pluripotent stem like cells (Takahashi and Yamanaka, 2006). These findings showed that somatic cells can be reprogrammed into pluripotent stem cells and that changes in epigenetic modifications can alter cell phenotypes through the regulation of gene expression patterns.

1.3.1. Cellular differentiation and reprogramming

Epigenetic modifications, as previous mentioned, change cell phenotypes, and the modifications can result in specialized cell types. Cellular differentiation is the process in which a less specialized cell becomes a more specialized cell type. It is accompanied by the loss of lineage potential and increasing functional restrictions.

Cell differentiation can be considered as programmed memory by epigenetics. The genomic information present in stem cells and in differentiated cells is the same; however, the epigenetic status of stem cells and differentiated cells are different. In the differentiation process, gene expression patterns are changed by epigenetic modification such as DNA methylation, histone modification, and chromatin remodeling. In ES cell development, self-renewal related genes are silenced and expression of developmental genes such as NEUROG1, PAX6, GATA4, CDX2 etc, are increased (Christophersen and Helin, 2010).

Before the discovery of the somatic cell reprogramming process, it had been known that stem cells can be differentiated into specialized cells by epigenetic control, and this was considered as irreversible. When the above mentioned work showed that it is possible to achieve somatic cell reprogramming into pluripotent stem like cells, it became clear that epigenetic processes are reversible, and that changes of cell phenotypes through the regulation of gene expression patterns can be accomplished.

Reprogramming is a process that induces somatic cells into pluripotent stem cell by epigenetic control. There are mainly three different approaches: nuclear transfer, cell fusion and the transduction of transcription factors. Transduction of transcription factors approach is also known as nuclear reprogramming. *Yamanaka* and *colleagues* have shown that somatic cells can be reprogrammed into pluripotent stem

cells by transduction of the four transcription factors (Oct4, Sox2, Klf4, and Myc) (Takahashi and Yamanaka, 2006).

In an extension of these observations, *Morrisey* and *colleagues* found that small non-coding microRNAs, especially the miR 302/367 cluster, is also able induce somatic cells into pluripotent stem cells through the regulation of epigenetic modifications (Anokye-Danso et al., 2011). Somatic cell reprogramming research showed that cellular differentiation and reprogramming processes are reversible through the epigenetic modifications.

1.3.2. Epigenetics and reprogramming

Yamanaka and *colleagues* found four transcription factors able to induce pluripotent stem cells from many different cell types (Papp and Plath, 2013). The reprogramming factors reset the epigenetic patterns of somatic cells, and modify them in a way which favors the pluripotent stem cell phenotype. Although it is clear that the reprogramming factors change somatic cell phenotypes by epigenetic modification, the details by which the reprogramming factors accomplish this task are still not perfectly clear.

Genome-wide analyses of ES cells have indicated that the transcription factor Nanog and the three reprogramming factors Oct4, Sox2, and Klf4 specify the ES cell identity by transcriptionally activating the self-renewal programs and by repressing lineage commitment pathways (Boyer et al., 2005; Pan and Thomson, 2007; Papp and Plath, 2013). Generally, pluripotency transcription factors interact with ES cell transcription factors and up-regulate ES cell specific gene expression. The ES cell related transcription factors form complexes and regulate ES cell pluripotency and self-renewal. However, when only one component of the pluripotency factors binds on the ES cell chromosome, target gene are often repressed, and the ES cells can undergo differentiation. This model suggests how the association of a gene with multiple transcription factors can increase its transcriptional output.

The four reprogramming factors do not act on the same set of genes. The target genes of cMyc are not shared by the other transcription factors. It indicates that the four reprogramming factors regulate ES cell specific gene expression in a combinatorial fashion by epigenetic modifications (Chen et al., 2008; Kim et al., 2008).

It has been shown that HDAC inhibitors, including valproic acid, enhance the induction of somatic cells into pluripotent stem cells by the four reprogramming factors (Huangfu et al., 2008). Moreover, the methylation of histone H3K9 has negative effects on somatic cell reprogramming, and inhibition of DNMT improves

the efficiency of somatic cell reprogramming (Ho et al., 2011; 2011b). Figure 13 shows the landmark events during the reprogramming process (Ho et al., 2011).

As mentioned above that reprogramming process is changing the somatic cell epigenetic landscape. During this process, somatic cell chromatin status is modified into ES cell chromatin by epigenetic modifications (Figure 13). Small molecules that affect such modifications, e.g. inhibitors of DNMTs or HDACs, can increase reprogramming efficiency. The eprogramming process is reversible by epigenetic manipulation, and addition of "epigenetic drugs", including DNMTs and HDACs inhibitors, in collaboration with the reprogramming factors can increase reprogramming efficiency by activation of various ES cell related genes.

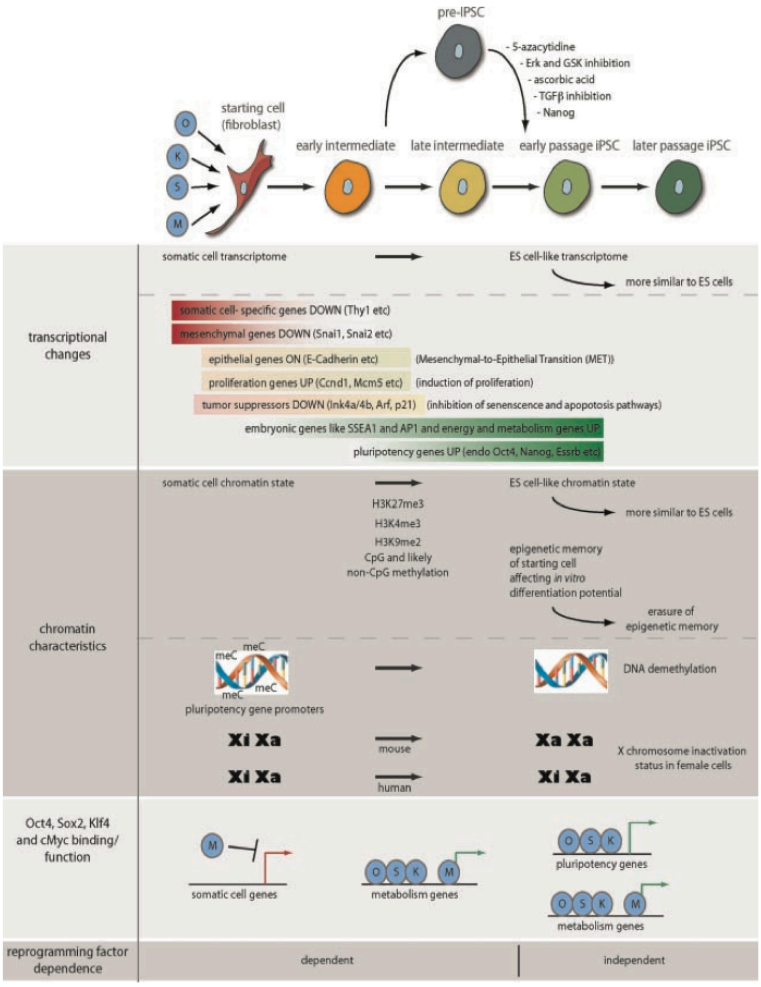


Figure 13. Landmark events on the path to induced pluripotency. It shows that the reprogramming factors triggers a cascade of reprogramming events, and these reprogramming factors regulate global gene expression patterns by epigenetic modifications (Ho et al., 2011).

1.3.3. Reprogramming of cancer phenotypes by epigenetic control

Cancer cells are characterized by de-differentiated phenotypes resulting from epigenetic marking and the over-expression of ES cell genes, including reprogramming factors, is associated with tumor malignancy and poor prognosis.

Reprogramming is a process by which epigenetic modifications of somatic cell are altered and a gene expression program is induced which confers the phenotypes of pluripotent stem like cells. Although cancer cells and stem cells share some similarities in their gene expression programs, cancer cells are clearly distinguishable from stem cells. The self-renewal capacity and proliferation of stem cell requires special medium components; cancer cells can maintain stem cell features, such as self-renewal, in normal serum. These features indicate that the partial expression of ES cell genes or induced pluripotency programs may cause incomplete regrogramming and could thus be contributing to the tumorgenicity of the cells.

Recent studies showed that even cancer cells can be reprogrammed into pluripotent stem cells. These cells loose transformation phenotypes by introduction into the embryonic stem cell microenvironment (Table 5).

Nuclear transfer was the first method applied to achieve cellular reprogramming of somatic cells (Kono, 1997). In this procedure, the nucleus of a somatic cell is transplanted into an enucleated oocyte. In the environment of the oocyte, the somatic cell nucleus is reprogrammed so that the cells derived from it are pluripotent. From this oocyte, a blastocyst is generated, from which embryonic stem cell lines are derived in tissue culture. This method suggests that the microenvironment of an oocyte modifies the somatic cell nucleus epigenetic markers.

Cancer researchers adopted these observations of developmental investigations and attempted to change the phenotypes of cancer cells epigenetically. Epigenetic therapeutic strategies are being developed. DNMTs or HDACs inhibitors can increase the expression of tumor suppressor genes through changing epigenetic imprints, and suppression of tumor cell traits.

Using nuclear transfer methods, researchers modified melanoma, medulloblastoma, breast cancer, and embryonal carcinoma cancer phenotypes into more normal cell phenotypes (Blelloch, 2004; Hochedlinger et al., 2004; Li et al., 2003; Postovit et al., 2008). Moreover, they also induced gastrointestinal cancer cells into pluripotent stem cells by using the transduction of genes encoding the four reprogramming factors (Miyoshi et al., 2010). Recently, Lin et al. also found that the microRNA 302/367 cluster, which also has been known as a reprogramming inducer, alters melanoma and prostate cancers into pluripotent stem cells when cultured with ES

cell conditioned medium *in vitro* (Lin et al., 2008). These results suggest that the epigenetic setting of aggressive tumor cells can be manipulated by the ES cell microenvironment or reprogramming inducers.

Table 5. List of published studies concerning cancer cell reprogramming (Nishikawa et al., 2012)**.**

Method	Type of cancer	Reference
Nuclear transfer	Medulloblastoma (Primary culture, mouse, Ptc1 geterozygous)	(Li et al., 2003)
Nuclear transfer (2-step cloning)	Melanoma RAS^{+} / Ink4a / $Arf^{-/-}$	(Hochedlinger et al., 2004)
Nuclear transfer	Embryonal carcinoma	(Blelloch, 2004)
Embryonic microenvironment	Metastatic melanoma, breast cancer	(Postovit et al., 2008)
microRNA 302 family	Melanoma (Colo), prostate	(Lin et al., 2008)
Defined transcription factor (OKM)	Melanoma (R545)	(Utikal et al., 2009)
Defined transcription factor (OSKM)	Gastrointestinal cancer (colon, liver, pancreatic)	(Miyoshi et al., 2010)
Defined transcription factor (OSKM)	KBM7 cells derived from blast crisis stage chronic myeloid leukemia	(Carette et al., 2010)
Defined transcription factor (OSLN)	A549 lung cancer	(Mathieu et al., 2011)
Oocyte extract	Breast cancer (cell line, MCF7, HCC1945)	(Allegrucci et al., 2011)

O, Oct4; S, Sox2, K, Klf4; M, c-Myc; L, Lin 28; N, Nanog.

1.4. Inflammatory cytokines and tumorigenesis

Inflammation is a response to injury or infection. There are two major types of inflammations. Acute inflammation is dominated by vascular changes and by action of neutrophils and leucocytes in the early stages of the wound healing process, and mononuclear phagocytes later on. Leucocytes adhere locally and emigrate into the tissue through the endothelial cell lining of the postcapillary venules. Plasma exudation from vessels may lead to tissue swelling, but the early vascular changes are independent of and not essential for the later cellular response. Another main type of inflammation is chronic inflammation. In chronic inflammation, where the stimulus is persistent, the characteristic cells are macrophages and lymphocytes. It is generally a response to a persistent antigenic stimulus. Inflammation also can affect the tumor microenvironment and exert influences on tumor proliferation, survival, and migration. Cancer cells secrete cytokines and chemokines which activate receptors conferring invasion, migration and metastasis (Figure 14) (Coussens and Werb, 2002; Perwez Hussain and Harris, 2007; Wu and Zhou, 2009).

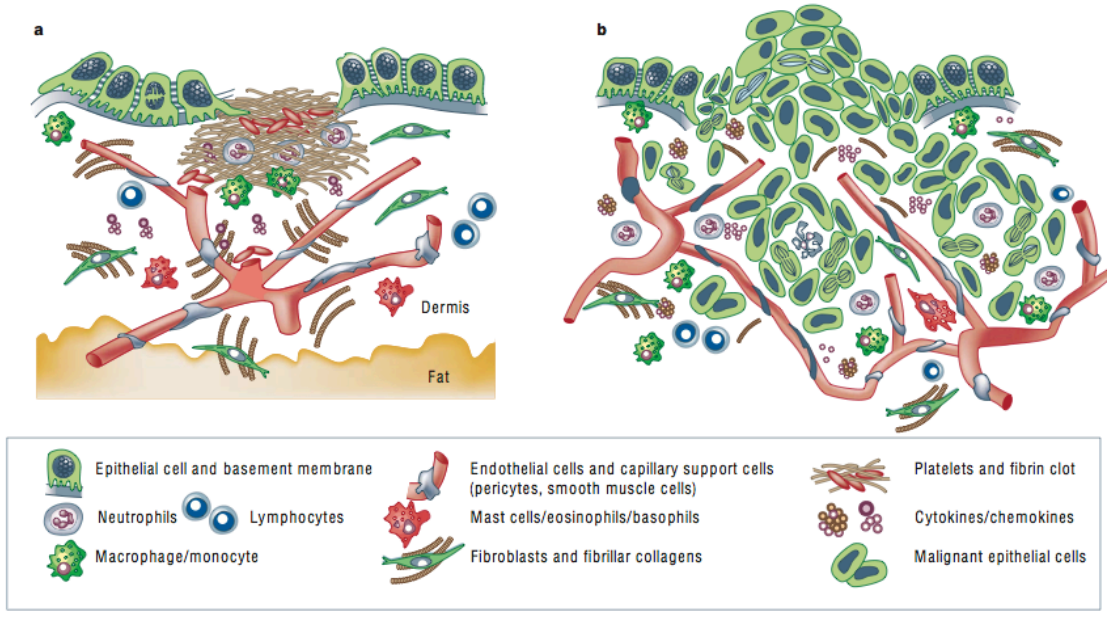


Figure 14. Wound healing versus invasive tumor growth. This figure shows that tumor use the cytokines and chemokines produced by immune cells for tumor migration and invasion (b) while normal tissues use them to remodel injured tissues through wound healing process (a) (Coussens and Werb, 2002).

Chronic inflammation can lead to an increase in cancer risk. In tumor tissues, various types of immune and inflammatory cells are present and these immune and inflammatory cells can affect the tumor properties. Immune cells affect malignant cells through the production of cytokines, chemokines, growth factors,

prostaglandins, and reactive oxygen and nitrogen species. Figure 15 shows the relationships between inflammation due to different causes and their consequences for tumor tissues. Inflammation can have opposing effects. It can restrict tumor growth and enhance the efficiency of therapy. It also can enhance tumor growth, tumor survival, neo-angiogenesis, metastasis, genomic mutations, and drug resistance to therapy. Chronic inflammation, tumor-associated inflammation, prolonged exposure to environmental irritants or obesity, and therapy-induced inflammation are mostly detrimental. In developing tumors, anti-tumorigenic and pro-tumorigenic immune and inflammatory mechanisms coexist; however, if the tumor is not rejected, the pro-tumorigenic effects dominate.

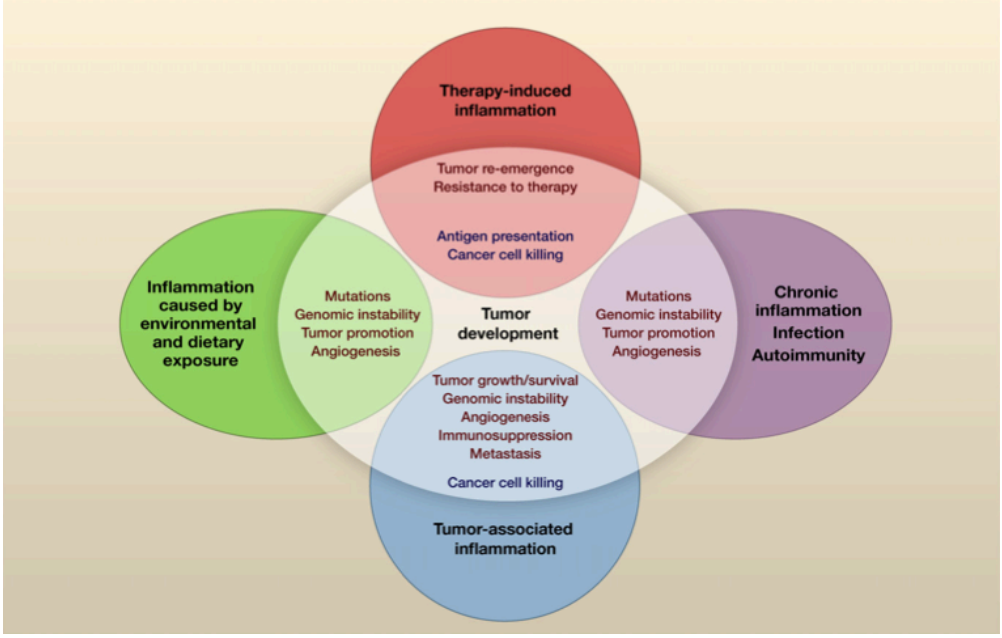


Figure 15. Types of inflammation in tumorigenesis and cancer. Various changes in inflammation network cause tumor development. Chronic inflammation can induce oncogenic mutations, tumor promotions, and angiogenesis in tumors. Response of tumors upon tumor associated inflammation causes enhancement of tumor growth, tumor survival, neoangiogenesis, immunosuppression, and tumor metastasis. Moreover, prolonged exposure to environmental irritants or obesity can also result in low-grade chronic inflammation that enhances tumor development. In some cases, therapy-induced inflammation can enhance antigen presentation, leading to immune-mediated tumor eradication; however, these inflammation can also induce tumor re-emergence, and increasing resistance to tumor therapy (Grivennikov et al., 2010).

1.4.1. Inflammatory cytokine signaling

Cytokines are released from tumor cells into the surrounding tissues and the released cytokines affect tumor progression. Cytokines are small proteins. They are released by cells, bind as ligands to cell surface receptors and affect the behavior of target cells. They comprise interleukins, lymphokines and related signaling molecules such as TNF and interferons.

Cancer cells can be affected directly by the cytokines released by immune cells, other cell types in the surrounding tissues, or by the tumor cells themselves. Although cytokines can have effects on tumor progression, they can also enhance the development of the immune system and result in increasing the anti-tumor effects by immune cells. Because of the complex roles in the tumor microenvironment, understanding the biological roles and mechanisms of the cytokines is necessary to develop drugs for cytokine-based cancer immunotherapy.

Typically, inflammatory cytokines are divided into two categories: pro-inflammatory cytokines (e.g. IL-1, IL-6, IL-8, TNF-α, IFN-γ) and anti-inflammatory cytokines (e.g. IL-4, IL-10, TGF-β, and BEGF) (Serefoglou et al., 2008). Theses pro- and anti-inflammatory cytokines activate oncogenic signaling pathways through stimulating functional receptors. Currently, seven cytokine receptor families are classified (Table 6): Type I cytokine receptors, Type II cytokine receptors, immunoglobulin superfamily receptors, tumor necrosis factor (TNF) receptors, G-protein coupled receptors (GPCR), transforming growth factor β (TGF-β) receptors, and IL-17 receptors (Lee and Margolin, 2011). Table 6 shows the pro- and anti-inflammatory cytokines and the functions of their receptors in cancer.

Type I receptors are associated with JAK (Janus kinases) / STAT (Signal transducers of activator of transcription) signaling pathway. gp130 is the best studied type I receptor in cancer. IL-6, leukemia inhibitory factor (LIF), and oncostatin M are well known cytokines that stimulate gp130. Stimulation of gp130 activates downstream signal pathways, such as the JAK/STAT3 pathway (Fischer and Hilfiker-Kleiner, 2007; Masuda et al., 2010; Sun et al., 2011; Wang et al., 2013).

Type II cytokine receptors can be stimulated by IFN-α, IFN-β, IFN-γ, and IL-10. Type II cytokine receptors are similar and contain tandem Ig-like domains. Type II cytokine receptors are connected with a tyrosine kinase of the JAK family. Thus, the stimulation of type II cytokine receptors also activates the JAK/STAT3 signaling pathway.

GPCR are cell surface receptors are a large family of cell surface receptors that are coupled to heterotrimeric G-proteins (GTP-binding proteins). All G-protein-coupled receptors seem to have seven membrane-spanning domains. IL-8 is well known cytokine, which stimulates a member of the GPCR family. Stimulation of GPCRs is associated with the PI3K/AKT signaling pathway which regulates tumor growth, transformation and metastasis (Osaki et al., 2004; Vanhaesebroeck et al., 2012; Vara et al., 2004).

Table 6. Inflammatory cytokines receptors, their ligands and functions (Lee and Margolin, 2011).

Receptor Family	Ligands	Function
Type I cytokine receptors	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, IL-21, IL-23, IL-27, Erythropoietin, GM-CSF, Growth hormone, Prolactin, Oncostatin M, LIF	Composed of multimeric chains. Signals through JAK / STAT pathway using common signaling chain. Contains cytokine-binding chains.
Type II cytokine receptors	IFN-α/β, IFN-γ, IL-10, IL-20, IL-22, IL-28	Immunoglobulin-like domains. Uses heterodimer and multimeric chains. Signals through JAK / STAT.
Immunoglobulin superfamily receptors	IL-1, CSF1, c-kit, IL-18	Shares homology with immunoglobulin structures. Signals through NF-kB.
IL-17 receptor	IL-17, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F	Signals through NF-kB.
G Protein coupled receptor (GPCR)	IL-8, CC chemokines, CXC chemokine	Functions to mediate cell activation and migration.
TGF-β receptors 1/2	TGF-β	Signals through SMAD.
Tumor necrosis factor receptors (TNFR)	CD27, CD30, CD40, CD120, Lymphotoxin-β	Functions as co-stimulatory and co-inhibitory receptors.

CD, cluster of differentiation; c-kit, mas/stem cell growth factor receptor; CSF, colony-stimulating factor; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; IL, interleukin; JAK, janus kinase; STAT, signal transducer and activator of transcription; TGF, transforming growth factor.

Figure 16 shows the major signal pathways that are regulated by inflammatory cytokine signaling. The JAK/STAT and NF-kb signaling pathways are associated with most cytokine receptors, and stimulation of the receptors by chronic inflammatory cytokines can promote tumor progression. As shown in Table 6, IL-6, IL-10, IL-12, and IL-23 bind to type I/II cytokine receptors, which are associated with the JAK family. Stimulation of those type I/II cytokine receptors phosphorylates JAK or other tyrosine kinases and results in the phosphorylation of STAT family. STAT3 phosphorylation can accelerate tumor cell proliferation and cause anti-apoptotic responses through the stimulation of type I /II cytokine receptors.

TNF-α and IL-17 bind to the TNFR or IL-17R, and activate the NF-kB signaling pathway through activation of TRAF2/6. In cancer cells, activation of the NF-kB signaling pathway by TNF-α, increases anti-apoptotic responses, cancer proliferation, cancer invasion and angiogenesis. However, TRAIL, a member of the TNF super

family, plays a different role in tumors. TRAIL is mainly produced by activated T cells and NK cells, which are killing tumors. Unlike TNF-α, TRAIL is able to induce apoptosis in various tumor cell types, but has only negligible effects on normal cells (Idriss and Naismith, 2000; Takeda et al., 2007; Yagita et al., 2004). However, not all tumor cells are TRAIL sensitive, and NF-kB activation through TNF-α or other pro-survival factors can confer resistance to TRAIL-mediated cytotoxicity on tumor cells. For these reasons, we still need to understand the role of the TNF super family to optimize cytokine-based immunotherapeutic strategies.

IL-6 is also a well-studied inflammatory cytokine in cancer. IL-6 binds to the IL6R-gp130 receptor, and mediates its intracellular action through theJAK1/STAT3 pathway. Activation of gp130 by IL-6 triggers phosphorylation of JAK1, then phosphorylated JAK1 activates STAT1 and STAT3 molecules. They induce the expression of a large number of genes, among them target genes which promote transformation. Many chronic inflammatory cytokines bind with various types of receptors that activate oncogenic transcription factors in cancer cells which can contribute to tumor progression.

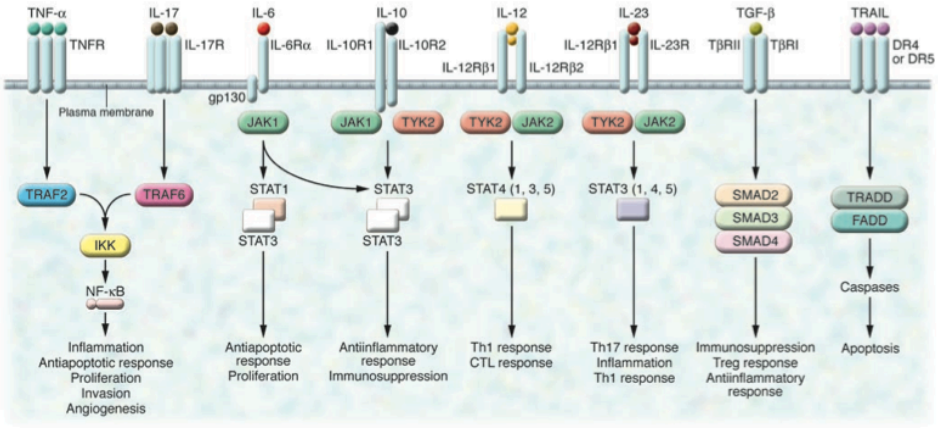


Figure 16. Signal transduction pathways and major biological responses of inflammation-modulating cytokines in cancer. Inflammatory cytokines stimulate their receptors, and the activated receptors then activate various oncogenic transcription factors in cancer cells results in increasing tumor progression (Lin and Karin, 2007).

1.4.2. Inflammation, tumor metastasis and epithelial mesenchymal transition (EMT)

Malignant tumors have a propensity to metastasize, a most detrimental event for tumor patients and inflammation and metastasis formation have been mechanistically linked. Cytokines, which activate type I/II cytokine receptors, can induce potentially oncogenic pathways such as JAK/STAT and NF-kB signaling.

These pathways can have central roles in tumor promotion and metastasis (Bollrath and Greten, 2009).

Inflammatory cytokines also contribute to metastasis formation through activation of epithelial mesenchymal transition (EMT) (López-Novoa and Nieto, 2009; Wu and Zhou, 2009; Zhou et al., 2012). EMT is a process that has initially been found in embryonic development and morphogenesis, chronic degeneration and fibrosis of mature organs, but also in cancer progression and metastasis formation (Eastham et al., 2007; Godde et al., 2010; Jiang et al., 2011; Nieto, 2002).

During embryogenesis, the EMT program regulates tissue-remodeling events: mesoderm formation, neural crest development, heart valve development, secondary palate formation and male Muellerian duct regression.

EMT plays an important role not only in embryonic development, but also in tumor invasion and metastasis formation. The process is characterized through the expression of mesenchymal marker genes. The hallmark of the EMT process is loss of E-cadherin expression, which has an important role in the epithelial phenotype. E-cadherin is a cell-cell adhesion molecule that forms epithelial adherent junctions. Loss of E-cadherin expression is associated with tumor grade and stage, because it results of diminished cell-cell adhesion. Several transcription factors have been discovered which regulate EMT, including mesenchymal marker genes of the Snail/Slug family, Twist, E12/E47, ZEB1 and SIP1. These factors suppress the expression of genes encoding cadherins, claudins, integrins, mucins, plakophilin, occludin and ZO1 to induce EMT.

The EMT process also can be controlled by extrinsic signals that cells receive from their microenvironment. TGF-β is an anti-inflammatory cytokine produced by cancer cells, myeloid cells, and T lymphocytes. TGF-β can act as a cytostatic cytokine that serves to arrest cell cycle progression and a tumor suppressor. However, TGF-β signaling is also an important regulator of EMT and metastasis (Yang and Weinberg, 2008). As shown in Table 6, TGF-β binds to the TGF-β receptor 1/2, which are activating Smad molecules. Upon stimulating TGF-β receptor, SMADs are phosphorylated, especially SMAD 2/3. SMAD 2/3 form a complex with SMAD4, and the complex translocates into nucleus to function as a transcription factor. The activation of SMADs through TGF-β signaling increases the expression of Slug and suppresses E-cadherin, and results In a promotion of the EMT process (Yang and Weinberg, 2008; Zavadil and ttinger, 2005).

The STAT3 and NF-kB signaling pathways are also involved in EMT induced by pro-inflammatory signals. IL-6, LIF, oncostatin M, and INF, activate JAK/STAT signaling through stimulation of type I/II cytokine receptors. Especially, STAT3 signaling is

associated with the expression of MMP-2 (Matrix metalloproteinase 2), which plays an important role in the process of invasion and metastasis of many malignant tumors. Moreover, STAT3 directly binds to the second proximal STAT3-binding site on the human Twist promoter and activates its transcription (Devarajan and Huang, 2009).

Interleukin-1 (IL-1) and TNF-α also have been reported to enhance EMT via activation of NF-kB signaling. Both inflammatory cytokines target the IKK complex, which comprises two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). The canonical NF-kB pathway depends on IKKγ and IKKβ kinase activity, and leads to the phosphorylation of the inhibitory IkBα, which is bound to NF-kB dimers in unstimulated cells. Phosphorylated IkBs are polyubiquitinated and subsequently degraded by the proteasome. NF-kB, in a complex with p50 and RelA, translocates to nucleus. Its target genes include genes encoding chemokines, cell survival, and innate immunity related genes. NF-kB signaling mediated chemokine secretion further recruits immune cells that release pro-inflammatory cytokines such as IL-1, TNF-α, and IL-6. These released pro-inflammatory cytokines activate again STAT3 and NF-kB signaling pathways, and this results in an enhancement of EMT in tumors (Bollrath and Greten, 2009). NF-kB not only regulates the NF-kB/IL-6/STAT3/EMT axis but also the expression of mesenchymal marker genes such as Vimentin, Tenascin-C, and Twist (Bollrath and Greten, 2009; Huber et al., 2004; Thiery and Sleeman, 2006).

Figure 17 shows the network of inflammatory cytokines, NF-kB and STAT3 signaling pathways and the EMT process. Pro-inflammatory cytokines bind to type I/II cytokine receptors, immunoglobulin superfamily receptors, or IL-17 receptor and activate NF-kB and STAT3 signaling pathways. The activation of these signaling pathways results in the enhancment of EMT.

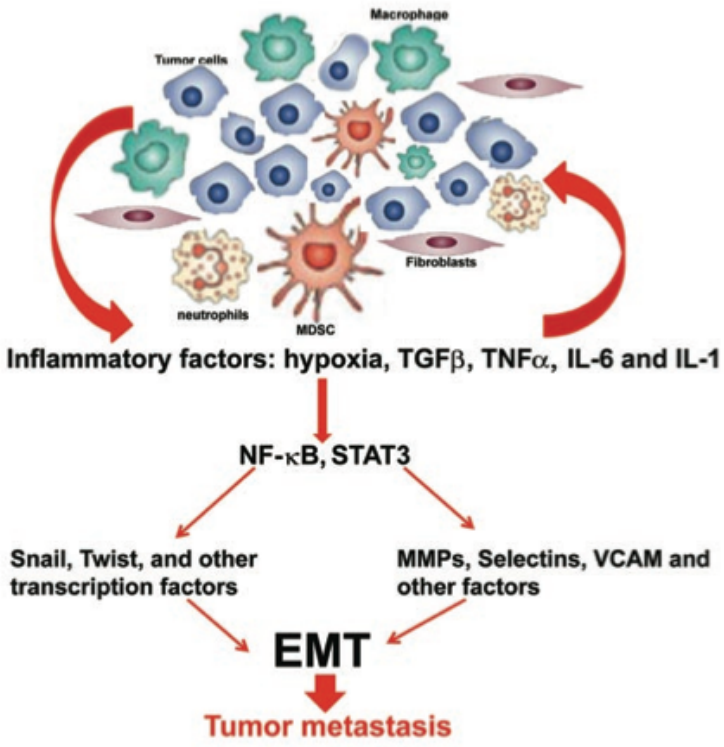


Figure 17. The network between inflammatory cytokines, NF-kB and STAT3 signaling pathways, and EMT. Tumor microenvironment secrets inflammatory cytokines to cancer cells, then NF-kB and STAT3 signaling pathways are activated resulting in increasing the EMT process in cancers (Wu and Zhou, 2009).

Recently, IL-8 has been identified as an important regulator of tumor progression. IL-8 recognizes a GPCR and triggers down stream signaling pathways which induce cell proliferation, survival, angiogenesis and invasion (Waugh and Wilson, 2008). IL-8 is also an inducer of EMT and establishes autocrine and paracrine loops in tumor metastasis (Palena et al., 2012). IL-8 is mainly released from monocytes and endothelial cells. However, recent reports show that IL-8 also can be secreted from other cell types, including fibroblasts, keratinocytes, and tumor cells (Xie, 2001). Brachyury, a T-box transcription factor, is a regulator of EMT in human carcinomas. It has been reported that brachyury induced EMT is characterized by enhanced IL-8/IL-8 receptor signaling and maintains the mesenchymal phenotype of brachyury overexpressing human tumor cells. In addition, overexpression of IL-8 in epithelial cancer cells increases brachyury mediated EMT in cancer cells. Conditioned medium from brachyury over-expressing mesenchymal tumor cells enhanced EMT of epithelial like cancer cells, including breast MCF7 and T47D luminal cancer cells. It increased the expression of brachyury, Snail and Slug (Fernando et al., 2011). These data suggest that IL-8 can enhance EMT in cancer cells by both autocrine and paracrine mechanisms (Palena et al., 2012).

1.5. MicroRNAs

High-throughput transcriptomic analyses has shown that eukaryotic genomes transcribe up to 90% of the genomic DNA (Kaikkonen et al., 2011). Only 1 ~ 2% of these transcripts encode proteins, whereas the vast majority of the genetic information is transcribed as non-coding RNAs (ncRNAs). Various ncRNAs are present in eukaryotic cells including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), long ncRNAs (lncRNAs), enhancer RNAs (eRNAs), and promoter-associated RNAs (PARs) (Kaikkonen et al., 2011). MicroRNAs are small non-coding RNAs (ncRNAs) that regulate gene expression post-transcriptionally. They consist of 21-25 nucleotides in length. A microRNA is partially complementary to one or more mRNA. The interaction of these RNA species suppresses translation and results in alterations in cellular phenotypes. Table 7 shows types and features of ncRNAs in eukaryotic genomes.

Table 7. Regulatory ncRNAs produced from eukaryotic genomes and their functions (Kaikkonen et al., 2011)**.**

Type	Length (nt)	Function
miRNA	20-24	Perfect complementary: Ago-2 mediated cleavage of mRNA Non-perfect complementary: Suppression of translation or mRNA degradation Minor functions: Transcriptional silencing / Translational activation
piRNA	24-31	Silencing of transposable elements in the germline
siRNA	20-24	Perfect match: Endonucleocytic cleavage Non-perfect match or endonuclease-inactive RISC: Translational repression or exonucleocytic degradation Other functions supported by Ago proteins: Induction of heterochromatin formation Silencing of the same locus from which they are derived
PAR	16-200	Partly unknown but indications of transcriptional regulation (example interaction with Polycomb group of proteins)
eRNA	100-9000	Mostly unknown but plays a role in transcriptional gene activation
lncRNA	>200	Chromatin remodeling Transcriptional regulation Post-transcriptional regulation Precursors for siRNAs Component of nuclear organelles

1.5.1. Biogenesis and function of microRNAs

MicroRNAs are transcribed from intragenic or intergenic regions. MicroRNAs are produced by cleavage of double stranded RNA arising from small hairpins within RNA that is mostly single stranded. Figure 18 shows the biogenesis of microRNAs from genomic DNAs. miRNAs are transcribed by polymerase II into primary transcripts, called pri-miRNA. The pri-miRNA undergoes further processing by the ribonucleases *Drosha*, thereby resulting in a hairpin intermediate of about 70-100 nucleotides, called pre-miRNA. Exportin 5 transport the pre-miRNA from the nucleus to the cytosol, then the pre-miRNA is processed by another ribonuclease, *Dicer*, into a mature double-stranded miRNA of variable length (20 ~ 24 nucleotides), called miRNA duplex.

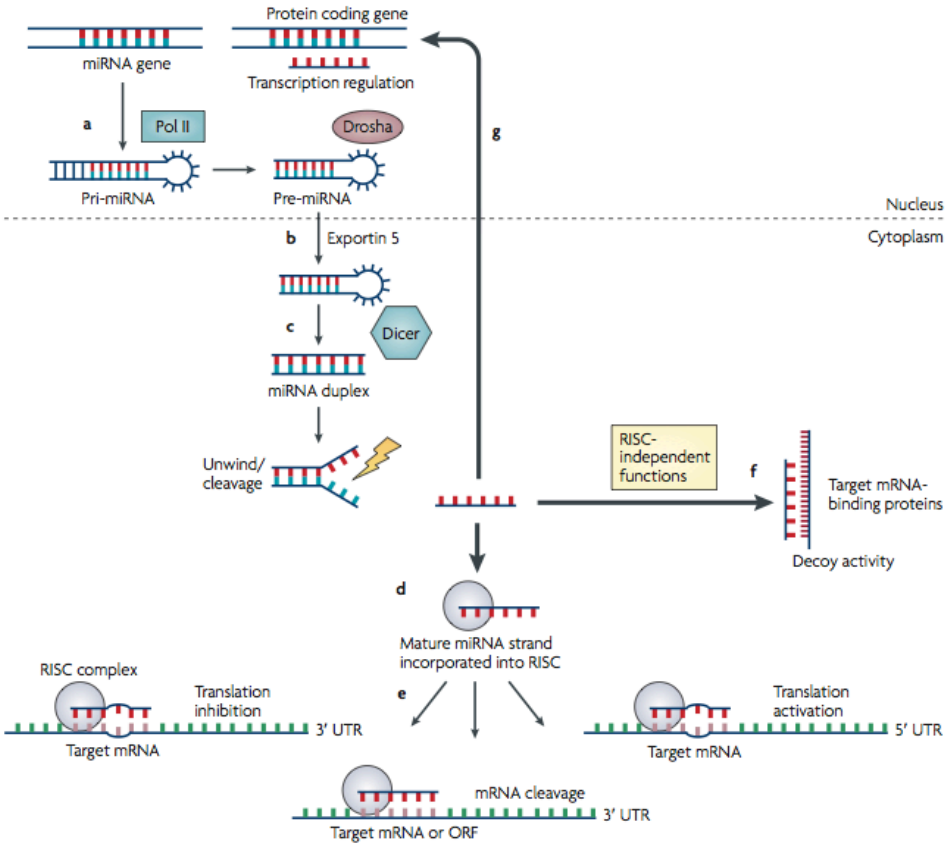


Figure 18. MicroRNA biogenesis and effector pathways. The gene of microRNA is processed into mature microRNAs by the actions of polymerase II, Drosha, and Dicer. Mature microRNA is incorporated into RISC that regulate translation of genes (Garzon et al., 2010).

After the double stranded structure is unwound into single strands, one mature miRNA strand is incorporated into a RNA induced silencing complex (RISC). The sequence recognized by the miRNA is usually present in the 3' UTR of the target. It suppresses translation of target mRNA by the action of translation inhibition or

mRNA cleavage. miRNAs can also directly bind to proteins, in particular RNA-binding proteins, without RISC formation, in a sequence dependent manner. The binding to proteins prevents these proteins from binding to their RNA targets and result in the suppression of protein translation. miRNAs can also have translational activation function. In some cases, miRNAs can bind to the open reading frame sequences or 5' UTR. These binding events are associated with translational activation functions of miRNAs (Stark et al., 2007; Ørom et al., 2008).

1.5.2. MicroRNA can cause epigenetic alterations in human cancer

Epigenetic modifications regulate gene expression patterns in embryonic development and cancer progression and miRNAs play important roles in the establishment of epigenetic programs during organismal development (Ren et al., 2009; Tzur et al., 2008). miRNAs thereby also regulate transcriptional gene expression patterns, signaling pathways and cellular phenotypes. Aberrant expression patterns of miRNAs are often detected in tumor cells when they are compared to the normal tissues. The de-regulated expression of miRNAs can partially be explained by epigenetic silencing of miRNA encoding sequences (Kala et al., 2013; Li et al., 2012; Wang and Shang, 2013).

De-regulation of miRNA expression patterns in cancer cells can yield effects on tumor progressions, similar to alteration of epigenetic modifications. For example, expression of miR-10b is regulated by Twist, and its over-expression increases EMT of breast cancer cells (Ma et al., 2007); on the contrary, miR-200c, miR-373 and miR-335 have been reported as metastasis suppressors in neuroblastoma and gastric cancer through the inhibition of ZEB1 and BMI1 (Png et al., 2011; Xu et al., 2012). miRNA also affect cancer cell drug sensitivity. The expression of miRNAs can increase drug sensitivity. miR-519c increases drug sensitivity of colon cancer cells by regulation of the ABCG2 gene (To et al., 2009). When the miR-519c is overexpressed, colon cancer has more sensitivity to mitoxantrone. Another miRNA, miR-15b and miR-16 negatively regulate the Bcl-2 protein level which also results in increased anti-cancer drug sensitivity in gastric cancer cells (Xia et al., 2008). Over-expression of miR-15b and miR-16 increases the sensitivity toward vincristine, Adriamycin, 5-fluorouracil, cisplatin, mitomycin, and etoposide. miR-451 increases doxorubicin sensitivity of breast cancer cells through the regulation of Mdr1/P-glycoprotein expression (Kovalchuk et al., 2008). These studies suggest that epigenetic alterations in cancer cells can cause the suppression of distinct miRNAs with tumor suppressor functions. The inhibition of these miRNAs enhances tumorigenesis and increases the

resistance to chemotherapeutic agents. Figure 19 shows that certain miRNAs can also function in cancer progression. MiRNAs regulate tumorigenesis, cancer invasion and metastasis and drug resistance through the inhibition of particular mRNA and signaling pathways.

Figure 19. MicroRNA regulate cellular functions during cancer progression. MicroRNAs have oncogenic and tumor suppressive functions in cancer. Expression of microRNAs effects tumor progression through regulating various signaling molecules, which are related with tumorigenesis, tumor invasion and metastasis, and drug resistance (Chen et al., 2012).

Epigenetic modifications can effect the expression of miRNAs, but miRNAs have also a reciprocal effect, and are themselves regulators of epigenetic modifications. This is achieved through the targeting of epigenetic regulators such as DNMTs or HDACs. Figure 20 and Table 8 show the suppression of epigenetic modulators by miRNAs. Epigenetic modifications, including DNA methylation, histone acetylation/de-acetylation, and suppression of gene expressions by polycomb group proteins, are regulated by the action of enzymes such as DNMTs, Polycomb repressive complexes (PRCs), and HDACs (Gruber and Zavolan, 2013). MiRNAs in turn regulate the expression of those enzymes. Epigenetic regulators are themselves subjected to regulation by epigenetic modifications with downstream consequences for the expression of tumor suppressor genes and oncogenes.

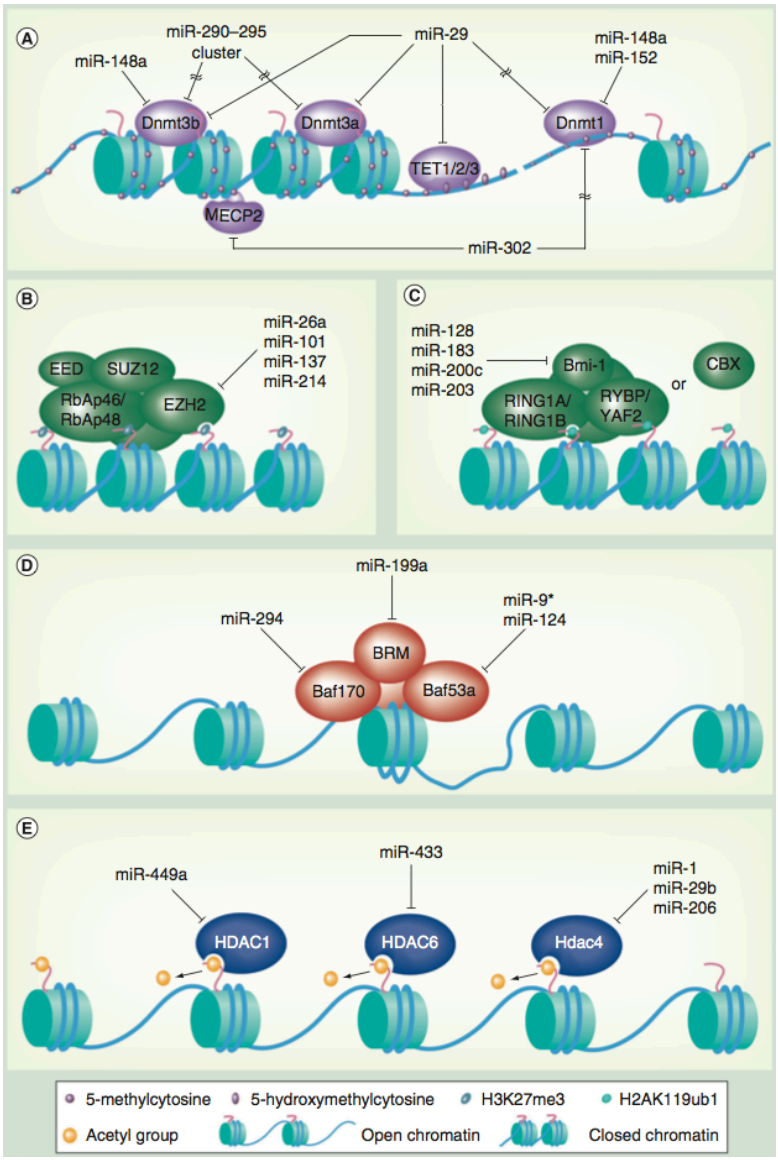


Figure 20. Epigenetic regulators that are targeted by miRNAs. MicroRNAs regulate directly or indirectly expression of epigenetic modulators such as (A) DNMTs, (B) PRC1, (C) PRC2, (D) SWI/SNF complex subunits and (E) HDACs (Gruber and Zavolan, 2013).

Table 8. List of microRNAs that regulate epigenetic regulators (Gruber and Zavolan, 2013)**.**

Target	miRNA	Epigenetic modification
MECP2	hsa-miR-302.	Metyl CpG binding protein 2. Complex with HDACs.
DNMTs	hsa-miR-148a, hsa-miR-29a/b/c, hsa-miR-152, hsa-miR-302. mmu-miR-290/295 cluster.	DNA methylation.
Tets (Ten-eleven translocation enzymes)	hsa-miR-29a, hsa-miR-26a.	DNA methylation.

EZH2	hsa-miR-26a, hsa-miR-101. mmu-miR-137, mmu-miR-214.	Histone methylation.
BMI-1	hsa-miR-128. mmu-miR-128, mmu-miR-183, mmu-miR-200c, mmu-miR-203.	Polycomb complex protein. Histone H3K27 methylation.
BAF170	mmu-miR-294.	SWI/SNF proteins. Regulation of chromatin remodeling.
BRM	hsa-miR-199a.	
BAF53a	mmu-miR-9, mmu-miR-124.	
HDACs	hsa-miR-449a, hsa-miR-433. mmu-miR-1, mmu-miR-29b, mmu-miR-206.	Histone de-acetylation

has: Homo sapiens; mmu: Mus musculus.

1.5.3. The role of the miRNA 302/367 cluster in the reprogramming process

During embryonic development, epigenetic modifications are most important in the regulation of cell fate. ES cells transcribe a limited number of miRNAs that are restricted to the pluripotent state and rapidly decrease during differentiation (Houbaviy et al., 2003). *Marson et al.* showed the expression of reprogramming factors and the expression of stem cell specific miRNAs are functionally linked (Marson et al., 2008). Chromatin immunoprecipitation experiments and enforced expression of reprogramming factors have shown that the factors bind to stem cell specific miRNA promoters and induce the transcription of stem cell specific miRNAs, including the miR-302/367 cluster and the miR-290/371 cluster (Marson et al., 2008).

The expression of the miRNA 302/367 cluster (miR 302/367 cluster), which is used in this study, enhances stem cell pluripotency and ES cell specific gene expression. It exerts its effects through the inhibition of cell cycle related genes, EMT regulators and epigenetic regulators (Figure 21) (Onder and Daley, 2011). The miR 302/367 cluster induces G1-S phase transition through inhibition of the Cyclin dependent kinase inhibitor CDkn1a/p21 which prevents the inactivation of p27 and arrests cells in the G1 phase (Card et al., 2008). In addition, the miR 302/367 cluster increases mesenchymal to epithelial transition (MET), the reverse process of EMT, by inhibition of the TGF-β receptor 2 and enhanced expression of E-cadherin during the reprogramming process (Liao et al., 2011). MET initiates the reprogramming process

upon transduction of the four reprogramming factors. It also enhances the endogenous expression of reprogramming factors in somatic cells (Li et al., 2010).
The miRNA 302/367 cluster also regulates epigenetic modifications in cells; it directly regulates the expression of methyl binding domain (MBD), which inhibits target gene expressions through binding to methylated DNA. Over-expression of the miR 302/367 cluster suppresses expression of MBD and results in an increase of the expression of pluripotency genes (Ryul Lee et al., 2012). In addition, the microRNA 302/367 cluster indirectly regulates histone methylation through inhibition of BMP receptors (Kang et al., 2012; Krishnakumar and Blelloch, 2013).
Since *Yamanaka* and *colleagues* identified the four reprogramming transcription factors, the possibility has been considered that reprogrammed cells might acquire tumorigenic properties. Two of the factors, Myc and Sox2, had previously been recognized as oncogenes. A most interesting aspect emerged from the comparison of iPS cells induced by the ectopic expression of the reprogramming factors and the expression of the miRNA 302/367 cluster. It was found that factor induced cells can cause teratomas upon transplantation whereas miRNA 302/367 cluster induced cells do not (Koide et al., 2012; Lin et al., 2010; Lin and Ying, 2013). In addition, it has been shown that the microRNA 302/367 cluster expression might evebn have tumor suppressive functions in skin, cervical and breast cancer cells. This is most likely due to the inhibition of AKT signaling (Cai et al., 2012; Liang et al., 2012; Lin et al., 2008).
However, not all signaling events induced upon miRNA 302/367 cluster expression in normal and cancer cells have been characterizes. AKT is an important signaling molecule which enhances pluripotency and self-renew in stem cells, AKT can also regulate tumorigenesis and EMT in cancer cells (Bellacosa et al., 2005; Boccaccio and Comoglio, 2006; Nakamura et al., 2008; Watanabe et al., 2006). The precise details of the effects of miRNA 302/367 cluster in the reprogramming process are still not completely known. Opposing observations have been made. Activation of AKT signaling seems to enhance the reprogramming efficiency during the reprogramming process triggered upon the transduction of transcription factors. Phosphorylated AKT seems to decrease the reprogramming efficiency in the reprogramming process upon somatic cell nuclear transfer (Nakamura et al., 2008). These data suggest that the miRNA 302/367 cluster mediated reprogramming process might rely on different mechanisms than reprogramming induced by the four factors. The miR 302/367 cluster mediated reprogramming may more closely resemble the process occurring upon somatic cell nuclear transfer.
The miR 302/367 cluster largely regulates epigenetic modifications in somatic cells and enhances somatic cell reprogramming into pluripotent stem cells accompanied

by the suppression of tumorigenesis. For this reason, we used the miRNA 302/367 cluster to influence glioblastoma cancer cell phenotypes and prod the cells towards a more normal behaviour.

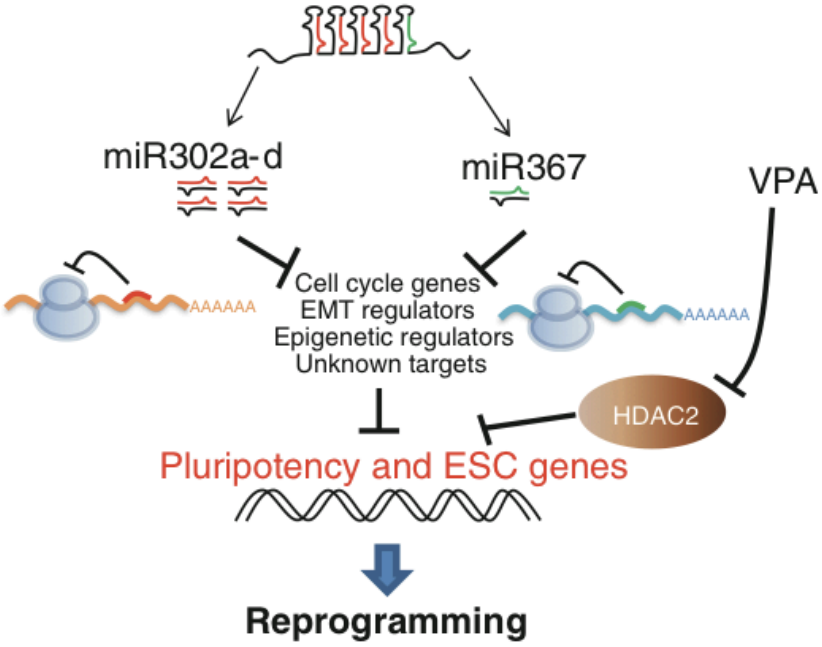


Figure 21. microRNA 302/367 cluster mediated reprogramming. The microRNA 302/367 cluster enhances reprogramming process through inhibition of cell cycle genes, EMT regulators, and epigenetic regulators in normal cells with special conditioned medium (Onder and Daley, 2011).

1.6. Glioblastoma multiform (GBM)

Glioblastoma multiforme (GBM; WHO classification name "Glioblastoma") is the most common and aggressive malignant brain tumor in humans. Treatment of glioblastoma includes chemotherapy, radiation, and surgery. The median survival interval for GBM patients is about 15 months after diagnosis. The etiology of GBM is unknown. Only a few environmental and genetic risk factors are known. These include exposure to ionizing radiation and smoking, hyper-activation of EGF signaling, loss of MGMT expression, TP53 mutations and loss of PTEN function.

High-grade glioblastoma patients have aberrations in EGFR signaling and elevated activity of oncogenic signaling through the PI3K/AKT, STAT3 and Ras/MAPK signaling pathways (Hatanpaa et al., 2010). In addition, 75% of secondary glioblastomas show a loss-of-function of MGMT via methylation of the CpG islands in the MGMT promoter region. This reduces the cytotoxicity of alkylating chemotherapeutic agents (Dunn et al., 2009). Loss of function mutations of PTEN

are also frequently found in GBM. This increases the activation of AKT signaling (Baeza et al., 2003). GBM cells are affected by genetic and epigenetic alterations.

1.6.1. Epigenetic alterations in GBM

GBM cells harbor epigenetic alterations, a feature associated with GBM grade and prognosis. Table 9 shows the genetic alterations, epigenetic alterations, and tumor tissue related protein expression profiles in primary and secondary GBM (Ohgaki and Kleihues, 2007).

Table 9. Genetic and epigenetic changes in primary and secondary GBM (Ohgaki and Kleihues, 2007).

	Primary GBM	Secondary GBM
Genetic alterations		
TP 53 mutations	28%	65%
EGFR amplification	36%	8%
PTEN mutation	25%	4%
$p16^{INK4a}$ deletion	31%	19%
LOH 1p	12%	15%
LOH 10p	47%	8%
LOH 10q	47%	54%
LOH 13q	12%	38%
LOH 19q	6%	54%
LOH 22q	41%	82%
Promoter methylation		
$p14^{ARF}$	6%	31%
$p16^{INK4a}$	3%	19%
RB1	14%	43%
MGMT	36%	75%
TIMP-3	28%	71%
Expression profiles		
Fas (APO-1 / CD95)	100%	21%
Survivin	83%	46%
MMP-9	69%	14%
EGFR	63%	10%
MDM2	31%	0%
VEGF	High	Low

VEGF fms-related tyrosine kinase 1	High	Low
IGFBP2	High	Low
Tenascin-X-precursor	High	Low
Enolase 1	High	Low
Centrosome-associated protein 350	High	Low
TP53	37%	97%
ASCL 1	33%	88%
Loss of TIMP-3	17%	64%
PDGF-AB	Low	High
ERCC6	Low	High
DUOX2	Low	High
HNRPA3	Low	High
WNT-11 protein precursor	Low	High
Cadherin-related tumor suppressor	Low	High
ADAMTS-19	Low	High

As shown in Table 9, both primary and secondary GBM have mutations in tumor suppressor genes and epigenetic alterations have been detected in both primary and secondary GBM. The origin of GBM is still unclear. However, the promoter methylation and gene expression profiles of primary and secondary GBM strongly suggest that the epigenetic alterations occur in different types of GBM. Secondary GBMs have a higher overall frequency of promoter methylation when compared to primary GBMs, this is at least true for the promoters of $p14^{ARF}$, $p16^{INK4a}$, RB1, MGMT, and TIMP3 (Table 9). Moreover, BMI-1 has frequent copy number alterations in both low- and high-grade gliomas, and BMI-1 deletion is strongly associated with poor prognosis in gliblastoma patients (Häyry et al., 2008). HDACs are down-regulated in high-grade GBM when compared to low-grade astrocytomas and normal brain tissues, and hyper-acetylation of histone H3 and H4 were frequently found in high-grade GBM (Lucio-Eterovic et al., 2008). These studies strongly suggest that GBM malignancy is highly associated with epigenetic alterations, and that epigenetic therapeutic strategies might become useful.

1.7. Aim of this study

Cancer is not only a genetic disease, but also epigenetic factors contribute to its etiology. Cancer cells have de-differentiation phenotypes, express reprogramming factors and stem cell genetic programs. These features could be based on epigenetic alterations (Ghosh et al., 2011; Schoenhals et al., 2009b). The expression of reprogramming factors is highly associated with tumor grade and prognosis (Schoenhals et al., 2009a).

Gioblastoma multiforme is a most aggressive cancer in humans. Many epigenetic alterations have been detected in GBM patients, and they are associated with glioma-grade. High-grade gliomas exhibit particular histone modifications correlated with an unfavorable prognosis in glioblastoma patients (Häyry et al., 2008; Lucio-Eterovic et al., 2008). Reprogramming factor expressions and the target genes regulated by these factors have been studied in gliomas of different grades. Both groups are highly expressed in high-grade gliomas such as GBM grade4 when compared to low-grade gliomas and normal brain tissues (Ben-Porath et al., 2008). Those studies suggest that GBM aggressiveness and prognosis are associated with epigenetic alterations stemming from the action of the reprogramming factors and that epigenetic therapeutic strategies couls possibly become beneficial.

Cellular differentiation of embryonic stem cells is accompanied by a loss of lineage potential and increasing functional restrictions. Since *Yamanaka* and *colleagues* found four transcription factors able to reprogram mammalian somatic cells into induced pluripotent stem cells. This is most likely achieved through epigenetic modifications (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In addition, *Morrisey* and *colleagues* found that miRNAs can also trigger somatic cell reprogramming into pluripotent stem cells, under special medium conditions (Anokye-Danso et al., 2011). Although the signaling events which confer transcription factor mediated reprogramming or miRNA mediated reprogramming are still only partially known, the experiments have clearly shown that the reprogramming processes is reversible through the manipulation of epigenetic parameters.

MiRNAs are small non-coding RNA molecules that consist of 21-25 nucleotides in length. MicroRNAs regulate the expression of large numbers of genes during embryonic development, but also in cancer cells. The miR 302/367 cluster is able to induce somatic cells into pluripotent stem cells, these cells are non-tumorigenic when they are kept in a special conditioned medium (Lin and Ying, 2013). A small number of publications have addressed the functions exerted by miR 302/367 cluster

expression in the reprogramming process of somatic cells and tumor cells, but the mechanisms involved are still largely unknown.

miRNAs can regulate tumor microenvironments through the regulation of the expression and secretion of inflammatory cytokines (Grivennikov et al., 2010). Inflammatory cytokines are highly associated with tumor invasiveness and metastasis and the regulation of epithelial to mesenchymal transition (López-Novoa and Nieto, 2009; Waerner et al., 2006; Wu and Zhou, 2009). Stimulation of type I/II cytokine receptors and G protein coupled receptors, activate oncogenic signaling pathway such as JAK/STAT3 and NF-kB signaling pathways. These oncogenic signaling pathways can increase tumorigenesis and metastasis.

Figure 22 shows the strategy of this study. GBM exhibits epigenetic alterations that promote tumorigenesis, drug resistance and tumor invasiveness. For these reasons, we investigated the possibility to reprogramm GBM cells through miRNA 302/367 cluster expression the ensueing epigenetic modifications into a non-tumorigenic state. The reprogrammed GBM cells were investigated for manifestations of the transformed phenotype. In reprogrammed cells all major transformation parameters were suppressed and an extensive cellular normalisation was observed.

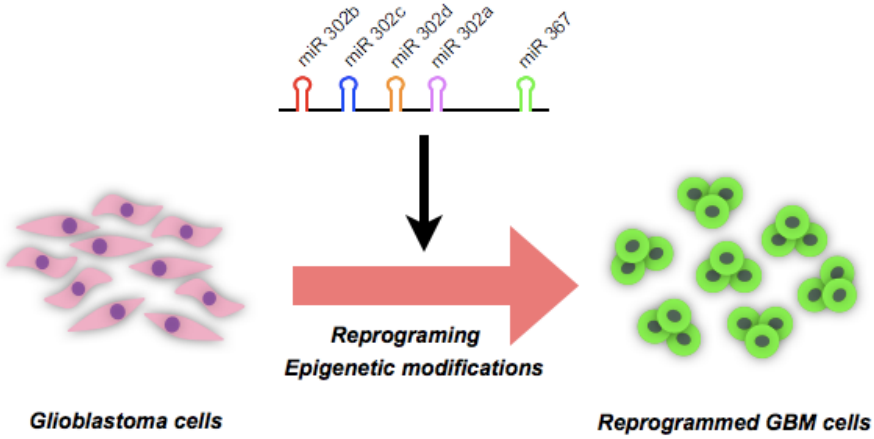


Figure 22. Aim of this study. The hypothesis of this study is reprogramming of glioblastoma cancer cells to lose their transformation phenotypes upon induction of the microRNA 302/367 cluster.

2. Materials

2.1. Cell lines

The following cell lines were used in this work and are in alphabetical order.

Table 10. Summary of human cell lines, their culture medium and origin

Cell line	Culture medium	Origin	Source
BJ	DMEM+++	Foreskin fibroblasts	Stemgent; Bergisch Gladbach
LN464	DMEM+++	Glioblastoma	Sabrina Genssler; AG Wels, GSH
MCF-7	DMEM+++	Breast cancer	ATCC; Wesel
MCF-10A	Growing medium	Healthy breast tissue	ATCC; Wesel
MDA-MB-231	DMEM+++	Breast cancer	ATCC; Wesel
MDA-MB-468	DMEM+++	Breast cancer	ATCC; Wesel
SF767	DMEM+++	Glioblastoma	Sabrina Genssler; AG Wels, GSH
SK-BR-3	DMEM+++	Breast cancer	Sabrina Genssler; AG Wels, GSH
SKMG-3	DMEM+++	Glioblastoma	Sabrina Genssler; AG Wels, GSH
U178	DMEM+++	Glioblastoma	Sabrina Genssler; AG Wels, GSH
U373	DMEM+++, 1X MEM Non Essential Amino Acids	Glioblastoma	ATCC; Wesel
U87MG	DMEM+++	Glioblastoma	ATCC; Wesel
UW28	DMEM+++	Glioblastoma	Sabrina Genssler; AG Wels, GSH
pLVET-tTR-KRAB vector infected cells	Tetracycline-free medium		

+++: DMEM including 10% fetal calf serum; 10ug/ml streptomycin, 100U/ml penicillin and 2uM glutamine

Growing medium: DMEM/F-12 including 5% FCS, 100U/ml penicillin, 100µg/ml streptomycin 2mM glutamine 20ng/ml hEGF 100ng/ml Choleratoxin, 10µg/ml Insulin and 500 ng/ml Hydrocortison

Tetracycline-free medium: DMEM including 10% Tet System Approved FBS (Clontech), 10ug/ml streptomycin, 100U/ml penicillin and 2uM glutamine

2.2. Bacteria

Table 11. Summary of bacterial strains used in this study

Strain	genotype	producer	resistence
One Shot® TOP10F`chemically Competent *E.coli*	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-	Invitrogen (Life Technologies)	TetR

Top10 bacteria were grown in LB medium

LB(Luria Bertani-Broth) medium:

5g/l yeast extract, 10g/l NaCl, 10g/l Trypton with pH 7.5

2.3. Antibiotics

Table 12. Summary of antibiotics used in this study

Antibiotics	Producer	Final concentration
Ampicillin	AppliChem GmbH; Darmstadt	10ug/ml
Kanamycin	AppliChem GmbH; Darmstadt	50ug/ml
Doxycycline hyclate	Sigma Aldrich Chemie GmbH; Steinheim	2ug/ml

2.4. Oligonucleotides

Table 13. Summary of oligonucleotides used in this study

Oligonucleotides	Sequence 5' > 3'
Cloning miR 302/367 Not1 fwd	CCCAGTAGAGCGGCCGCCGCGGATCCAGGACCTACTTTCCCCAGAGC
Cloning miR 302/367 Not1 rev	AGATCTCAGCGCCGGCGCCGCTCGAGTTTAACCAGTTTAACCACAAC
miR 302b stem-loop fwd	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGAAG
miR 302c stem-loop fwd	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAGGT
miR 302d stem-loop fwd	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAAGT

miR 302a stem-loop fwd	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCAAG
miR 367 stem-loop fwd	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGTTG
U6 Stem-loop fwd	CGCTTCACGAATTTGCGTGTCAT
miR 302b fwd	GGCGGCACTTTAACATGGGAATG
miR 302c fwd	GGCCGTGCTTTAACATGGGG
miR 302a fwd	GGCCGGACTTAAACGTGGTTGTA
miR 302d fwd	GTCCGGACTTTAACATGGAGGCA
miR 367 fwd	AGCCGGACTGTTGCTAACATGC
miR universal RT-PCR rev	GCTTCGGCAGCACATATACTAAAAT
U6 RT-PCR rev	Same with U6 stem-loop fwd
OCT3/4 fwd	AGCAAAACCCGGAGGAGT
OCT3/4 rev	CCACATCGGCCTGTGTATATC
SOX2 fwd	GCCGAGTGGAAACTTTTGTCG
SOX2 rev	GCAGCGTGTACTTATCCTTCTT
KLF4 fwd	ATTGGACCCGGTGTACATTC
KLF4 rev	AGCACGAACTTGCCCATC
NANOG fwd	ATGCCTCACACGGAGACTGT
NANOG rev	AGGGCTGTCCTGAATAAGCA
LIN28 fwd	CTGTCCAAATGCAAGTGAGG
LIN28 rev	GCAGGTTGTAGGGTGATTCC
cMyc fwd	CACCAGCAGCGACTCTGA
cMyc rev	GATCCAGACTCTGACCTTTTGC
STAT3 fwd	AGCAGCTTGACACACGGTA
STAT3 rev	AAACACCAAAGTGGCATGTGA
ERα fwd	ACAAGGGAAGTATGGCTATGGA
ERα rev	GGTCTTTTCGTATCCCACCTTTC
ERβ fwd	GCTCAATTCCAGTATGTACCCTC
ERβ rev	CCATGCCCTTGTTACTCGCA

PR fwd	ACATGGTAGCTGTGGGAAGG
PR rev	GCTAAGCCAGCAAGAAATGG
Cyp19a1 fwd	ATGGTTTTGGAAATGCTGAACCC
Cyp19a1 rev	AGGACCTGGTATTGAGGATGTG
EGFR fwd	GGTGTGTGCAGATCGCAAAG
EGFR rev	GACATGCTGCGGTGTTTTCAC
HER2 fwd	CCAGGACCTGCTGAACTGG
HER2 rev	TGTACGAGCCGCACATCC
HER3 fwd	ACATCGTGAGGGACCGAGAT
HER3 rev	CCATTACACTGAGGAGCACAGAT
HER4 fwd	CAGCCCAGCGATTCTCAGTC
HER4 rev	CCCATGACAACCTCACAGTTTT
Survivin fwd	GGACCACCGCATCTCTACAT
Survivin rev	GACAGAAAGGAAAGCGCAAC
CCND1 fwd	GTGCTGCGAAGTGGAAACC
CCND1 rev	ATCCAGGTGGCGACGATCT
BCL-XL fwd	GAGCTGGTGGTTGACTTTCTC
BCL-XL rev	GGGGCCTCAGTCCTGTTCT
TCF3 fwd	TATTTCGCCGAAGTGAGAAGGC
TCF3 rev	GGGGTCCGTTGGAGAGGTA
Dax1 fwd	AGCACAAATCAAGCGCAGG
Dax1 rev	GAAGCGCAGCGTCTTCAAC
Sall4 fwd	CCAGGGAATGACGAGGTGG
Sall4 rev	GAACTCCGCACAGCATTTCTC
Jarid2 fwd	AGCAGGTCAACACGGGAGA
Jarid2 rev	TCGAGTGACTCCGTTTACCTTA
Mtf2 fwd	GTCCACCTGGCCCATATACAA
Mtf2 rev	CCGTGAAATCCACATCTGAGG
Suz12 fwd	CCCACAGGTAAAAAGCAGGTG

Suz12 rev	GAAACTGCAAGGGACGGGAA
Rnf2 fwd	CACAGCCCTTAGAAGTGGCAA
Rnf2 rev	GTCTGGCCTTAGTGATCTTTTGG
Max fwd	TGGAGAAGGCGAGGTCAAGT
Max rev	CCCCATCGAAGGCAGAGAT
Trrap fwd	GACAGTGAGACTCGAACACATT
Trrap rev	CAAGGGCACAAACTCAGCAAC
E2F1 fwd	CATCCCAGGAGGTCACTTCTG
E2F1 rev	GACAACAGCGGTTCTTGCTC
E2F4 fwd	ATCGGGCTAATCGAGAAAAAGTC
E2F4 rev	TGCTGGTCTAGTTCTTGCTCC
N-cadherin fwd	AGCCAACCTTAACTGAGGAGT
N-cadherin rev	GGCAAGTTGATTGGAGGGATG
Snail fwd	ACCACTATGCCGCGCTCTT
Snail rev	GGTCGTAGGGCTGCTGGAA
Slug fwd	TGTTGCAGTGAGGGCAAGAA
Slug rev	GACCCTGGTTGCTTCAAGGA
Vimentin fwd	TCTACGAGGAGGAGATGCGG
Vimentin rev	GGTCAAGACGTGCCAGAGAC
E-cadherin fwd	TCCCATCAGCTGCCCAGAAA
E-cadherin rev	TGACTCCTGTGTTCCTGTTA
IL6 fwd	CCTGAACCTTCCAAAGATGGC
IL6 rev	TTCACCAGGCAAGTCTCCTCA
IL8 fwd	TTTTGCCAAGGAGTGCTAAAGA
IL8 rev	AACCCTCTGCACCCAGTTTTC
MCP1 fwd	CAGCCAGATGCAATCAATGCC
MCP1 rev	TGGAATCCTGAACCCACTTCT
GAPDH fwd	TCGGAGTGAACGGATTTG
GAPDH rev	CCTGGAAGATGGTGATGG

S18 fwd	GGCTACCACATCCAAGGAAG
S18 rev	TCCAATGGATCCTCGCGGAA

2.5. Plasmids

Table 14. Summary of plasmid DNA used in this study

Plasmids	Description	Source
pLVET-tTR-KRAB vector	Mammalian expression vector, Tet-regulated (Tet-on) lentiviral vector for transgene, hEF-1 alpha promoter, GFP expression, Ampicillin resistance	Addgene, Szulc et al., 2006
pLVET-miR 302/367 cluster	Transgene: A part of LARP7 genomic DNA which containing the microRNA 302/367 cluster whole sequence with Not1 restriction site	This study
pCMVΔ8.91	Mammalian expression vector, Expression of gag, pol, rev and tat gene. Derived from HIV1 genome, CMV promoter, Ampicillin resistance	Dr.M.Grez, GSH
pMD2.VSV-G	Mammalian expression vector, Expression of envelope protein for Vesicular Stomatitis Virus (VSV-G), CMV promoter, Ampicillin resistance	Dr.M.Grez, GSH

2.6. Antibodies

Table 15. Summary of antibodies used in this study

Antigen	Species	Producer
Actin (#2066)	Rabbit	Sigma Aldrich
Lamin B1 (ab16048)	Rabbit	Abcam
b-Tubulin	Mouse	Sigma Aldrich
GFP (#2555)	Rabbit	Cell signaling technology

STAT3 (#sc-482)	Rabbit	Santa Cruz
pSTAT3^{Tyr705} (#9145,XP)	Rabbit	Cell signaling technology
PIK3CA (p100a) (#4249)	Rabbit	Cell signaling technology
PIK3R1 (p85a) (#4257)	Rabbit	Cell signaling technology
AKT (#4691)	Rabbit	Cell signaling technology
pAKT^{ser473} (#4060)	Rabbit	Cell signaling technology
NANOG (#4903)	Rabbit	Cell signaling technology
OCT-4A (#2840)	Rabbit	Cell signaling technology
SOX2 (#3579)	Rabbit	Cell signaling technology
KLF4 (#4038)	Rabbit	Cell signaling technology
cMYC (#5605)	Rabbit	Cell signaling technology
LIN28 (#3695)	Rabbit	Cell signaling technology
E-Cadherin	Rabbit	Cell signaling technology
Vimentin	Rabbit	Cell signaling technology
b-catenin	Rabbit	Cell signaling technology
Ki67[SP6] (ab16667)	Rabbit	Abcam
Mouse-HRP	HRP-conjugated 2nd Antibody	Amersham (GE Healthcare)
Rabbit-HRP (#7074)	HRP-conjugated 2nd Antibody	Cell signaling technology

Mouse-HRP secondary antibody was diluted 1:5000 in 3% BSA in TBS-T and Rabbit-HRP secondary antibody was diluted 1:3000 in 3% BSA in TBS-T. Primary antibody attached membranes were incubated with HRP-conjugated 2nd antibodies for 1 hours at room temperature

2.7. Mouse strain (NSG mice)

Table 16. Summary of NSG mice used in this study

Strain	Description	Origin
NSG(Humanized mice)	NOD scid IL2 receptor gamma chain knockout mice. This strain is the most highly	Charles River Laboratories, 976333 Sulzfeld, Germany

	immunodeficient mice, engrafting the widest range of human cells and tissues. For these reasons, this strain used for cancer xenograft modeling, stem cell biology, humanized mice and infectious disease research	

2.8. Chemicals

Table 17. List of used reagents

chemical/reagent	Supplier
Acetic acid	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Ampicilin	AppliChem, Darmstadt Germany
APS	Sigma-Aldrich, Munich, Germany
Bradford reagent	Bio-Rad Lbaoratories GmbH, Munich, Germany
BSA	Sigma-Aldrich, Munich, Germany
CaCl2	Sigma-Aldrich, Munich, Germany
Chloroqine	Sigma-Aldrich, Munich, Germany
DMEM	Lonza Group Ltd, Basel, Switzerland
DMSO	Carl Roth GmbH + Co KG, Karlsruhe, Germany
DNA ladders	Fermentas GmbH, St. Leon-Rot, Germany
dNTPs	Fermentas GmbH, St. Leon-Rot, Germany
DPBS	Lonza Group Ltd, Basel, Switzerland
DTT	AppliChem, Darmstadt Germany
ECL	Amersham (GE Healthcare)
Extracellular matrix (ECM) gel	Sigma-Aldrich, Munich, Germany
EDTA	AppliChem, Darmstadt Germany
EGF (human)	BD Bioscience Heidelberg, Germany
Ethidiumbromid solution	AppliChem, Darmstadt Germany
Ethanol	Carl Roth GmbH + Co KG, Karlsruhe, Germany

FCS	Life Technologies GmbH, Darmstadt, Germany
Formalin	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Glycerol	AppliChem, Darmstadt Germany
isopropanol	Carl Roth GmbH + Co KG, Karlsruhe, Germany
KCL	Carl Roth GmbH + Co KG, Karlsruhe, Germany
KH2PO4	AppliChem, Darmstadt Germany
KHCO3	Carl Roth GmbH + Co KG, Karlsruhe, Germany
L-Glutamin	PAA Laboratories GmbH, Pasching, Germany
MgCl2	Sigma-Aldrich, Munich, Germany
NaCl	AppliChem, Darmstadt Germany
NaOH	AppliChem, Darmstadt Germany
NP40	Sigma-Aldrich, Munich, Germany
PBS	Lonza Group Ltd, Basel, Switzerland
Penicillin/Streptomycin	PAA Laboratories GmbH, Pasching, Germany
Polyacrylamid Rotiphorese Gel 30	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Protease inhibitor cocktail III & IIV	Merck KGaA, Darmstadt, Germany
Protein ladder	Fermentas GmbH St. Leon-Rot, Germany
Restriction enzymes	New England Biolabs GmbH, Frankfurt a M. Germany
SDS	Carl Roth GmbH + Co KG, Karlsruhe, Germany
Sodiumacetate	Sigma-Aldrich, Munich, Germany
TEMED	Sigma-Aldrich, Munich, Germany
Tris-HCL	AppliChem, Darmstadt Germany
Triton X-114	Sigma-Aldrich, Munich, Germany
Trypsin-EDTA	Life Technologies GmbH, Darmstadt, Germany
trypton	AppliChem, Darmstadt Germany
Tween-20	Carl Roth GmbH + Co KG, Karlsruhe, Germany
trypton	AppliChem, Darmstadt Germany
urea	AppliChem, Darmstadt Germany
yeast extract	AppliChem, Darmstadt Germany

ß-Mercaptoethanol	Carl Roth GmbH + Co KG, Karlsruhe, Germany

2.9. Buffer and solutions

Table 18. List of used buffers

Buffer	Contents	Usage
5X SDS sample buffer	60 uM Tris-Cl (pH 6.8), 25% glycerol, 2% SDS, 5% b-mercaptoethanol, 0.1 % bromophenol blue, distilled water	Western blot
10X gel running buffer	30 g Tris, 144 g Glycine, 10 g SDS, distilled water up to 1L	Western blot
10X TE buffer	100 mM Tris-Cl (pH7.5), 10 mM EDTA (pH 8.0), distilled water	Buffer for dissolving plasmids DNA and oligonucleotides
10% (W/V) Ammonium Persulfate (APS)	1 g Ammonium persulfate, distilled water up to 10 mL	SDS page gel
Crystal violet	0.5% crystal violet solution in 25% methanol.	Staining of membranes of living cells
2.5M $CaCl_2$	2.5 M $CaCl_2$ in distilled water,	Ca_2PO_4 transfection
Hypotonic Buffer	20 mM Tris-Cl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$	Cellular protein fractionation
Cell Extraction Buffer	100 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM EGTA, 0.1% SDS in distilled water	Cellular protein fractionation
RIPA buffer (1% PBTx) (Radio-immunoprecipitation assay)	50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 % triton x-100, PBS	Cell lysis
Semi-dry-transfer buffer	5.82 g Tris, 2.93 g Glycin, distilled water up to 1L	Western blot
Solution A	Rotiphorese Gel 30 (37, 5:1) (30 % Acrylamide)	SDS page gel
Solution B (pH 8.8)	1.5 M Tris-Cl, 0.4% SDS, distilled water	SDS page gel
Solution C (pH 6.8)	0.5 M Tris-Cl, 0.4% SDS, distilled water	SDS page gel
2X HBSS	0.28M NaCl, 0.05M HEPES, 1.5mM Na2HPO4 in distilled water, pH 7.05 adjusted with 5N NaOH, Solutions were filtered with 0.45um nitrocellulose filter, Store at 4°c	Ca_2PO_4 transfection

2.10. Kits and ready-to-use solutions

Table 19. Summary of used kits and ready-to-use solutions

Name	Purpose	Producer
Cell Proliferation Kit (CCK-8)	Cell proliferation, Drug sensitivity	Sigma-Aldrich, Munich, Germany
Cytokine Array	Detection of soluble cytokines	Hoelzel Diagnostika (Raybiotech), Koeln, Germany
First Strand cDNA Synthesis Kit	cDNA synthesis from isolated mRNA	Fermentas GmbH St. Leon-Rot, Germany
Lipofectamine LTX & Plus Reagent	Plasmid DNA transfection into cultured cells	Life Technologies GmbH, Darmstadt, Germany
miRVana™ miRNA Isolation Kit	Isolation of mature microRNAs from cultured cells	Life Technologies GmbH, Darmstadt, Germany
NucleoBond Xtra maxi	Isolation and purification of plasmid DNA with large scale	Macherey-Nagel GmbH & Co. KG Dureren, Germany
NucleoSpin Extract II	Isolation of DNA from bacteria culture	Macherey-Nagel GmbH & Co. KG Dureren, Germany
NucleoSpin Plasmid	Purification of plasmid and linear DNA	Macherey-Nagel GmbH & Co. KG Dureren, Germany
NucleoSpin® RNA II	Isolation and purification of mRNA from cultured cells and tissues	Macherey-Nagel GmbH & Co. KG Dureren, Germany
SuperScript III – Reverse Transcriptase Kit	cDNA synthesis from isolated mature microRNAs	Life Technologies GmbH, Darmstadt, Germany
Immunoperoxidase staining	Immuno-staining of frozen tissue section	Life Technologies GmbH, Darmstadt, Germany

2.11. Machines and labware

Table 20. List of used machines and labware

name	purpose	Producer
Balance table	-	Mettler-Toledo; Gießen, Germany
Bench centrifuge	-	Eppendorf AG, Hamburg, Germany
Blotting-apparatus	Protein transfer western Blot	Amersham/ GE Healthcare Europe GmbH, Freiburg Germany
Blotting paper	Western blot transfer	A. Hartenstein GmbH, Würzburg Germany
Cell culture plates	-	BD Biosciences Heidelberg, Germany Sarstedt AG & Co, Nümbrecht, Germany
Centrifuge (Avanti J-20 and J-12)	-	Eppendorf AG, Hamburg, Germany
Centrifuge (MIKRO 220R & Rotanta 460R)	-	Hettich Mühlheim, Germany
CO_2 incubators	Incubation cells	Binder GmbH Tuttlingen, Germany

Confocal laserscanning microscope (Leica DM IRBE)	-	Leica Mikrosytsems Vetrieb GmbH Wetzlar, Germany
Cryo tubes	Freezing of cells	Sarstedt AG & Co, Nümbrecht, Germany
cuvettes	Measuring protein or DNA content in a photometer	Sarstedt AG & Co, Nümbrecht, Germany
DNA gelelectrophoresis chambers	Electrophoresis of DNA gels	Bio-Rad Laboratories GmbH, Munich, Germany
developermachine	Developing films	G. Beetz Röntgenartikel Langen, Germany
Fine scale balance	Weighing small amounts	Mettler-Toledo, Gießen, Germany
Film cassette	For western blot films	CAWO photochemical Werk, Schrobenhausen, Germany
Fluorescence microscope Nikon Eclipse TE300	-	Nikon Instruments Europe B.V., Kingston, United Kingdom
Fridges & freezers	-	Liebherr-Intenational GmbH, Biberach an der Riss Germany
Gelelectrophoresis chamber	SDS gelelectrophoresis	Bio-Rad Laboratories GmbH, Munich, Germany
Glass slide	-	Carl Roth GmbH + Co KG, Karlsruhe, Germany
HandyStep Electronic	-	Brand GmbH & CoKG, Wertheim Germany
Homogeniser (Ultra Turrax T25)	Disintegration of tissue	IKA® Werke GmbH & Co KG, Staufen Germany
Heating Block	-	Eppendorf AG, Hamburg, Germany
Incubators	Incubation of bacteria and yeast	Heraeus Instruments GmbH, Hanau, Germany
Magnetic stirrer	-	IKA®-Werke GmbH & Co. KG Staufen, Germany
Nitrocellulose membrane	Protein transfer western blot	Whatman GmbH, Dassel, Germany
PCR tubes	-	Peqlab Biotechnologie GmbH Erlangen, Nürnberg
Petri dishes	-	Sarstedt AG & Co, Nümbrecht, Germany
Phase contrast microscope Nikon Eclipse TS100	-	Nikon GmbH, Düsseldorf, Germany
Pipettes	-	Gilson International, Limburg, Germany
Pipette tips	-	Sarstedt AG & Co, Nümbrecht, Germany
Reaction tubes (1,5ml)	-	Sarstedt AG & Co, Nümbrecht, Germany
Reaction tubes (2ml)	-	Sarstedt AG & Co, Nümbrecht, Germany
Roentgen films (Fujifilm SuperRX)	-	Fujiifilm, Düesseldorf, Germany
Sequencer (3130 genetic Analyser)	-	Applied Biosystems Germany GmbH Darmstadt, Germany

Scalpels	-	Servoprax GmbH Wesel, Germany
Snake skin membrane	Dialysis for protein purification	Thermo Fisher scientific GmbH Dreieich, Germany
Sonifier (Branson 250)	-	Branson Emerson Industrial Automation, DanburyCT, USA
Sterile filter system	-	Millipore Ireland Ltd, Tullaagreen Ireland
Sterile workflow hood	-	Gelaire Flow Laboratories, Aberdeen, Australia
Thermo cycler	-	Biometra GmbH, Goettingen, Germany
UV table	Visualise ethidiumbromide in DNA gels	Bio-Rad Laboratories GmbH, München, Germany

3. Methods

3.1. Cell culture methods

All potential, therapeutic compounds are being evaluated pre-clinically in cell culture and animal models. Cell culture experiments provide valuable information about basic properties of compounds which then have to be verified and extended in experimental animals. In this thesis experiments were carried out which tumor cells were transduced with a gene encoding the microRNA 302/367 cluster. Parental tumor cells and transduced cells were compared with repect to signalling and protein expression patterns. The work yielded insights into the effects of the microRNA 302/367 cluster on the cell morphology, viability, drug sensitivity, invasiveness and the growth behaviour upon transplantation into mice.

3.1.1. Cultivation of eukaryotic cell lines

Cells can be conserved for longer periods if they are frozen in a solution of FCS/DMEM/DMSO (5:4:1) at -80°C or liquid nitrogen. In order to re-culture them, they are thawed and subsequently mixed with 3ml culture medium. Then they are centrifuged at 1.500rpm for 5min. The supernatant contains cell-toxic DMSO and is discarded. Cell pellets are than seeded in 10ml of pre-warmed medium in a 10cm culture dish.

3.1.2. Passaging cells

Cells are living organisms, which need nutrition and certain conditions to grow. In order to prevent mutations or selection processes, cells have to be kept at an optimal cell density. When they are regularly split, immortalized cells can be kept for very long periods of time.
Adherent cells were grown to a maximal density of 70-80%. Then they were split depending on their growth rates. Fast growing cells were split in a ratio 1:10 (100µl of 1ml trypsin) every 2-3 days.
The medium was aspirated and plates were washed with 5ml 1x PBS for each 10cm dish. This was done to remove residual medium because the inherent FCS content inhibits the enzymatic function of trypsin. Then PBS was aspirated and 1ml trypsin

per 10cm plate was added. The plate was incubated at 37°C for about 5min. After that, the tryspin including the detached cells was pipetted several times to detach all cells and gain equal distribution. Depending on the split ratio trypsinised cells were dispersed onto different plates containing 10ml fresh medium. Cells cultured in smaller plates were split accordingly but with adapted volumes of reagents.

3.1.3. *In vitro* experiments

3.1.3.1. Quantification of viable cell number assay

With the CCK-8 test from Sigma Aldrich it is possible to quantify viable cell numbers and assess proliferation and cytotoxicity and compare parental cells and cells over-expressing the miRNA 302/367 cluster.
The method is based on the ability of living cells to cleave tetrazolium into formazan. Formazan formation is than visualized by absorption in an ELISA reader at the difference between 490-650nm. For this experiment $2x10^3$ cells per well in 100µl medium were seeded. Over-expression of the microRNA 302/367 cluster started from the second day on. To measure the viable cell numbers, 10ul of CCK-8 solution was added according to manufacturer's guidelines. After 4 hours of incubation the absorption difference between 490nm and 650nm was measured with an ELISA reader. The absorption directly correlates to the cell number of living cells.

3.1.3.2. Cell invasion assay and crystal violet staining

The cell invasion assay has been used to study the interactions between tumor cells and extracellular matrix (ECM). ECM contains biological factors important for the survival and growth of tumor cells. Tumor cells can secret enzymes and these enzymes release certain components of the EMC. ECM gel mimic the ECM environment. Metastatic tumors have strong invasive properties, this can be mimicked in ECM gels, due to their motility and the enzymatic activity degrading ECM components. Invading living cells can be stained with crystal violet. This dye stains cell walls purple and thereby visualizes the number of invaded cells on the bottom of Millicell Cell Culture Inserts. Millicell Cell Culture Inserts were coated with 2mg/mL concentration of ECM gel. ECM gel was diluted in ice-cold serum-free DMEM. To coat the Millicell Cell Culture Inserts with the ECM gel, 100uL of diluted ECM gel was added directly on the upper compartment of the Millicell Cell Culture

Inserts, and then immediately incubated on the plate, with insert and ECM gel inside, at 37°C for 2 hours. The lower compartment, a well of the 12 well plate, was filled with 1mL of culture medium. After solidifying the ECM gel, 100uL of $3X10^4$ea/mL in serum-free DMEM tumor cells were seeded on the ECM gel coated Millecell Cell Culture Inserts, and then the plate was incubated at 37°C in a 5% CO_2 incubator for 16~18hrs. After the incubation period, non-invaded cells, remained on the upper compartment of the insert, they were gently removed with a cotton swab. The invaded cells, in the lower compartment of the insert, were fixed with 3.7% paraform aldehyde for 10min, followed by staining with 1% crystal violet in 2% ethanol for an additional 20min. Afterwards the cells are washed 2 times with 1X PBS to remove unbound crystal violet. After drying the Millicell Cell Culture Inserts at room temperature, stained invaded cells were scanned or analysed under the microscope.

3.1.3.3. Fluorescence activated cell sorting (FACS)

Cells get activated by electrons and are bypassing a laser. Because of the miRNA 302/367 cluster encoding plasmid DNA also directs the expression of GFP, the GFP signal was used for FACS sorting. GFP is fluorescent protein which then allows the identification of microRNA 302/367 cluster expressing cells. Cells differ in their size, shape or granularity. This means every cell population is behaving differently in FACS analysis and optimal settings have to be established.

3.1.3.4. Transfection of DNA

To produce viral particles, which are encoding the miRNA 302/367 cluster, the Ca_2PO_4 transfection methods were used. Cells were seeded in a 10cm plate on day 1 and left to attach overnight. The next day Xµg plasmid DNA is mixed with 450µl H_2O and 50µl 2.5M $CaCl_2$. 500µl of 2xHBS is added dropwise and the solution is vortexed for another minute. Before adding the plasmid DNA, chloroquin is added at a final concentration of 10µM. After incubation at RT for 30min the plasmid mix is added to the cells, also dropwise. The next day the medium is changed again. Then it takes approximately two days until the cells express the introduced construct.

3.1.3.5. Immunocytochemistry

1X 104 cells were seeded on Poly-L-lysine coated round shape coverslip in a well of 12 well plates. The next day, cells were washed 2 times with 1X PBS. After the washing steps, cells were fixed with 500uL of 37% para-formaldehyde for 15min at room temperature. Fixed cells were washed 2 times with 1X PBS for 5 min at room temperature on a rocker. Then cells were permeabilized with 500uL of 0.2% Triton X-100 in PBS for 10min at room temperature. Cells were washed 3 times with 500uL PBS for 5min. 0.5% BSA in 0.1% Tween-20 TBS was used as a blocking solution. Permeabilized cells were blocked with 250uL of the blocking solution for 1hr at room temperature, and then 100uL of 1:50 diluted primary antibody in PBS was added directly after discarding the blocking solution. Cells were incubated with the primary antibody overnight at 4°C. The next day, cells were washed 3 times with 1X PBS for 5min on a rocker. The washed cells were incubated directly with 100uL of Alexa conjugated secondary antibody with 1:100 dilution factors for 1hr in the dark at room temperature. After incubation with a secondary antibody, cells were washed 3 times with 1X PBS for 5min in the dark at room temperature. Then the coverslip with the stained cells was mounted on a slide glass and sealed with nail polish. Cells were scanned and analysed under the fluorescence microscope.

3.1.3.6. Soft agar colony formation assays

Cancer cells can form colonies in soft agar because of their anchorage independent growth ability. The cell lines used in this study have an anchorage independent growth phenotype, so they can generate colonies in soft agar. For this assay, cells are cultured in soft agar medium for 4 weeks. While the cells are growing in the soft agar, they were fed daily with fresh medium. All steps for base and top agar preparation are done under sterile conditions with cell culture grade water. For base agar preparation, 1% (W/V) DNA grade agarose in distilled water was melted in a microwave oven and cooled to 40°C in a waterbath. To equilibrate the 1% agar with cell culture medium, 2X DMEM complete cell culture medium (20% FCS, 2X P/S, 2X Glu) was incubated at 40°C for 30min. The 1% agar and 2X DMEM complete cell culture medium were mixed in equal volumes under the cleanbench to give 0.5% agar in 1X complete cell culture medium. 1mL of the mixture was immediately plated in a well of 6 well plates. These plates were incubated in the cleanbench for 10min to allow the agar to solidify. For top agar preparation with cells, 0.7% o(W/V) DNA

grade agarose in distilled water was melted in a microwave oven and cooled to 40°C in a waterbath. Similar to the base agar preparation, 2X DMEM complete cell culture medium was incubated at 40°C for 30min. To seed the cells with top agar, cells were washed twice with 1X PBS and trypsinized. After trypsinisation cells, the cells were passed through a 40um pore size nylon mesh Cell Strainer to remove aggregated cells. Then cells were counted in a haemocytometer and adjusted as 1X 10^5 cells/ml. 100ul of the cell suspension was added into 10ml of 2X DEME complete cell culture medium which had been incubated at 40°C. The 2X complete cell culture medium contained adjusted cell numbers and was mixed with 0.7% agar of the same volume. To avoid agarose premature hardening, only did one tube was prepared at a time. This mixture was gently mixed by swirling and 1.5ml was added to the base agar plated. After seeding the cells, 0.5ml of 1X complete medium was added in a well of 6well plates. Then the cells were incubated at 37°C in 5% Co_2 incubator for 28days. The medium was changed every day, and the cells were observed under the microscope to evaluate colony formation in soft agar. When the control cells were grown to a visible size, the cells were stained with crystal violet and analysed.

3.1.3.7. Tumor sphere formation assays

The tumor sphere formation assay is similar to the soft agar colony assay. Cancer cells can divide and generate tumor spheres under 3D culture conditions. To analyse the tumor sphere formation *in vitro*, cancer cells were plated on 10cm dishes on day 1. The next day, cells were washed 2 times with 1X PBS and trypsinized. After washing the trypsinized cells in complete cell culture medium, the medium was discarded and the cells were suspended in 1ml tumor sphere medium (20ng/ml Epidermal Growth Factor, 10ng/ml Basic Fibroblast Growth Factor, 5ug/ml Insulin, 1X B27 supplement and 0.4% Bovine Serum Albumin in Phenol red free DMEM/F12). Suspended cells were passed through a 40um pore size nylon mesh Cell Strainer to remove aggregated cells, and then single cells were counted with a haemocytometer. The cells were adjusted to 15 cells/ul with tumor sphere medium, before seeding 200ul of suspended cells on the ultra-low attachment 6 well plate, 3ml tumor sphere medium was added to each well of the 6 well plates. The cells were incubated for 5 days at 37°C in 5% Co_2 incubator, and tumor sphere formation was followed under the microscope.

3.1.3.8. Cytokine array

Expression and secretion of cytokines establish cellular communication and are mediated via the activation of signalling pathways. Inflammatory cytokines can activate oncogenic signalling pathways in tumor cells. The secretion of cytokines from cancer cells changes and influences the tumor microenvironment and can establish paracrine or autocrine loops. For example, IL6 can be secreted from cancer cells and its secretion can induce the oncogenic STAT3 signaling in cancer cells and heterotypic neighboring cells. To study the consequences of miRNA 302/367 cluster expression on cytokine secretion by U87MG cells, we measured cytokines in the supernatant of these cells with a cytokine array (RayBiotech) and followed the manufacturer's instructions. 1X 10^6 cells were seeded on 10cm dish with complete cell culture medium. The next day, the complete cell culture medium was discarded and the cells were washed twice with 1X PBS. Then serum free DMEM medium was added to the cells for 2 days. 2 days later, the medium was collected into 50mL falcon tubes and the collected medium was centrifuged briefly to remove cells. The supernatant was transferred into a 15ml falcon tube, and stored at -20°C. The medium was thawed at room temperature to perform the cytokine array analysis. First, the human antibody array membranes were incubated with 2ml blocking buffer (provided with the kit) in an 8 well tray (provided with the kit) for 30min at room temperature. Then, the blocking buffer was discarded, and the membranes were incubated with 1 ml of the collected supernatant medium overnight at 4°C. The next day, the medium was discarded, and the human antibody array was washed 3 times with 2 ml of 1X Wash Buffer I (20X Wash Buffer I, provided with the kit, and diluted ato a 1X concentration with distilled water) at room temperature and shaking for 5min. The procedure was repeated with Wash Buffer II. To prepare the working solution for the primary antibody, 100 ul of 1X block buffer was added into Biotin-Conjugated Anti-Cytokines tube (provided with the kit), and gently mixed. The Biotin-Conjugated Anti-Cytokines were transferred into a tube containing 2 ml of 1X blocking buffer. 1 ml of the diluted Biotin-Conjugated Anti-Cytokines were added to each array membrane, and then the membranes were incubated at room temperature for 2 hours. The membranes were washed again with 1X Wash Buffer I and 1X Wash Buffer II. IRDYE 800CW Streptavidin was used as a secondary antibody. The IRDYE 800CW Streptavidin was diluted in Blocking buffer as 1:1000 ratio, and then the membranes were incubated with 2ml of the diluted IRDYE 800CW Streptavidin for 2 hours at room temperature in the dark. The membranes again with 1X Wash Buffer I and 1X Wash Buffer II, and Odyssey was used to detect the florescence signals of the

membranes. The membranes were scanned, and density of the each cytokine was analysed using the Odyssey software.

3.2. Biomolecular techniques

3.2.1. DNA cloning procedures

Cloning methods have been well established and thoroughly described. The gene of interest can be excised with restriction enzymes or the desirable restriction sites can be inserted flanking the gene of. This method revolutionized the field of molecular biology because it made it possible to engineer gene sequences.

3.2.2. Isolation of genomic DNA

I cloned a part of the LARP7 gene contains the miRNA 302/367 cluster. For this purpose I used MEF cell genomic DNA as a template for a polymerase chain reaction (PCR). MEF cells were plated on a 10 cm dish, the cells were collected in the 50 ml falcon tube by centrifugation for 5 min at 500 X g. The cells were washed twice with 10 ml ice cold 1X PBS and 1X 10^8 cells were re-suspended in 500 ul digestion buffer (100mM NaCl, 10mM TrisCl-pH8, 25mM EDTA-pH8, 0.5% SDS and 0.1 mg/ml proteinase K in PBS). The digestion buffer and the cells were incubated with shaking at 50°C for 18 hours in tightly capped tubes. To discard proteins and RNase activity, 500 ul of phenol/ chloroform/isoamyl alcohol mixture (25:24:1) was added to the tubes with the genomic. Then the tubes were mixed vigorously by shaking, not vortexed, and centrifuged for 10 min at 1700 X g at room temperature. The top, aqueous phase of the tubes, containing the genomic DNA, was carefully transferred to fresh tubes. To purify and precipitate the genomic DNA, 1/10 volume of 3M sodium acetate added to the aqueous phase, followed by 2 volumes of 100% ethanol. Genomic DNA recovered by centrifugation at 1700 X g for 2 min at 4°C. The supernatant was discarded, and 450 ul of isopropanol was added to the pellet. After 1 hour incubation at -20°C, the tubes were centrifuged 1700 X g for 30 min at 4°C. The ethanol precipitate was recovered, the supernatant was discarded. The DNA was washed with 700 ul of 70% ethanol. The genomic DNA pellet was dried and re-suspended in 1X TE buffer and stored in 1X TE buffer at -20°C.

3.2.3. Polymerase chain reaction (PCR)

The polymerase chain reaction was invented in 1983 and served as a most important tool for molecular biologists (Mullis, 1990). This procedure is based on an enzyme from *Bacillus themophilus aquaticus* and achieves an amplification of the copy numbers of DNA molecules in an exponential fashion. It is performed in three steps. In the first step double stranded DNA molecule is denatured, which leads to two single stranded molecules. This is done at 95°C. In a second step DNA primers are annealed to the single stranded DNA. The primers are designed, complementary sequences. The amplification requires a forward and a reverse reaction. The temperature for this step varies significantly, because each primer has its own temperature optimum. Finally the product is elongated at 72°C by incorporating dNTPs (desoxyribonucleosidtriphosphate) into the newly built strand. dNTPs are the building blocks for the new strand. Several ingredients are needed to carry out a PCR reaction, primers, dNTPs and the enzyme. To ensure the optimal pH for the polymerase a buffer is used. $MgCl_2$ is required as cofactor for the enzyme and *bovine serum albumin* (BSA) builds a complex around the enzyme and the dNTPs to protect the reaction.

The polymerase chain reaction can be used to amplify a gene of interest from a genomic DNA sequence or to detect and amplify DNA. In this thesis, I modified the PCR procedure to fit my objectives. The conditions of the PCR for genomic DNA are described below.

Table 21. Components of the PCR reaction

Component	Volume
Genomic DNA	1 ul (100ng)
10X PCR buffer	5 ul
10 uM Forward primer	2.5 ul
10 uM Reverse primer	2.5 ul
10 mM dNTP-mix	1 ul
DMSO	2.5 ul (5%)
Distilled Water	35.5 ul (up to 50 ul)
Total	50 ul

Table 22. Procedure of the genomic DNA PCR reaction

Procedure	Temperature	Time	Cycle
Pre-denaturation	95°C	6 min	1 cycle
Cooling on ice	Ice	2 min	1 cycle
Polymerase mixture (Taqpolymerase: 0.15 ul Pfupolymerase: 0.4 ul)	Ice		1 cycle
Continue pre-denaturation	95°C	3 min	1 cycle
Denaturation	95°C	30 sec	35 cycle
Annealing	55°C	30 sec	
Extension	72°C	2 min	
Storage	4°C	Forever	1 cycle

3.2.4. Restriction enzyme digestion

Restriction enzymes are able to very specifically cleave DNA at specific recognition sites. All enzymes have a unique palindromic recognition sequences. The enzymes produce blunt ends or sticky ends, which can then be religated. Restrictions sites can be very useful to delete or insert specific DNA fragments in vectors with multiple cloning sites. It is easy to manipulate them by mutation or by inserting certain restrictions sites into DNA sequences.
In order to insert the gene of interest into a certain plasmid, restriction sites can be used. The vector plasmid and the gene of interest need to be restricted with the same restriction enzyme. The restriction site should not be present within the gene of interest more than once, to prevent unwanted fragmentation.
Usually restriction digests are performed at 37°C. Depending on the enzyme the duration of the restriction digest has to be adjusted. EcoR1 is cutting very fast, for example, whereas RsrII is a slow cutting enzyme. Some enzymes also need BSA as a cofactor for optimal function.

The usual protocol is the following:
Max. 1/10 vol enzymes
1/10 vol buffer (1,2,3 or 4)
1/10 vol BSA (for some enzymes needed as a cofactor)
up to 20µl or 30µl with ddH_2O

Incubation time for the restriction digests at 37°C can be some hours or overnight depending on the size of the plasmid, the purpose of the restriction digest and the enzymes.

3.2.5. Dephosphorylation of DNA (Cipping)

To prevent that the vector is religating with itself rather than with the insert, in cases where only one restriction enzyme is used, the vector has to be dephosphorylated (also known as cipped). This is an enzymatic reaction of the calf intestinal phosphatase (CIP) or the arctic shrimp phosphatase (SAP). The reaction is terminated by heat inactivation at 65°C for 15min. These phosphatases remove phosphate groups from DNA and prevent self-ligation.
The usual protocol is the following:

+ 1µl phosphatase to your restricted DNA
+ 5µl phosphatase specific buffer
Fill up with ddH_2O up to 50µl
Incubate at 37°C for 30min and if necessary heat inactivate at 65°C for 10min.

3.2.6. DNA Ligation

In order to ligate two ends of a vector produced by restriction digestion, mainly the T4 ligase is used. This enzyme catalyses the formation of phosphodiester bonds. The ligation is usually performed at 16°C overnight or at 4°C for longer periods of time. Self-ligation is tested with only the restricted empty vector. The molar ratio between vector and insert should be 1:4.
5-10ng insert
x ng vector
2µl buffer
Up to 20µl with ddH_2O
1µl T4 ligase
Incubate at 4°C over the weekend or at 16°C overnight.

3.2.6.1. Quick DNA ligation

The quick ligase reaction enables the researcher to perform ligation and transformation in one day.
50ng vector
up to 3 molar fold DNA
add H_2O up to 10µl
1µl quick ligase
10µl quick ligation buffer
5-10min at RT and then chill the reaction on ice and move on to transformation

3.2.7. Bacterial DNA transformation

Transformation is used to introduce the ligated product (the gene of interest) into bacteria. The bacteria then amplify the plasmid which subsequently can be isolated .
This entry into bacteria can be induced by electroporation or heat shock. Here the latter method was used. For the transformation 100µl Top10 F` bacteria per transformation were thawed on ice for 15min. Then 5µl H_2O and 5µl of the ligation product were transferred into a 1.5ml tube on ice. 100µl bacteria were added and carefully mixed without vortexing. The mixture was kept on ice for max. 30min. Thereafter the mixture was heat shocked at 42°C for 1min and subsequently incubated on ice for 2min. Then 1ml room temperature LB medium was added. This solution was than incubated at 37°C for 1hour while shaking at 350rpm. Finally the mixture was centrifuged for 1min at 12.000rpm and the supernatant was removed, except for 50µl. These 50µl were used to resuspend the bacterial pellet and put on agar plates containing the antibiotic needed for this special plasmid. The plates were incubated at 37°C overnight.

3.2.7.1. Transformation and the quick ligation protocol

50µl of competent bacteria per reaction were thawed on ice. 2µl ligation product (approx. 5ng) were added and mixed very carefully without vortexing. The reaction was left on ice for 30min. Afterwards the heat shock was performed at 42°C for 30sec. Then 950µl pre-warmed LB medium containing no antibiotics was added and the reaction was incubated at 37°C with 250rpm for one hour. Finally 50-100µl were plated on the corresponding selection plate and incubated at 37°C overnight.

3.2.8. DNA isolation from plasmids

After successful transformation DNA was isolated from a representative number of clones. For this purpose individual clones were picked, transferred into 5ml of LB medium containing the corresponding antibiotic and incubated at 200rpm at 37°C overnight. The next day the samples are centrifuged at 4.000rpm for 20min. The DNA was isolated with a Macherey & Nagel NucleoSpin Plasmid Kit. This kit is based on SDS/alkaline lysis in the first step. After applying the optimal conditions with another buffer, which leads to pellet formation of unwanted parts (e.g. genomic DNA), the sample is loaded on a silica membrane. Here another buffer washes off salts and metabolites. Finally the plasmid DNA is eluted in a slight alkaline buffer with low ion content. The exact protocol was performed according to manufacturer's guidelines.
After the isolation a test restriction digest was performed. A small portion of the DNA (5µl) is restricted with the same enzymes used for the cloning procedure for 2-3hours and than loaded on a DNA gel. The samples that are restricted successfully are then analysed by sequencing.

3.2.8.1. Plasmid isolation (maxi preps)

If the correct DNA sequence was found, the clone was incubated in a larger volume of LB-medium containing the right antibiotic (200ml-400ml). This depended if the plasmid is a low (>20 plasmids per bacteria) or high (< several hundred plasmids per bacteria) copy plasmid. The method was based on the same principle as in the small scale DNA isolation, but this protocol ensures high purity and high concentrations of the plasmid of interest. The isolation was also performed with the NucleoBond Xtra maxi kit from Macherey & Nagel. The DNA isolated here could either be used as stock for further transformations or directly used for cell transfection.

3.2.9. DNA sequencing

Sequencing is based on the PCR technique. With one primer only either reverse or forward single stranded molecules were generated to be able to sequence the desired sequence from both directions. Cycle sequencing was done to mark the sequences with fluorescent ddNTPs (didesoxyribonukleosidtriphosphate). This reaction is based on the dideoxy sequencing or Sanger sequencing. DdNTPs lack a hydroxygroup at

3`position in addition to the one that is missing in dNTPs. If a ddNTP was incorporated into the new sequence the elongation process stops. This prevented the bond between the hydroxygroup and the 5`phosphate of the next nucleotide. Normally there is a condensation reaction between the 3`hydroxygroup of one base with the 5` phosphate group of the next base that is about to be incorporated. This reaction led to the characteristic phosphodiesterbond. As the synthesis stopped with the incorporation of a ddNTP in every chain all of them have different lengths.
The fluorescent dye of the ddNTPs was specially labeled with zwitterionic dRhodamines for each base and enables the sequencer to detect the ends of the sequences. The sequencer used the terminator dyes, which are attached to the dideoxy nucleoside triphosphate. The sequences were analysed with software from DNA Star Lasergene.

3.3. Protein analyses

3.3.1. Isolation of total protein fractions from cell lysates

I evaluated the effects of the cellular manipulations on the level of protein expression. For these experiments protein extracts were derived usually using RIPA (Radio-immunoprecipitation assay) buffer. Cells were kept on ice, the medium was aspirated and the cells were washed with PBS, then an acidic solution wash and again with PBS. No medium or protein reagents were left on the cells. RIPA buffer was added , the volumes were adjusted to cell density, well size and the morphology of the cells. The cells were scraped from the plate and transferred into a 1.5ml Eppendorf tube. The cells were left on ice for 20min to lyse. The lysate was sonicated (output cycle 2-3, duty cycle 20-30%) and kept on ice for 20min afterwards. After the incubation, the cell lysates were vortexed and centrifuged for 20min with 14.000rpm at 4°C. The soluble proteins were present in the supernatant, and this fraction was transferred to a fresh 1.5ml Eppendorf tube. Until further analysis, the protein fraction was kept at -20°C in small aliquots.

3.3.2. Cellular protein fractionation

Subcellular protein fractions can be obtained to characterize the presence of a particular protein in different compartments of the cell. The localization of proteins in subcellular fractioons can be determined by Western blot analysis. To fractionate

the cytosol from nuclei nuclear, Hypotonic Buffer (see buffer recipe) and Cell Extraction Buffer (see buffer recipe) were used. 5 X 10^6 cells were collected in 1X PBS by centrifugation, and then the cells were washed twice with cold PBS. After removing the supernatant, the cell pellet was transferred into a prechilled microcentrifuge tube with 500ul Hypotonic Buffer, and then the pellet was gently re-suspended by pipetting. The re-suspended pellet was incubated on ice for 15 min. After the incubation, 25 ul of NP40 was added directly and the tubes were vortexed for 10 seconds at the highest setting. The cytosolic membranes are lysed, but the nuclear membrane stays intact. Then the tubes were centrifuged to separate the cytosolic proteins from the cell nuclei. The supernatant, containing the cytoplasmic fraction, was carefully transferred to new tubes. The pellet fraction, containing the nuclei, was re-suspended in 50 ul of Cell Extraction Buffer. For Western blot analysis, the cytoplasmic fraction was stored at -20°C. The re-suspended nuclear pellet was incubated on ice for 30 min and vortexed at 10 min internals. After the incubation, the tubes were centrifuged for 30 min at 14,000 X g at 4°C. The supernatant, containing the nuclear protein fraction, was transferred to a new tube and the proteins were stored at -20°C.

3.3.3. Measuring protein concentrations

Several methods are available to measure protein concentrations. The Bradford Solution consisting of Coomassie brilliant blue G-250 as staining reagent provides one option. This reagent forms a complex with proteins and shifts the absorption maximum from 465nm to 595nm. This complexation is occurs after 5min of incubation. 1ml of diluted Bradford solution is mixed with 1-2µl protein solution. The protein content is determined with a photometer at 595nm comparing the measured sample with a control sample containing no protein. The amount of protein can now be calculated and used in further experiments.

3.3.4. Western blot analysis

The Western blot technique is used to visualize individual proteins upon size fractionation on gels. It is also referred to as immune-blotting, because it visualizes proteins via antibody detection.
After protein isolation and protein concentration measurements, the proteins are mixed with 5X SDS sample buffer to yield a final concentration of 1X SDS sample

buffer. The mixture is heated in a heating block to 100°C for 10 min, resulting in protein denaturation. After the denaturation step, proteins were loaded onto SDS-PAGE gels in a tank filled with 1X electrophoresis buffer. For the preparation of SDS-PAGE gels, 8~15% concentrations of polyacrylamide gel were used from 30% polyacrylamide stock solutions (Solution A; acrylamide : bisacrylamide = 29 : 1). Plates of polyacrylamide gels with a 1.5 mm comb were made with Solution A, Solution B, Solution C (see the recipe table) and distilled water. For the SDS-PAGE stacking gel, 0.67 ml of Solution A, 1 ml of Solution C and 2.3 ml distilled water were mixed and 60 ul of 10% Ammonium persulfate and 6 ul of TEMED were added to solidify the gel. To make gels with different acrylamide percentages in the running gel, each of the components were mixed as described below in the Table, and then 60 ul of 10% Ammonium persulfate and 6 ul of TEMED were added to solidify the gel. Electrophoresis was performed around 1.5 ~ 1 hours at 80 – 120 volt, or until the protein markers had reached the bottom of the gel. After finishing the electrophoresis, the gel was washed with water to remove the SDS. The washed gel was incubated in 20% methanol contained Semi-dry-transfer buffer for 20 min. For the transfer of the gel to a Nitrocellulose membrane, the membrane was incubated for 30 min in 20% methanol contained Semi-dry-transfer. After the incubation of the membrane and the gel, proteins on the gel were transferred to the membrane by using Trans-Blot SD Cell. Thick Blot Paper soaked in the Semi-dry-transfer buffer was used. Then the wet Thick Blot Paper, Nitrocellulose membrane and gel were sandwiched in the following order: 2 layers of wet Thick Blot Paper / Nitrocellulose membrane / gel / 2 papers of wet blot paper. Before running the Trans-Blot-SD Cell, air bubbles between the gel and membrane were removed by rolling them out with a pipet. The transfer was performed for 1 hour 15 min at 12 voltages. After finishing the transfer, the membrane was washed with 1X PBS for 5 min on a shaker. For blocking, the membrane was incubated in 5% BSA TBS-T or 5% Skim milk TBS-T for 1 hour at room temperature with rocking. The blocked membrane was briefly washed 1 time with 1X TBS, and then incubated with primary antibody, diluted in 3% BSA TBST, overnight at 4°C. The next day, the primary antibody attached to the membrane was washed 3 times with TBST for 5 min. Then the membrane was incubated with HRP conjugated secondary antibody, diluted in 3% BSA TBST, to detect the specific protein. After incubation of the membrane with secondary antibody, the unbound and non-specific bound secondary antibody washed out by TBST with same as previous primary antibody washing step. The HRP coupled protein is than revealed by chemiluminoscence and detected on a photographic film.

Table 23. Composition of SDS-PAGE running gel and stacking gel

	Running gel				
Solutions	6%	8%	10%	12%	15%
Solution A	1.8 ml	2.4 ml	3.0 ml	3.6 ml	4.5 ml
Solution B	2.5 ml				
D.W.	5.7 ml	5.1 ml	4.5 ml	3.9 ml	3.0 ml
Solutions	Stacking gel				
Solution A	0.67 ml				
Solution C	1 ml				
D.W.	2.3 ml				

3.3.5. Protein profiling

Proteomics is the large-scale study of proteins of cells or tissues. Quantitative proteomics has traditionally been performed by 2D gel electrophoresis. 2D gel electrophoresis allows protein quantitation depending on staining intensity. The development of methods based on mass spectrometric analysis are more quantitative and allow the identification and quantitation of individual proteins in complex protein mixtures of cells or tissues. In this study, we used stable isotope labelling with amino acids in cell culture, SILAC, based protein profiling of U87MG cells. We compared protein lysates of cells grown in medium containing arginine and lysine labeled with carbon-13 atoms (13C) and cells grown in medium containing these amino acids with regular carbon-12 (12C) isotopes. Control cells were cultured in medium with amino acids with the light isotopes and the miRNA 302/367 cluster expressing cells were cultured in medium with amino acids containing the heavy isotopes. The cells were lysed and sonicated to fragment DNA. The lysates were loaded on 1D page gel to fractionate the total protein in 20 fractions according to size and the individual size fractions were digested with trypsin. Mass spectrometry was performed to identify and quantify the proteins and the data were analysed by MaxQuant software.

3.4. Gene transfer experiments with viral vectors

To delivery genes into target cells, lentiviral gene transfer vector were used. Delivery is highly efficient and independent from replication of the infected cells. The use of lentiviral vectors was carried out with caution in a S2 safety level laboratory.

3.4.1. Transfection of proviral DNA into 293T cells

For efficient transfer of genes into eukaryotic cells, replication-deficient viral vectors are normally used. These vectors allow proviral DNA integration, but will not support the production of viral particles. The generation of replication-deficient transfer vectors requires the elimination of trans-acting *gag*, *pol* and *env* genes from the viral genome. Instead of these genes, a gene of interest can be inserted and transferred into the host genome. The *gag*, *pol,* and *env* genes are encoded on two separate plasmids, provide the encoded proteins in trans and allowthe assembly of retroviral particles. The co- transfection of the three plasmids into 293T packaging cells then leads to the production of viral particles, which are released into the media. The viral particles contain a RNA genome encoding the gene to be transferred, but not the viral *gag*, *pol* and *env* genes. Therefore, the virus is able to transfer and integrate the foreign gene into the host genome, but is unable to replicate and form new viral particles upon infection of the target cells. The efficiency of viral infection can be monitored through the expression of a GFP marker gene also encoded within the viral genome by florescence microscopy.

3.4.2. Production of viral particles

For the production of viral particles HEK 293T cells were used. The cells were washed once with 1x PBS, trypsinized, centrifuged (1,500 rpm, 3 min, RT), resuspended and counted in a Neubauer cell counting chamber. The cells were seeded at low density and incubated until they reached 80 % confluency. HEK 293T cells were transfected by the calcium phosphate method using 10 µg transfer vector, 6.5 µg pCMVΔR8.91 (gag-pol plasmid), 3.5 µg pMD2.VSVG (env plasmid) in a total volume of 1 ml. The plasmids were diluted in 450 µl dH_2O and 50 µl $CaCl_2$ was added. Then 500 µl 2x HBS was added dropwise to the mixture which was simultaneously vortexed for 1 min. The samples were incubated for 30 min at RT to allow the formation of precipitates containing DNA-calcium phosphate crystals

which bind to the cell surface and are finally taken up into the cell by endocytosis. Two hours before transfection the medium was changed to medium containing 1 µl of a 100 mM chloroquine solution (final concentration: 10 µM) and the DNA precipitates were added. The following day the cells were washed once with pre-warmed 1x PBS and 5 ml of fresh media was added. 48 h and 72 h after transfection the medium of all transfections containing the viral particles were pooled, centrifuged (1,000 rpm, 5 min), filtered through a 0.22 µm filter and aliquots were stored at -80 °C. Aliquots were then used for titration and subsequent transduction of cells or were ultra- centrifuged to concentrate the viral particles.

3.4.3. Ultra centrifugation of viral supernatants

To obtain high viral titers (> 108 TU/ml) the viral particles were concentrated by ultracentrifugation at 20,500 rpm (51,610 g) at 4 °C for 2 h and 20 min. The pelleted viral particles were resuspended in 600 µl DMEM, aliquoted and stored at -80 °C. Concentration of viral particles by ultracentrifugation is only possible for lentiviral particles, but not for retroviral particles, because they will be damaged during ultracentrifugation.

3.4.4. Titration of viral supernatants

For the determination of viral titers 105 HEK 293T cells per well were seeded in a 24-well plate. The next day the medium was removed and 250 µl DMEM containing polybrene (stock: 0.8 mg/ml, diluted 1:50 in medium) was added. Dilutions of the viral particles ranging from 10-2 to 10-6 were prepared in 250 µl DMEM each in a second 24-well plate. From these dilutions 250 µl were added to the 293T cells. The plate containing 293T cells and the added viral particles was then centrifuged (2,000 rpm, 30 min, 30 °C) to facilitate docking of the viral particles to the cells and subsequent entry. The following day the medium was changed. The GFP expressing cells were quantified three days after transduction by FACS analysis. To calculate the viral titer the following formula was used:

$$\mathbf{TU/ml = (\% \ GFP \ positive \ cells \ / \ 100) \ X \ 2^t \ X \ 10^5 \ X \ 2 \ X \ 10^x}$$

TU/ml: Transducing units per ml, % GFP positive cells: statistically one viral integration per cell, when 15 % of the cells are GFP positive, t: time since seeding in days, x: Dilution in which 15 % of the cells are GFP positive.

3.5. Experiments with RNA

Ribonucleic acid is much more sensitive to the action of nucleases than DNA T prevent degradation of RNA by RNases special precautions have to be taken and gloves, special RNAse free tips and tubes should be used.

3.5.1. Isolation of RNA

In this work I tried different methods of RNA isolation, but the experiments shown in this work were performed with the Nucleo Spin RNA isolation Kit. The procedure was performed according to manufacturer's protocol. The method is based on cell lysis with a buffer containing guanidine thiocyanate. This denatures the cellular content rapidly and destroys RNases. Then the RNA sample is bound on a silica membrane in an ethanol containing buffer and washed several times. The kit included also an enzyme for DNA digestion. Afterwards the RNA was eluted and stored at -80°C.

3.5.2. Isolation of microRNA

To isolate miRNAs from cells and tissues, the miRVana miRNA isolation kit was used in this study. The procedure was performed according to manufacturer's protocol. The first step is to disrupt samples in a denaturing lysis buffer, and then RNAs are purified by using Acid-Phenol:Chloroform extraction which provides a robust purification that also removes most DNA. After the RNA purification, very small RNAs, which is less than around 200 bases, are isolated by glass-fiber filtration. During this isolation step, small RNAs are binding on the glass-fiber filter, and the filter is washed 3 times with ethanol containing buffer. A provided elution buffer is used to elute small RNAs from the glass-fiber filter.

3.5.3. Measuring RNA concentrations

The NanoDrop was used to measure the RNA concentration and ensure sufficient purity for further experiments. The optimal purity of RNA is defined by a ratio of absorbance 260/280nm ration of > 2. If the value is below 2 the RNA is contaminated with aromatic substances used in the purification process.

3.5.3. Stem-loop RT-PCR

To evaluate the expression of microRNAs, stem-loop RT-PCR was performed in this study. Because miRNAs are typically less than 20 nt in length, they are hard to detect with standard and quantitative PCR methods. For this reason, I used stem-loop RT-PCR to detect the expression of the microRNA 302/367 cluster. It is based on the addition of a stem-loop structure during the synthesis of cDNA from the miRNAs with stem-loop contained primers. For the cDNA synthesis, 44 nt of the stem-loop sequence (*5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT AC-3'*) was added in front of 8~9 nt of the mature microRNA 5' sequence (Chen et al., 2005). By using the stem-loop sequence contained primer, the template cDNA of each miRNA for the RT-PCR was synthesized following isolation of the miRNAs and PCR. Because the stem-loop sequence has a high GC content, I used SuperScript III kit, which provides M-MLV Reverse Transcriptase that has been engineered to reduce RNase H activity and increased thermal stability, for the PCR reactions. The PCR reaction procedure was as following:

Table 24. **Stem-loop RT-PCR miRNA and Primer mixture** (total 25 ul)

RNA/Primer mixture component	Volume
Total isolated mature microRNA	1 ~ 5 ug (ul)
2uM Stem-loop primer	1 ul
10mM dNTP mix	1 ul
DEPC-treated water	up to 25 ul

Mix and briefly centrifuge.
Incubate the mixture at 65°C for 5 min (Denaturation of stem-loop primer and mature microRNA).
Immediately incubate on ice more than 2 min.
Add following total 10 ul of cDNA synthesis mixture directly into the RNA/primer mixture.

cDNA synthesis mixture component	Volume
10X RT Buffer	2 ul
25mM $MgCl_2$	4 ul
0.1M DTT	2 ul

RNase OUT	1 ul
SuperScript RT III	1 ul

Mix gently and collect by brief centrifugation.
Run PCR as following condition:

Temperature	Time
25°C	10 min
50°C	50 min
85°C	5 min
4°C	∞

After collecting the reactions by brief centrifugation, 1 ul of RNase H added. The reactions were incubated for 20 min at 37°C, stored at -20°C, and used for normal RT-PCR with SYBR Green Mix®.

3.5.4. Complementary DNA (cDNA) synthesis from mRNA

In this work components from Fermentas were used for cDNA synthesis from mRNA. First mRNA has to be reverse transcribed into cDNA. Here whole mRNA was used as a template. Random hexamer primers and reverse transcriptase from molony murine leukaemia virus (MMLV-RT) were used for reverse transcription. As this enzyme has RNase H activity, an RNase inhibitor (RiboLock) was used.
The protocol was as following:
1µg RNA (in 10µl)
+2µl Random Hexamer Primers
5min 70°C
Put on ice and spinned down
Per reaction:
4µl RT-buffer
+ 2µl 10mM dNTPs
+1µl RNAse inhibitor (MBI)
+1µl Mmulv H-enzyme
added to RNA/primer mix
1 hour at 37°C and then heated up to 70°C for 10min
put on ice again and used in RT-PCR in an 1:20 dilution

3.5.5. Real Time Polymerase Chain Reaction

This type of PCR is used to detect transcription levels of individual genes in cells. In all experiments shown SYBR Green from Fermentas was used. This reagent binds to double stranded DNA and the resulting complex has an emission between 494nm and 521nm. The SYBR® Green Mix includes a Maxima® Hot Start Polymerase, dNTPs and a buffer containing KCL and $(NH_4)_2SO_4$ to ensure specific primer annealing. In addition, dUTP was included to control for contaminations caused by carry over. One drawback of this reagent is that it produces non-specific signals, because it binds to any double stranded DNA. This PCR has therefore to be controlled in biological as well as methodological replications sufficient to establish statistical relevance.

According to manufacturers instructions the PCR was prepared the following:
10 µl Maxima SYBR Green Mix®
1 ul 20 uM of forward primer
1 ul 20 uM of reverse primer
2 ul cDNA (adjusted as 1 ug)
0.03 ul of Rox
Up to 20 ul water

First, adjust 2 ul of cDNA put into an well of FrameStar 480 96 well plate (4titude). Then 18 ul of the mixture of SYBR / Primers / Rox / Water added into the well. All steps were done on ice to delay the SYBR Green enzyme activity. After putting the samples into the well, the plate was sealed with QPCR seal (Peqlab). Before running the RT-PCR, the plate was centrifuged at 4°C, 400 X g for 5 min to spin down the mixtures to the bottom of the plate. The PCR was run on LightCycler 480 from Roche and analysed with LCS480 1.5.0.39.
Program steps:

Program name	Target (°C)	Acquisition Mode	Hold	Ramp Rate (°C/s)	Acquisitions (per°C)	Cycle	Analysis Mode
Pre-incubation	95	None	10 min	4.40	X	1	None
Amplification	95	None	15 sec	4.40	X	50	Quantification
	60	Single	30 sec	2.20	X		
Melting curves	95	None	5 sec	4.40	X	1	Melting Curves
	65	None	1 min	2.20	X		

	97	Continuous		0.11	5		
Cooling	40	None	30 sec	2.20	X		None

For the stem-loop RT-PCR, the mixture component and RT-PCR program were same, except the amplification step of the program. Because the microRNA sequence length is short, the amplification time was reduced:

Program name	Target (°C)	Acquisition Mode	Hold	Ramp Rate (°C/s)	Acquisitions (per°C)	Cycle	Analysis Mode
Amplification	95	None	5 sec	4.40	X	50	Quantification
	60	Single	10 sec	2.20	X		

3.6. Experiments with animals

Experiments that have been performed *in vitro* are indicative and instructive, but might not reflect the in vivo situation in a comprehensive manner. In cancer research the ultimate goal is to bring drugs to clinical applications and improve the lot of patients. The bridge between *in vitro* and clinics are *in vivo* experiments in animal models. In this study humanized NSG mice were used to investigate the effects of the miR 302/367 cluster expression on U87MG tumor growth and metastasis *in vivo*.

3.6.1. Working with mice

Male humanized NSG (NOD *scid* IL2 receptor gamma chain knockout mice) mice were purchased from Charles River Laboratories International Inc. (Sulzfeld, Germany) or were obtained from our in-house breeding stock. 21 male humanized NSG mice were obtained from the Georg Speyer Haus animal facility.
All animal experiments were carried out according to German government guidelines for animal protection (Regierungspräsidium Darmstadt).

3.6.2. Tumor transplantation experiments

Humanized NSG mice were used in the tumor transplantation experiments. For the tumor transplantation model, cells were cultured and left to grow until confluent. Then they were trypsinised, counted, adjusted to 1 x 10^8 in 100µl PBS. To inject the tumor into the right hind flank of NSG mice, tumor cells are mixed with matrigel (BD

Matrigel™ Basement Menbrane Matrix Growth Factor Reduced, Cat #354230, Lot #3010866) as following order and composition:

Order	Component	Volume (Final 200 µl)
0	Autoclaved effendorf tube on ice	
1	1X cold PBS	150 µl
2	1 X 10^8 cells in 100 µl PBS	10 µl
3	Matrigel	40 µl (Final 20%)

The above mixture was prepared on ice before the injection into NSG mice, and 100 µl of the mixtures was immediately injected into the right hind flank of NSG mice. Tumor volumes were measured in regular intervals up to 30 days after inoculation by using vernier caliper. The volume was calculated by the Ellipsoid equation ($\pi/6$ *X Length X Width*^{2}). 30days after the injection, mice were killed and dissected. Tumor, liver and lung tissues were embedded with Frozen Tissue Matrix (Tissue Tek O.C.T.™) or frozen directly in cryotubes for further study.

3.6.3. Immunohistochemistry (Immunoperoxidase staining)

To evaluate metastatic growth of tumor cells in liver, immunoperoxidase staining was performed with a Ki-67 specific antibody. Frozen tissues were in 4-10 µm sections and mounted on APES (aminoprophyltriethoxysilane) coated glass slides. The frozen tissue sections were stored at -80 °C before performing immunoperoxidase staining. For immunoperoxidase staining, the slides containing frozen tissue sections were thawed at room temperature for 5 min. Then the tissues on the slides fixed with precooled acetone at 4°C for 10 min. After the fixation, the slides were washed 3 times carefully with TBS. Histostain®-Plus 3rd Gen IHC Detection Kit was used to detect primary antibody with DAB Chromogen on the tissues. At first, the tissues incubated with peroxidase quenching solution for 5 min to block endogenous peroxidase activities and immediately rinsed with distilled water. Then the slides were dipped twice in TBS for each 2 min. Because I performed immunoperoxidase staining with liver tissue, which has high endogenous peroxidase activity, I used Peroxo-Block™ (Invitrogen) as an extra endogenous peroxidase inhibition solution. The tissues were incubated with the Peroxo-Block™ for 45 sec at room temperature, and immediately washed with distilled water. To block the non-specific binding of primary anti-body, the tissues were incubated with Blocking Solution for 10 min at

room temperature. Then the solution was drained and 1:1000 diluted primary anti-body in 10% normal goat serum in TBS was added and incubated for 1 hour at room temperature. After the primary anti-body incubation, the slides were dipped 3 times into 0.05% tween 20 TBS (0.05% TBS-T) for 2min. Then the tissues were incubated with 100 ul of secondary anti-body (Reagent C) for 10 min at room temperature to conjugate them with streptavidin peroxidase. After the conjugation with the secondary anti-body, tissues were rinsed 3 times with 0.05% TBS-T for 2 min. For the conjugation with streptavidin peroxidase, 100 ul of Reagent D from the kit was added and incubated for 10 min at room temperature. To visualize Ki-67 immunoperoxidase staining, the tissues were incubated with DAB Chromogen solution (Reagent E1 and E2) for 5 min at room temperature followed by a rinsing step. After the incubation with DAB Chromogen, the tissues were washed with tap water, and then the nuclei of tissue cells were stained with haematoxylin. Tissues were incubated with aqueous haematoxylin for 5 min at room temperature, and then the slides were washed twice in a bath of distilled water for 5 min. After mounting the samples with non-aqueous permanent mounting medium, the samples were scanned and analyzed under the microscope.

4. Results

4.1. Expression of reprogramming factors and stem cell genetic programs in cancer cell lines

In 2006, *Shinya Yamanaka* and *colleagues* found that somatic cells can be reprogrammed into pluripotent stem cells through the action of four defined reprogramming factors, Oct3/4 (Oct4), Sox2, KLF4 and Myc (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). These reprogramming factors are up-regulated and involved in the maintenance of pluripotency in embryonic stem (ES) cells. Researchers also found that the expression of reprogramming factors and their target genes are detectable in tumor cells and that they are associated with tumor grade and unfavorable prognosis (Ben-Porath et al., 2008; Schoenhals et al., 2009a). We extended these studies and analyzed the expression of reprogramming factors and stem cell genetic programs in GBM and breast cancer cell lines.

4.1.1. Expression of reprogramming factors in cancer cell lines

In this study, 4 different breast cancer cell lines and 7 different GBM cell lines were used and the levels of endogenously expressed reprogramming factors were measured. The normal mammary epithelial cell line (MCF-10A) was used as a control for the breast cancer cell lines, and the human foreskin cell line (BJ) was used as a control for the GBM cell lines. The expression of reprogramming factors and the activation of stem cell genetic programs in GBM cells were measured by RT-PCR and western blotting analysis were with specific primers and anti-bodies (Fig. 23, 24).
I detected high mRNA expression levels of reprogramming factors in four GBM cell lines when compared to BJ cell (Fig. 23). This confirms results obtained in gene expression analyses of tissues from patients (Ben-Porath et al., 2008; Schoenhals et al., 2009a). In the breast cancer cell lines, mRNA expression levels of reprogramming factors was up-regulated in MCF7 cells when compared to MCF-10A cells (Figure 23A). The western blot data show, that the MCF7 cells strongly express reprogramming factors, and confirm the RT-PCR data (Fig. 24B). In the GBM cell lines, Sox2 and Oct4 were expressed in all cell lines (Figure 24A). Nanog, Klf4 and Myc expression was found by RT-PCR. The RT-PCR data indicate mRNA expression of all reprogramming factors, but this is not seen by western blotting on the protein

level. This suggests a post-translational regulatory mechanism. I conclude that GBM cells are most likely partially de-differentiated through the expression of a subset of reprogramming factors.

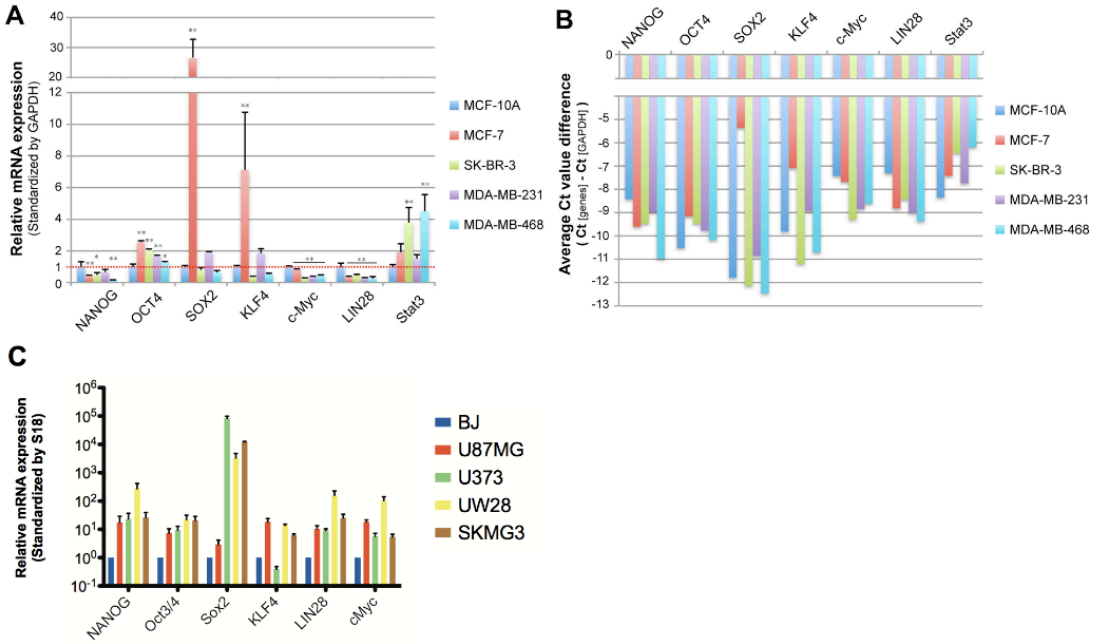


Figure 23. Expression of reprogramming factors in GBM and breast cancer cell lines. (A) mRNA expression level of reprogramming factors in breast cancer cell lines. Data were normalized to MCF-10A cells. (B) Average Ct value differences of the genes from that of GAPDH (Ct[genes of interest] – Ct[GAPDH]) are indicated to show th mRNA expression levels as compared with GAPDH expression in identical cell lines. (C) mRNA expression level of reprogramming factors in GBM cell lines. Data were normalized to BJ cells, and relative mRNA expression is standardized by an enodogenous control of S18. N=3. Error bars present mean ± S.D.

The signal transducer and activator of transcription 3 (STAT3) can assume the function of an oncogene, when its activation is deregulated in extent and duration. It can regulate stem cell and cancer stem cell proliferation and self-renew (Cartwright, 2005; Dahéron et al., 2004; Sherry et al., 2009). In somatic cell reprogramming, STAT3 enhances and promotes the reprogramming process by modifying epigenetic parameters (Tang et al., 2012). Clinical investigations show that STAT3 activation in tumor tissue is associated with an unfavorable prognosis in breast and glioblastoma cancer patients (Birner et al., 2010; Sato et al., 2011; Sheen-Chen et al., 2008).

PI3K/AKT signaling pathway deregulation can also have oncogenic effects. PI3K/AKT signaling regulates various cell phenotypes including cell apoptosis, growth, transformation and metabolism (Vivanco and Sawyers, 2002). Clinical data show that GBM often exhibit PTEN loss-of-function mutations and breast cancers have PI3K gain-of-function mutations. Both types of mutations result in an activation of AKT signaling (Baeza et al., 2003; Sun et al., 2001; Wang et al., 1997; Wu et al., 2005). PI3K/AKT signaling pathway is also involved in the maintenance of stem cell

pluripotency and its roles in cell proliferation and growth (Watanabe et al., 2006). The role of PI3K/AKT signaling is still controversial in the process of somatic cell reprogramming. Although activation of PI3K/AKT maintains ES cell pluripotency and enhances transcription factors mediated reprogramming efficacy, *Nakamura* and *colleagues* showed that activation of AKT signaling reduced the efficiency of reprogramming in somatic cell nuclear transfer experiments (Nakamura et al., 2008). For these reason, I also evaluated the expression and phosphorylation of STAT3, and the activation of PI3K/AKT signaling molecules by western blot analysis. Western blotting showed that STAT3 was expressed and phosphorylated in breast cancer cell lines (Figure 24B) and most GBM cell lines except UW28 and SF767 cells (Figure 24A). Expression of the reprogramming factors, and activation of STAT3 and PI3K/AKT signaling indicate that MCF7 cells and most glioblastoma cancer cell lines have de-differentiated phenotypes. These result are relevant in the light of clinical data reported earlier (Ben-Porath et al., 2008; Schoenhals et al., 2009a).

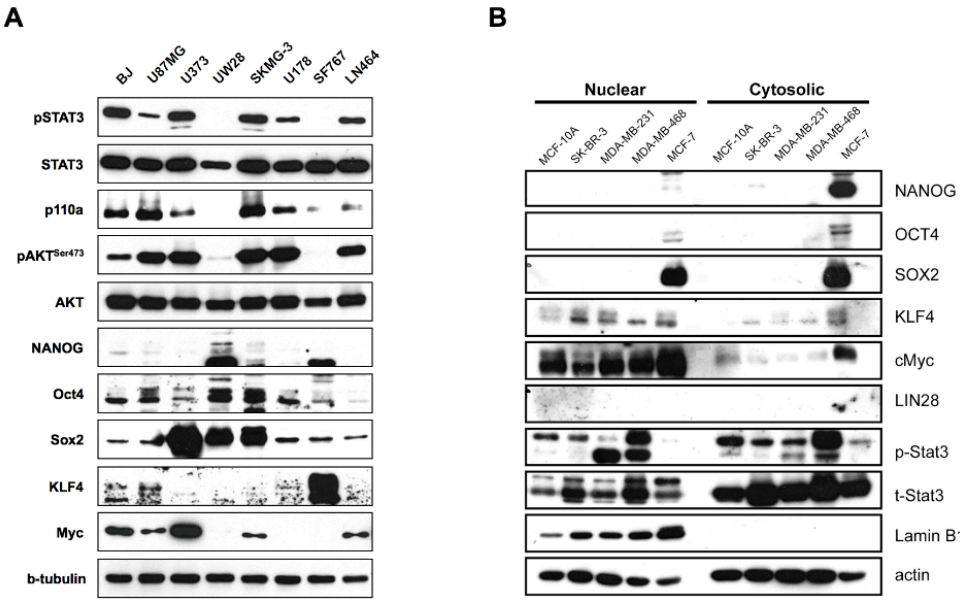


Figure 24. Protein expression level of reprogramming factors, pSTAT3, PI3K, and pAKT in GBM and breast cancer cell lines. (A) Western blot analysis of reprogramming factors, pSTAT3, PI3K, and pAKT in BJ normal cell line and GBM cell lines. b-tubulin was used as the loading control. (B) Western blot analysis of reprogramming factors and pSTAT3 in normal mammary epithelial cell, MCF-10A, and breast cancer cell lines. Cell lysates were fractionated into nuclear and cytosolic fractions. Nuclear envelope protein Lamin B1 used as a marker for nuclear fraction and actin was used as an endogenous loading control of the lysate.

4.1.2. mRNA expression of stem cell markers in glioblastoma cell lines

The signaling networks characteristic for stem cells or iPS cells are still incompletely defined, but in 2010, *Kim* and *colleagues* described important stem cell, gene

expression signatures (Kim et al., 2010). These authors found three main gene sets that regulate and maintain stem cell pluripotency and self-renewal. These three main gene sets are Core (Core pluripotency factors), PrC (Polycomb repressive complex factors) and Myc (Myc-related factors) genes and have been named the CPM modules (Kim et al., 2010).

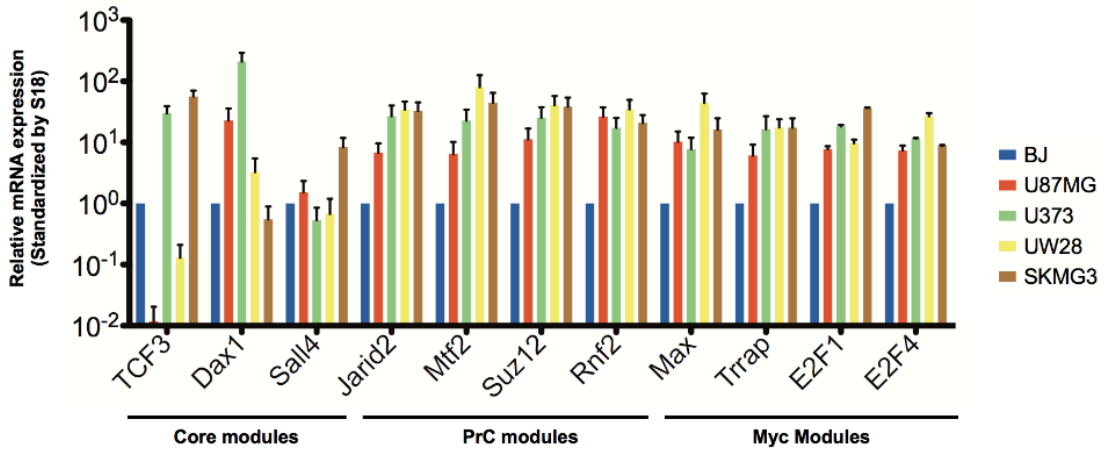


Figure 25. mRNA expression level of CPM modules in GBM cell lines. Expression was determined by RT-PCR. Data were normalized to BJ cells. N=3. Error bars represent mean ± S.D.

To assess the expression of genes of the CPM modules in cancer cells that indicate stem cell phenotypes, RT-PCR was performed with CPM module gene specific primers (Fig. 25). mRNAs of the PrC and Myc modules were highly expressed in the indicated glioblastoma cell lines when compared to BJ cell (Fig. 25). Core module genes were also highly expressed in glioblastoma cell lines, except the transcription factor 3 (TCF3), which has been identified as a regulator of stem cell fate (Cole et al., 2008).

Myc is the most well studied oncogene among the four reprogramming factors in cancers. Myc regulates many biological pathways, including stem cell self-renewal (Meyer and Penn, 2008).

PrC module genes are repressors in association with H3K27me3 (Surface et al., 2010). Absence or loss of function PrC proteins in ES cells diminishes their pluripotency potential and they fail to undergo differentiation (Surface et al., 2010). Over-expression of PrC proteins has been observed in various tumors (Simon and Lange, 2008), and the expression is strongly associated with the de-differentiated phenotypes of tumors (Ben-Porath et al., 2008).

In this study, mRNA expression levels of CPM modules were analyzed by RT-PCR with specific primer sets. Fig. 25 shows that glioblastoma cancer cells highly express CPM modules, with the exception of TCF3.

Expression of TCF3 in the absence of β-catenin, enhances embryonic stem cell differentiation by regulating the expression of NANOG and Oct4 (Nguyen et al., 2006; Ombrato et al., 2012; Pereira et al., 2006; Wu et al., 2012; Yi et al., 2011). We observed different expression levels of TCF3 mRNA in different glioblastoma cell lines (Fig. 25). This observation suggests that glioblastoma cancer cell lines do not represent fully differentiated or fully de-differentiated cells, but represent intermediate, partially de-differentiated phenotypes as they are frequently found in glioblastoma patients (Fig. 25) (Ben-Porath et al., 2008).

4.2. Expression of the microRNA 302/367 cluster in U87MG cells

The microRNA 302/367 cluster is preferentially expressed in human ES cells (Barroso-delJesus et al., 2008; Suh et al., 2004). The ectopic expression of the miR 302/367 cluster induces reprogramming of somatic cells, and enhances the somatic cell reprogramming efficiency of the reprogramming factors (Anokye-Danso et al., 2011).

Reprogramming of cells to the iPS state and the use of such cells in regenerative medicine are promising therapeutic options. However, there are still technical problems, related to the efficiency of the reprogramming process and the potential tumorigenicity of the manipulated cells obtained with the reprogramming factors. The microRNA 302/367 cluster plays an interesting role in the reprogramming process. The microRNA 302/367 cluster not only enhances the efficiency of the reprogramming process, but also prevents tumor formation by iPS cells obtained through the expression of the four transcription factors (Kuo et al., 2012; Liao et al., 2011; Lin et al., 2010).

We studied the possibility to change the transformed phenotype of glioblastoma cancer cells through the expression of the miR 302/367 cluster. For this purpose, we designed and cloned a part of LARP7 gene, including the microRNA 302/367 cluster, from MEF cells. Genomic DNA from MEF cells was isolated, and then a part of LARP7 gene, including the microRNA 302/367 cluster, was amplified with specific primers by PCR. The PCR product was then ligated into the pLVET-tTR-KRAB vector, a doxycycline inducible “tet-on” vector. Fig. 26A shows the map of the pLVET-tTR-KRAB-miR 302/367 cluster. After cloning the miRNA 302/367 cluster into the pLVET-tTR-KRAB vector, the plasmid DNA was transformed into Top10 competent cells. The plasmid DNA was isolated and sequenced to confirm the miRNA 302/367 cluster insertion in the vector. Because pLVET-tTR-KRAB vector is a lentiviral vector, it was transfected into 293T cells together with the lentivirus packaging plasmid

DNAs. The cells produced lentiviral particles encoding the microRNA 302/367 cluster. The cell lines we used in this study were infected with the miRNA 302/367 cluster encoding lentiviral particles.

To measure the expression of the microRNA 302/367 cluster in U87MG cells, stem-loop RT-PCR was carried out. Fig. 26B shows the expression of the miRNA 302/367 cluster upon induction of the cells with doxycycline for 10 days. The miRNA 302/367 cluster is expressed in the presence of doxycycline in the infected U87MG cells. Non-infected U87MG cells served as controls. To establish miRNA 302/367 cluster expressing cell lines, FACS sorting was performed and GFP expressing cells were obtained.

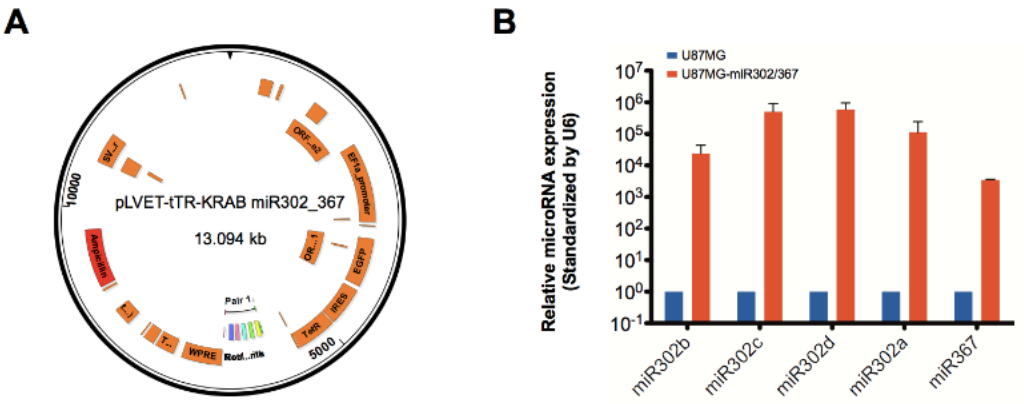


Figure 26. Expression vector of the miR 302/367 cluster. (A) For over expression of the miR 302/367 cluster, a part of the mouse genomic DNA containing the miR 302/367 cluster was cloned into the doxycycline inducible lintiviral vector. (B) U87MG cells were infected 2 times with the vector, and then the infected cells were growing in the 10% FCS DMEM medium with 2ug/mL doxycycline for 5 days. After 5 days, the miR 302/367 cluster infected cells were sorted out by FACs using GFP signals. To evaluate the miR 302/367 cluster expression level, Stem-Loop RT-PCR was performed. Data were standardized by U6 and normalized non-infected U87MG cells. N=3. Error bars represent mean ± S.D.

4.3. Activation of signaling pathways and stem cell characteristic genes upon expression of the miRNA 302/367 cluster

Micro RNAs are small non-coding molecules that regulate gene expression in cells (Inui et al., 2010; Wahid et al., 2010). miRNAs play important roles in fundamental cell processes, including cell development, differentiation, proliferation survival and death (Garzon et al., 2010). Mature miRNA can cause cleavage of mRNA or translational repression by incorporation into RISC (Bartel, 2009; Shukla et al., 2011). Because miRNAs can regulate and target components of signaling pathways, we performed protein profiling analysis to assess the influence of miR 302/367 cluster expression on signaling molecules in U87MG cells. These data showed that the miR 302/367 cluster strongly inhibits PI3K/AKT signaling components and the STAT3

signaling pathway. In addition, it influences the expression of genes indicative for stem cells, including the reprogramming factors in GBM cells.

4.3.1. Global protein profiling upon over-expression of the microRNA 302/367 cluster in U87MG cells

miRNAs inhibit or enhance expression of target proteins (Bartel, 2009; Bartel and Chen, 2004; Garzon et al., 2010; Shukla et al., 2011; Wahid et al., 2010). For this reason, protein profiling analyses were performed. The patterns of cellular proteins of U87MG cells were compared as a function of miRNA 302/367 cluster expression.
Figure 27A shows the design of the protein profiling experiments. The endogenous proteins of the two cell lines were labeled by the SILAC (Stable Isotope Labeling by Amino acids in Cell culture) method for 2 weeks. Then cells were the lysed with protein lysis buffer, the cell lysate sonicated and protein extracts were obtained. After determining the protein concentration by "NanoDrop", the labeled proteins from both cell extracts were mixed at a ratio of 1:1. The mixture was fractionated on a 1D page gel and different size fractions were obtained. Proteins from individual size fractions were digested with trypsin and the proteins were identified and quantified by mass spectrometry and MaxQuant software analyses.
The protein analyses yielded the identification of 4,450 proteins. 26 proteins were found to be significantly suppressed and 35 proteins strongly enhanced in U87MG cells expressing the miRNA 302/367 cluster (Figure 27B). Fig. 28 shows the list of proteins regulated by the miR 302/367 cluster expression in U87MG cells. A remarkable fraction of the suppressed proteins were associated with PI3K/AKT signaling (Fig. 28A). This corroborates our findings described above. The mRNA of the PI3 kinase catalytic domain, PI3 kinase regulatory domain and AKT1 are targets of the miRNA 302/367 cluster identified by the web database (www.microrna.org; www.mirdb.org; Fig. 28A). These proteins were also found to be suppressed in our protein profiling analysis (Fig. 28A). Protein profiling also shows that the miR 302/367 cluster significantly enhanced the expression of tumor suppressor genes, cytoskeleton organization genes and metabolism related genes and migration inhibitory factor CD9 (Fig. 28B). The protein expression affected by the miR 302/367 cluster in U87MG cells suggests that the miR 302/367 cluster might exert effects on the U87MG cell phenotypes through the regulation of nodal proteins such as PI3K/AKT signaling components.

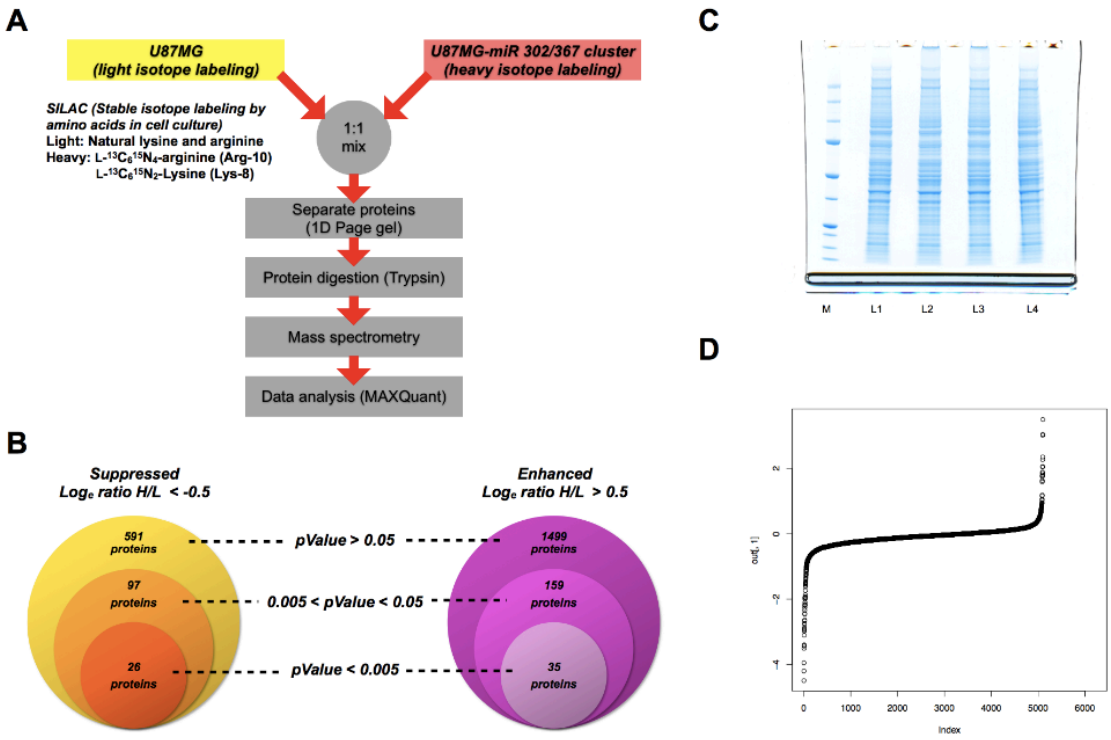


Figure 27. Scheme of protein profiling analysis. (A) Cells were labeled with different isotopes. Proteins in U87MG cells were labeled with natural lysine and arginine, called light isotope, and proteins in the miR 302/367 cluster over-expressed U87MG cells were labeled with Arg-10 and Lys-8, called heavy isotope. (C) Labeled proteins were mixed as 1:1 ratio, and then loaded on 1D-page gel to exclude with different size. (D) The size excluded proteins were digested by trypsin, and then the digested proteins were identified and quantified by mass spectrometry analysis and MaxQuant software. (B)(D) Total 4,450 proteins were identified. 591 proteins were down-regulated, and 1498 proteins were up-regulated by the miR 302/367 cluster.

A

Suppressed proteins upon over-expression of the microRNA 302/367 cluster

Gene	H/L	Gene	H/L	Gene	H/L
ACADL	-1.7	EPS8L2	-2.4	PDLIM5	-1.3
AKT1	*-1.8*	FHL3	-1.6	*PIK3C3*	*-0.8*
ANKH	-1.2	*FN1*	*-2.1*	*PIK3CB*	*-0.9*
APBB2	-1.8	GSTA4	-3.4	*PIK3R1*	*-1.4*
CDKN2C	-1.6	HSD17B8	-2.0	*PIK3R2*	*-1.6*
CHM	-2.9	MYO1D	-1.3	*PIK3R4*	*-1.2*
CRABP1	-2.1	NCAM1	-2.1	PLA2G4A	-2.1
CSRP2	-2.0	*NEDD4*	*-2.0*	XDH	-1.8
CYP51A1	-1.6	PAK1	-1.3		

Red	*PI3K/AKT signaling pathway related genes*
	Target predicted genes of miR 302 bcda
	Target predicted genes of miR 367

B

Enhanced proteins upon over-expression of the microRNA 302/367 cluster

Gene	H/L	Gene	H/L	Gene	H/L
ACYP2	*2.7*	*GALK1*	*2.0*	PROCR	2.9
B2M	3.1	HNRNPH3	1.8	PTPLAD1	1.4
BLVRB	*2.9*	ILVBL	1.7	SFXN1	2.2
CD9	*3.1*	LRPPRC	2.2	SH3BGRL3	1.6
COL6A3	*1.9*	*MAP1B*	*1.8*	SLIRP	2.0
DDAH2	2.1	*MTHFD2*	*1.5*	*STMN1*	*1.5*
ECE1	1.6	*MYBBP1A*	*1.7*	*STMN2*	*1.5*
ESYT1	1.5	NQO1	2.2	SYNGR2	3.1
FTL	*2.1*	*PEA15*	*1.4*	*TBCD*	*1.7*
FYCO1	1.3	PITHD1	1.8	*UCHL1*	*2.9*
G6PD	*1.4*	*PNP*	*2.0*	UQCRB	2.5
GALE	*1.7*	PRKDC	2.1		

Red	*Migrating inhibitory factor*
Blue	*Tumor suppressor*
Purple	*Cytoskeleton organization*
Green	*Metabolism*

Figure 28. List of up- and down-regulated proteins by the miR 302/367 cluster in U87MG cells. Protein profiling analysis showed that total 26 proteins were significantly down-regulated and total 35 proteins were significantly up-regulated upon over-expression of the miR 302/367 cluster in U87MG cells. (A) Most down-regulated proteins are highly associated with PI3K / AKT signaling pathway (Red italic). (B) Up-regulated proteins are associated with tumor suppressor (Blue), cell migration (Red), cytoskeleton organization (Purple), and metabolism (Green).

4.3.2. Inhibition of the PI3K/AKT and STAT3 signaling pathways upon expression of the miRNA 302/367 cluster in GBM cells

The protein profiling analyses indicated that the miRNA 302/367 cluster strongly suppresses components related to the PI3K/AKT signaling pathway (Fig. 28A). To confirm this observation, western blotting analyses were carried out and activated pathway components were visualized with PIK3CA and AKT specific anti-bodies (Fig. 29). The results show that expression of the miR 302/367 cluster inhibits the expression of the PI3 kinase catalytic domain, regulatory domain and the AKT molecule in GBM cell lines and human BJ cells (Fig. 29). The data confirm the protein profiling analyses. Moreover, inhibition of STAT3 phosphorylation was found to be a consequence of the miR 302/367 cluster expression in the GBM cell lines, but not in BJ cells (Fig. 29). On the contrary, the phosphorylation of STAT3 was up-regulated in BJ cells by the miR 302/367 cluster. The regulation of STAT3 activity by PI3K/AKT signaling pathway has not been entirely elucidated on the molecular level, but a functional connection has been established. The studies showed that STAT3 signaling can be activated through PI3K/BMX signaling (Hart et al., 2011; Vogt and Hart, 2011) or PTEN/AKT mediated signaling (la Iglesia et al., 2008). Although the mechanisms are still unclear, the miR 302/367 cluster strongly inhibits STAT3 activation in GBM cell lines.

Different observation, concerning the phosphorylation level of STAT3 as a function of miR 302/367 cluster expression, were made in BJ cells when compared to GBM cells. There could possibly be explained by the function of PTEN. Most of GBM patients have loss-of-function mutations in the PTEN gene (Baeza et al., 2003; Srividya et al., 2011; Wang et al., 1997). It has been suggested that the activation of the wild type PTEN gene increases the activation of STAT3 signaling via the PTEN/Akt/FOXO3/LIFRβ/STAT3 axis (la Iglesia et al., 2008). This observation and our findings suggest that although the miR 302/367 cluster directly inhibits PI3K/AKT signaling, the activation of AKT may restored through PTEN activation in PTEN wild-type cells by the inhibition of NEDD4, a PTEN negative regulator (Fig. 28A) (Amodio et al., 2010; Wang et al., 2007). Because of the PTEN mutation in GBM cancer cells, the effect of NEDD4 on PTEN may not be exerted upon expression of the miR 302/367 cluster. The stabilization of PTEN in normal cells, through the suppression of NEDD4, may increase the expression of LIFR□. This in turn could results in an increase in STAT3 phosphorylation in PTEN wild-type cells, but not in GBM cells with a loss-of-function PTEN mutation. Fig. 29 shows that the microRNA 302/367 cluster increases the phosphorylation of STAT3 in normal cells and suppressed it in GBM cell lines.

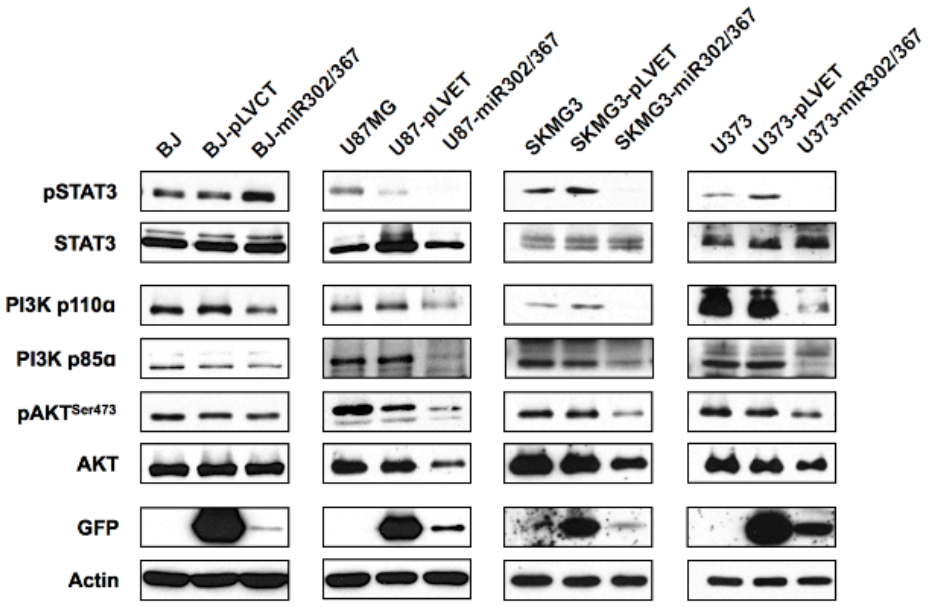


Figure 29. The microRNA 302/367 cluster inhibits PI3K / AKT and STAT3 signaling molecules in GBM cell lines. Western blot analysis shows the levels of pSTAT3, PI3K and AKT in BJ and GBM cell lines upon over-expression of the miR 302/367 cluster. Actin was used as the loading control.

4.3.3. Inhibition of endogenous reprogramming factor expression upon expression of the miRNA 302/367 cluster in GBM cells

The function of the miR 302/367 cluster in somatic cells is most likely associated with its ability to enhance the expression of reprogramming factors and stem cell related genetic programs (Kuo et al., 2012; Liao et al., 2011; Subramanyam et al., 2011). I evaluated the expression of the reprogramming factors and stem cell genetic programs upon expression of the miR 302/367 cluster in normal MEF cells and GBM cell lines. Western blotting analyses and RT-PCR experiments were performed. Fig. 30 shows that the expression of the reprogramming factors was increased in MEF cells upon expression the miR 302/367 cluster. However, the expression levels of the reprogramming factors were down-regulated in GBM cell lines. This indicates that the miR 302/367 cluster has different roles in normal and in cancer cells. As discussed above, it is conceivable that these differences in the expression of the reprogramming factors between normal and GBM cells, might be related to the PTEN status of the cells and the regulation of STAT3 activation. Alternatively, the different regulation of reprogramming factor expression might be caused by genetic mutations, e.g. in the miRNAs target sequences. However, the important finding of this study indicates that the activation of STAT3 is associated with the expression of the reprogramming factors.

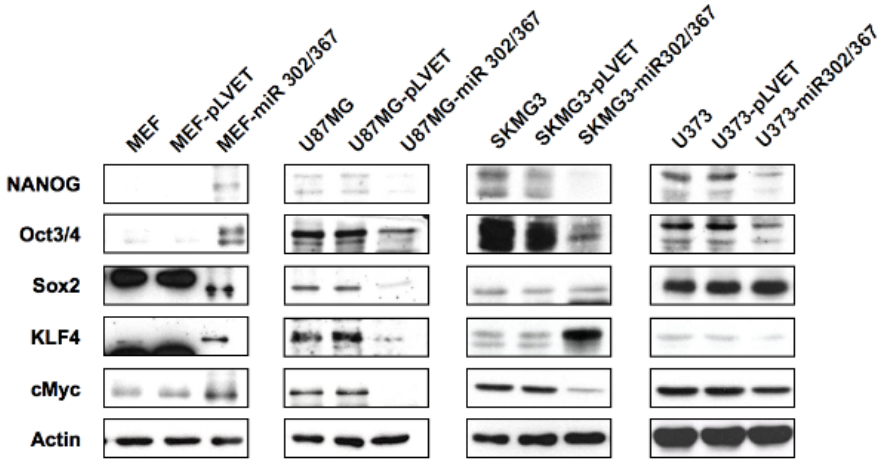


Figure 30. The microRNA 302/367 cluster inhibits expression of reprogramming factors in GBM cell lines but not normal MEF cells. Western blots shows the levels of the reprogramming factors in MEF and GBM cell lines upon over-expression of the miR 302/367 cluster. Actin was used as the loading control.

4.3.4. Inhibition of stem cell genetic programs by the miRNA 302/367 cluster in U87MG glioblastoma cells

Our data showed that the miR 302/367 cluster inhibits the endogenous expression of reprogramming factors in GBM cell lines. We also investigated the effects of miR 302/367 cluster expression on stem cell genetic programs. RT-PCR was performed with specific primers to evaluate the expression of stem cell genetic program related genes in U87MG cells. Fig. 31 shows that the microRNA 302/367 cluster strongly inhibited the expression of stem cell genetic program related genes in U87MG cells. The Myc module, which is related to stem cell pluripotency, self-renewal, and proliferation, was strongly down-regulated in U87MG cells; also, the core module, which regulates the expression of pluripotency related gene in stem cells, is strongly down-regulated, with the exception of TCF3 (Fig. 31). The PrC module is known as a gene set that modulates chromatin remodeling. In stem cells and differentiated cells, the PrC module is down-regulated (Kim and Orkin, 2011; Nagata et al., 2012; Widschwendter et al., 2006). The miR 302/367 cluster strongly suppressed the expression of the PrC module in U87MG cells. The Myc module and the core module, which are normally expressed in stem cells, but not in differentiated cells, were suppressed, and the PrC module, which is suppressed in differentiated cells and stem cells, but not in de-differentiated cells, was inhibited by the miR 302/367 cluster. These observations are consistent with a model in which the de-differentiated phenotypes of U87MG cells changed into a more differentiated state upon expression of the miR 302/367 cluster (Figure 31).

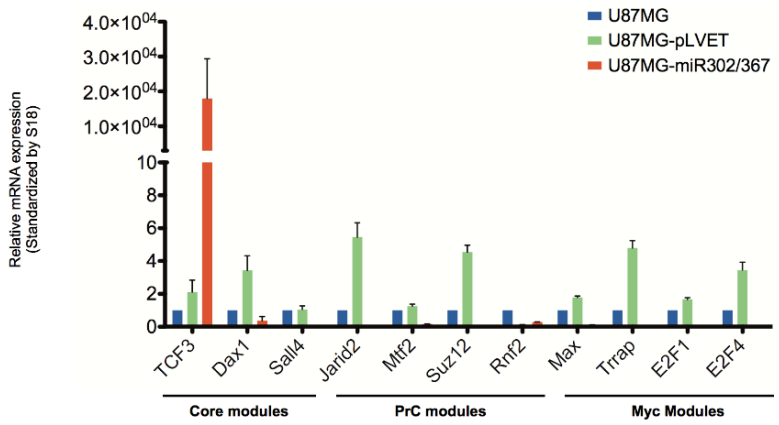


Figure 31. mRNA expression level of CPM modules in U87MG cells. Expression was determined by RT-PCR. Blue bars present non-infected U87MG cells, green bars present empty-vector infected U87MG cells, and red bars present the miR 302/367 cluster over-expressed U87MG cells. Data were standardized by S18 and normalized to BJ cells. N=3. Error bars represent mean ± S.D.

4.3.5. Inhibition of PI3K/AKT signaling by small molecular weight inhibitors suppresses reprogramming factor expression and STAT3 phosphorylation in cancer cells

I could show above that miR 302/367 cluster expression in U87MG cells results in the suppression of PI3K/AKT and STAT3 signaling and the downregulation of the endogenous reprogramming factors (Figure 28,29,30). This suggests that the expression of endogenous reprogramming factors may be regulated through PI3K/AKT and STAT3 signaling.

To investigate this model, U87MG cells and MCF7 cells were treated with the PI3K/AKT or STAT3 signaling inhibitors. The expression of the reprogramming factors and activation of STAT3 were strongly down-regulated in both cell lines upon exposure to the PI3K/AKT inhibitor LY294002. This compound inhibits the PI3 kinase catalytic domain (Fig. 32A). The same result was obtained when miR 302/367 cluster expressing GBM cell were treated with the inhibitor. This indicates that either the downregulation of PI3K/AKT signaling components by the miR 302/367 cluster or the inhibition of the PI3K enzymatic activity by a low molecular weight drug causes the suppression of the reprogramming factors.

Interestingly, the inhibition of STAT3 phosphorylation was also observed in the LY294002 treated cancer cells (Fig. 32A). It has been reported that PI3K/AKT signaling regulates activation of STAT3 through signaling events including the AKT/LIFRβ/STAT3 axis and the PI3K/BMX/STAT3 axis (Hart et al., 2011; la Iglesia et al., 2008; Vogt and Hart, 2011). Our data support the notion that PI3K and STAT3

signaling pathways are connected and show that LY294002 treatment inhibits the PI3K/STAT3 axis in cancer cells.

We also investigated if the expression of the reprogramming factors is affected by STAT3 inhibition. We treated MCF7 cells with Stattic, an established STAT3 inhibitor. Western blot analysis and RT-PCR were performed to assess the expression levels of the reprogramming factors (Fig. 32B, 33). The western blot data indicated, that Stattic is able to suppress the expression of the reprogramming factors in these cells (Fig. 32B). The RT-PCR confirmed this observation. The treatment with Stattic suppressed the mRNA expression of the reprogramming factors, with the exception of SOX2 (Fig. 33). Our data show that the expression of the reprogramming factors can be inhibited by LY294002 and Stattic in cancer cells. PI3K/AKT and STAT3 signaling probably regulate the reprogramming factor expression and subsequently the de-differentiated phenotypes of the cancer cells.

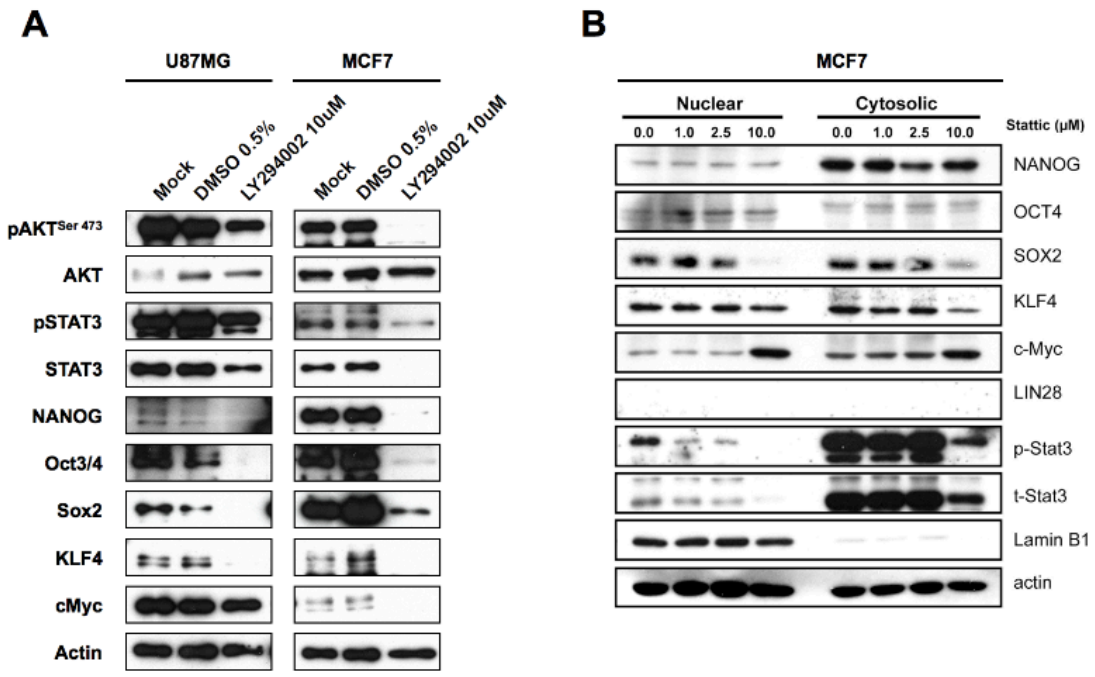


Figure 32. Inhibition of PI3K by LY294002 suppresses the expression of reprogramming factors through inhibition of AKT and STAT3 activation. (A) Western blot shows the levels of pAKT, pSTAT3 and reprogramming factors in U87MG and MCF7 cells 3 days after presence of PI3K inhibitor (LY294002) 10uM. Actin was used as the loading control. (B) STAT3 inhibitor (Stattic) was treated on MCF7 cells for 12 hours with indicated concentrations. Western blot shows the expression levels of the reprogramming factors upon treatment of Stattic with different concentrations in MCF7 cells. Lamin B1 was used as a marker for nuclear fraction and actin was used as an endogenous loading control of the lysates.

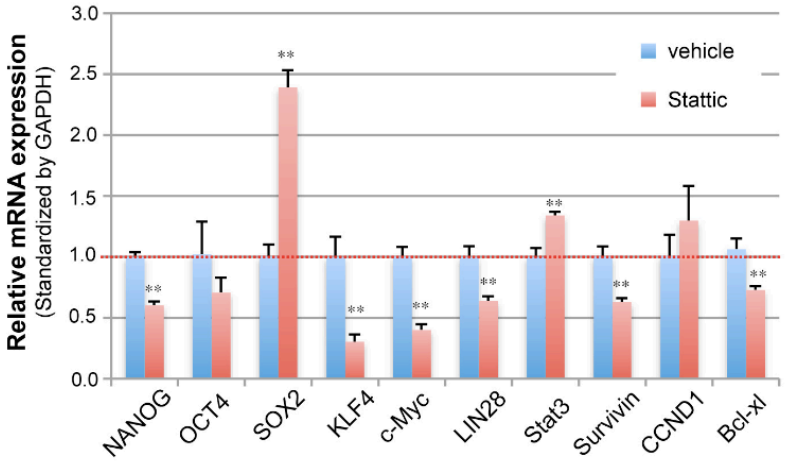


Figure 33. Effects of the STAT3 inhibitor Stattic on mRNA expression levels of the reprogramming factor genes in MCF7 cells. RT-PCR analyses of the reprogramming factors and STAT3 related genes were performed in MCF7 cells treated with or without 10 uM Stattic for 6 hours. Relative mRNA expression was standardized by GPADH and normalized by MCF-10A cells. N=3. Error bars represent mean ± S.D.

4.4. miRNA 302/367 cluster expression affects GBM cell phenotypes

The inappropriate activation of PI3K/AKT signaling can result in cellular transformation. It can trigger tumorigenic processes and de-regulate proliferation, apoptosis, glucose metabolism and mRNA translation (Vivanco and Sawyers, 2002). Because the miR 302/367 cluster strongly inhibits PI3K / AKT signaling in GBM cells (Fig. 28, 29) and suppresses the expression of the reprogramming factors (Fig. 30), the transformation related phenotypes of GBM cells were investigated. Cell morphology, invasiveness, proliferation, secretion of cytokines and drug sensitivity were compared in parental GBM cells and in GBM cells expressing the miR 302/367 cluster.

4.4.1. Effects of miRNA 302/367 cluster expression on the morphology, proliferation and drug sensitivity of U87MG cells

I evaluated the effects of miR 302/367 cluster expression on the morphology of U87MG cells. The cells were grown in culture medium containing 10% FCS for 20 days. U87MG cell morphology was evaluated by light microscopy. Fig. 34A shows the comparison of the morphologies of parental U87MG cells and cells upon expressing the miR 302/367 cluster. miR 302/367 cluster expressing U87MG cell assumed an epithelial like cell morphology. It should be noted that this change in morphology did not require special factor addition and occurred under regular cell culture conditions.

(Fig. 34A). It has been reported that PI3K/AKT signaling can induce the expression of mesenchmal marker genes and the cause epithelial to mesenchymal transition (EMT) (Larue and Bellacosa, 2005). EMT and the reverse process MET (mesenchymal to epithelial transition) (Raghu Kalluri, 2009) are characterized by changes in cell morphology through rearrangements of the cytoskeleton (Nakaya and Sheng, 2013; Savagner, 2010; Yilmaz and Christofori, 2009). We suggest that the miRNA 302/367 cluster induced changes in the U87MG cell morphology may be initiated through the inhibition of PI3K/AKT signaling, result in the downregulation of mesenchymal marker gene expression and thus cause the initiation of MET.

We also assessed the effects of PI3K/AKT signaling inhibition on U87MG cell proliferation directly. Proliferation of the cells was quantitated with the CCK-8 cell counting kit. We observed that *in vitro* the proliferation of U87MG cells was not affected upon expression of the miR 302/367 cluster (Fig. 34B).

Drug sensitivity is a most important parameter for the treatment of cancer patients. We evaluated if miRNA 302/367 cluster expression in U87MG cells might have an effect on this cellular property. The cells were exposed to increasing concentrations of the JAK inhibitor AG490 or the STAT3 inhibitor S3I-201 for 72 hours (Fig. 34C). After this period cell viabilities were measured with the CCK-8 cell counting kit. The JAK inhibitor AG490 had no effects on cell viability of U87MG and U87MG-pLVET cells, but the miR302/367 cluster expressing U87MG cells shown a markedly decreased cellular viability (Fig. 34C).

Exposure to high doses of the STAT3 inhibitor S3I-201, 15 uM, for 72 hours negatively affected the viability of U87MG and U87MG-pLVET cells, but low doses of S3I-201 had little effect (Fig. 34C). The miR302/367 cluster expressing U87MG cells were much more sensitive and low doses of S3I-201, 0.015 uM, affected the cells similarly as the parental cells treated with the high dosis of S3I-201, 15 uM (Fig. 34C). These data show that the miRNA 302/367 cluster expression in U87MG increases the sensitivity for JAK and STAT3 inhibitors.

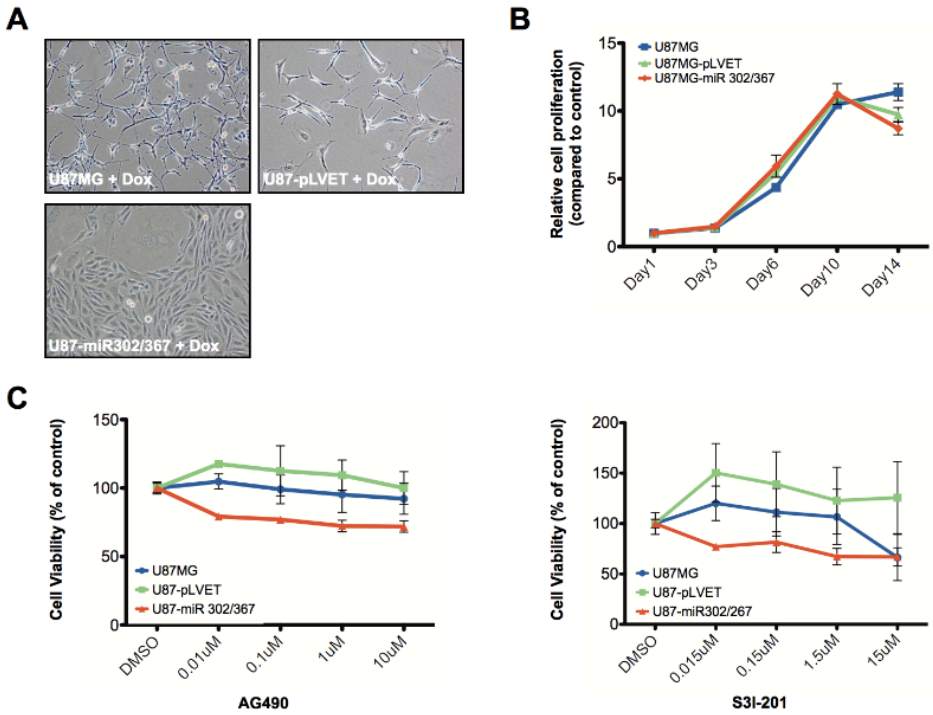


Figure 34. The miR 302/367 cluster changes the morphology of U87MG cells towards epithelial like cells, and increases JAK and STAT3 inhibitor sensitivities. (A) U87MG cells morphological changes by the long-term culture with presence of the miR 302/367 cluster were observed under microscope. Cells were cultured for 30 days with 2 ug/mL doxycycline. (B) Cell proliferation was determined by CCK-8 asay kit. Data were normalized to Day 1. N=3. Error bars represent mean ± S.D. (C) Drug sensitivities of U87-pLVET and U87-miR 302/367 to AG490 (JAK inhibitor) and S3I-201 (STAT3 inhibitor) were determined by CCk-8 assay kit, and compared with non-infected U87MG cells. Cells were treat with indicated drug doses for 72 hours. Data were standardized by DMSO treated group. N=3. Error bars represent mean ± S.D.

4.4.2. Effects of miRNA 302/367 cluster expression on cellular invasiveness and epithelial and mesenchymal gene markers in glioblastoma cells

EMT is often found at the leading front of invading primary tumor tissues and has been suspected to correlate with metastasis formation (Jing et al., 2011; Voulgari and Pintzas, 2009). It is characterized by changes in cell morphology and a more mesenchymal like appearance of the tumor cells. These cells also show an enhanced cell migratory capacity and invasiveness (Kalluri, 2009). EMT is strongly connected with cancer cell motility and invasiveness through the regulation of mesenchymal marker genes. They reduce cell polarity and result in a loss of cell-cell adhesion (Gunasinghe et al., 2012; Wang and Shang, 2013; Yilmaz and Christofori, 2009).

EMT not only plays a role in cancer invasiveness, but also has a crucial role in stem cell development and somatic cell reprogramming (Esteban et al., 2012; Hawkins et al., 2012; Li et al., 2010; Lim and Thiery, 2012; Nakaya and Sheng, 2013). During embryonic development, EMT regulates formation of the three germ layers, endoderm, mesoderm and ectoderm (Thiery et al., 2009).

MET is a process which is associated with cellular reprogramming by the *Yamanaka* factors (Li et al., 2010). *Li* and *colleagues* found that the *Yamanaka* factors block TGFβ signaling, and this results in the suppression of EMT. KLF4 increases the expression of E-cadherin, and thus contributes to MET during MEF cell reprogramming (Li et al., 2010). This is corroborated by experiments which showed that the suppression of E-cadherin expression by a specific shRNA prevents reprogramming by the *Yamanaka* factors and the induction of pluripotent stem cells from MEF (Li et al., 2010). miR302/367 cluster expression increases MET during somatic cell reprogramming, and up-regulates pluripotent and ES cell specific gene expression (Lamouille et al., 2013; Liao et al., 2011).

Our observations which describe morphology changes of U87MG upon expression of the miR302/367 cluster, suggest that the miR302/367 cluster might revert EMT and induce MET in U87MG cells. For this reason we evaluated the expression of genes indicative of EMT in GBM cells upon expression of the miR302/367 cluster. RT-PCR, western blotting and immunocytochemistry experiments were performed.

N-cadherin, Snail, Slug and Vimentin specific primers were used in the RT-PCR experiments to measure the expression of mesenchymal marker genes and E-cadherin specific primer pairs were used to follow an epithelial marker gene. The results of the RT-PCR analysis showed that mRNA expression of mesenchymal marker genes was strongly down-regulated in the miR302/367 cluster expressing U87MG cells; the epithelial marker gene E-cadherin was up-regulated when the cells were compared to parental or vector infected U87MG control cells (Fig. 35C).

The Western blotting experiments confirmed the RT-PCR analysis. Up-regulation of E-cadherin and down-regulation of vimentin and b-catenin were detected in the miR302/367 cluster expressing GBM cells (Fig. 35D). Immunocytochemistry analysis with vimentin specific anti-bodies also showed that the miR302/367 cluster suppressed the mesenchymal marker gene expression in U87MG cells. Vimentin was not detected in the miR302/367 cluster expressing U87MG cells when compared to non-infected or vector infected U87MG control cells (Fig. 36). These data strongly indicate that the miR 302/367 cluster inhibits signaling molecules indicative of EMT in GBM cells.

We investigated if these changes in gene expression might exert an effect on U87MG cell invasiveness *in vitro*. Cell invasion assays were performed and the ablity of cells to penetrate a layer of extra cellular matrix components (ECM) was measured. ECM contains many molecules which support cell growth and invasion of cancer cells. The *in vitro* ECM-based cell invasion assays try to mimck the microenvironmental conditions which cancer cells encounter *in vivo*. Plate inserts are coated with ECM

resembling basal membranes. The cells are seeded on the ECM coated plate inserts, and then incubated for 18 hours. The invading cells, which are attached to the lower side of the plate inserts, can then be quantitated by staining with crystal violet and used as a measure of cell invasiveness.

Fig. 35A shows the crystal violet staining of invading U87MG cells, and Fig. 35B the quantitation of the staining intensities. miR302/367 cluster expression strongly suppressed U87MG cell invasiveness when compared to non-infected and vector infected U87MG control cells (Fig. 35A, B).

These experiments suggest that the miRNA 302/367 cluster expression suppresses the U87MG cell invasiveness through the inhibition of EMT. This is accompanied by the down-regulation of mesenchymal marker and up-regulation of epithelial marker gene expression.

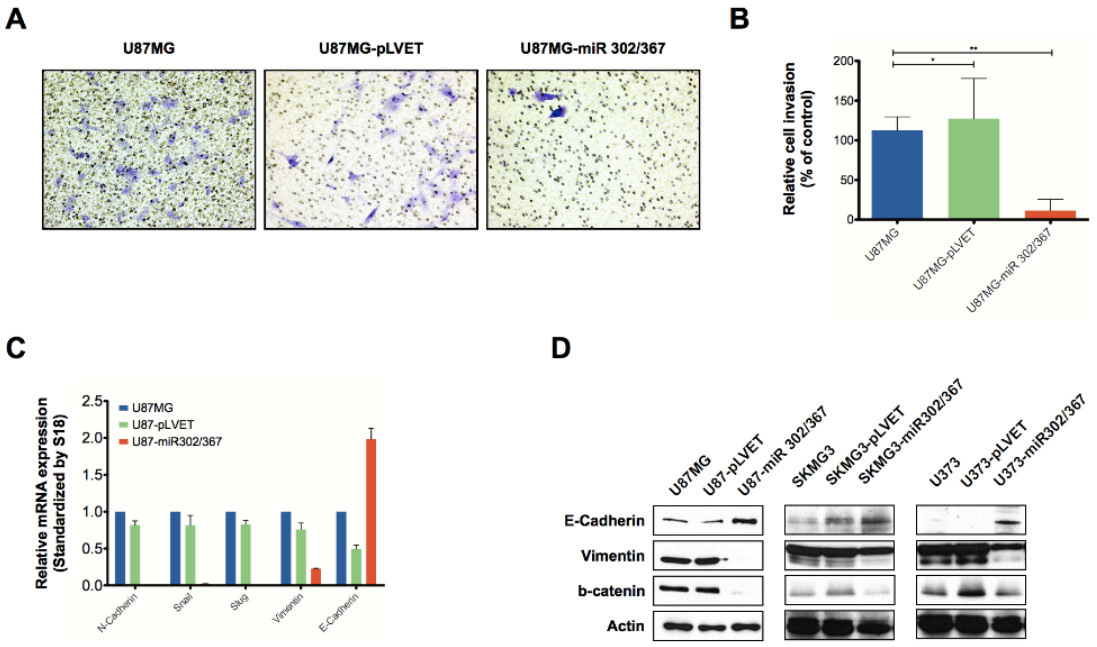


Figure 35. The miR302/367 cluster expression inhibits U87MG cell invasiveness through the regulation of marker genes for epithelial to mesenchymal transition (EMT). (A) To evaluate U87MG cell invasiveness upon over-expression of the miR 302/367 cluster, extracellular matrix (ECM) cell invasion assay carried out. Cells were plated on 2 mg/mL doxycycline in normal DMEM medium for 16 hours in 5%, CO_2, 37°C incubator. (B) Invaded cells were stained with crystal violet and quantified. N=6. Error bars represent mean ± S.D. *$P<0.1$. **$P<0.01$. (C) mRNA expression of EMT marker genes in U87MG cells upon induction of the miR 302/367 cluster was determined by RT-PCR. N=3. Error bars represent mean ± S.D. (D) Protein levels of EMT marker genes in GBM cell lines upon induction of the miR 302/367 cluster were determined by western blot analysis. Actin was used as the loading control.

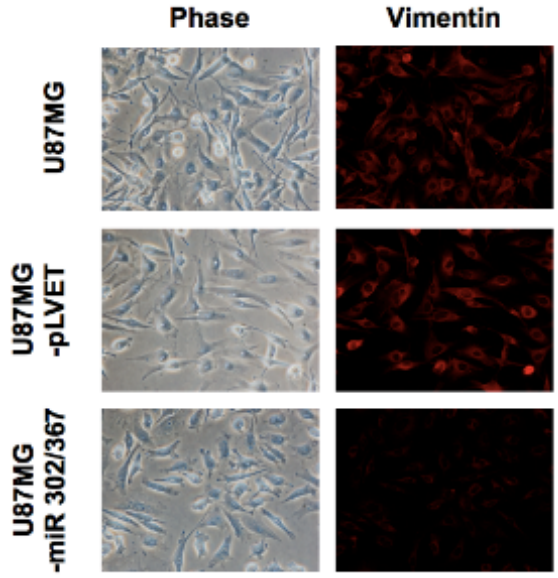


Figure 36. The miR302/367 cluster expression inhibits vimentin, a mesenchymal marker gene, in U87MG cells. Immunohistochemistry was performed to detect expression of a mesenchymal marker, vimentin.

4.4.3. Effects of miRNA302/367 cluster on GBM colony formation *in vitro*

In contrast to normal cells, tumor cells have the ability to grow anchorage-independently in culture. Soft agar growth assays and tumor sphere formation assays are useful tools to evaluate the anchorage-independent growth potential. Soft agar growth assays and tumor sphere formation assays are performed under 3D culture conditions, and resemble in some aspects the *in vivo* situation. In this study, soft agar growth assays with U87MG cells and tumor sphere formation assays with for SKMG3 and U373 cells were carried out. The GBM cell lines, used in this study, have previously been shown to be able to form colonies or sphere under 3D culture conditions. In the soft agar growth assay, around 0.1% ~ 0.2% of U87MG and vector infected U87MG cells formed into tumor colonies. No colonies were detected when miR302/367 cluster expressing U87MG cells were assayed (Fig. 37A, B). SKMG3 and U373 cells yielded similar results. Tumor sphere formation was detected when non-infected or vector infected SKMG3 and U373 cells were assayed, but miR302/367 cluster expressing SKMG3 and U373 cells had no or very little tumor sphere formation potential (Fig. 37C). These data show that the miR302/367 cluster expression suppresses the ability of these tumor cells to grow in an anchorage-independent fashion *in vitro*.

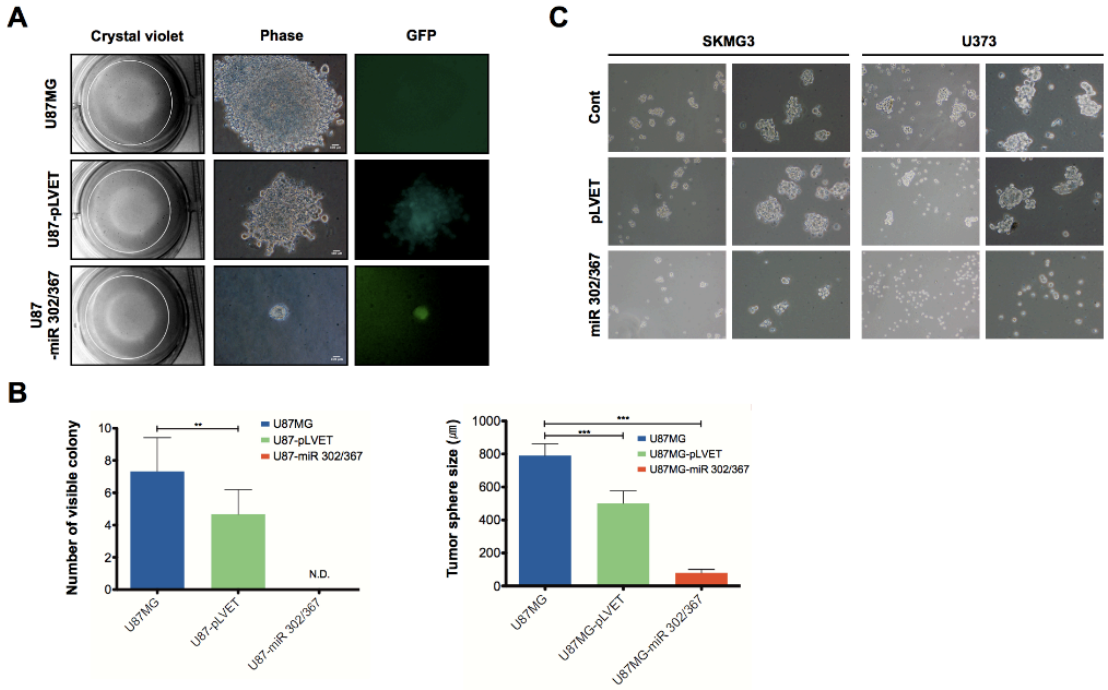


Figure 37. miR302/367 cluster expression inhibits colony formation of U87MG cells in soft agar and floating cell culture. (A) Soft agar colony assay was performed to evaluate U87MG colony formation *in vitro*. Cells were incubated in soft agar for 30 days with supplied daily fresh 10% FCS DMEM media containing 2 ug/mL doxycycline in 5%, CO_2, 37°C incubator. (B) Colonies were stained with crystal violet, and quantified the numer of visible colonies (over 200um size) under microscope (Left). Each colony size were determined by the Image J software, and whole colony size were quantified. Error bars represent mean ± S.D. $^{**}P<0.1$, $^{***}P<0.001$. (C) Tumor sphere assay was performed to evaluate SKMG3 and U373 tumor sphere formation *in vitro*. Cells were incubated for 5 days in 5%, CO_2, 37°C incubator with conditioned medium including EGF, FGF, Insulin, and B27 supplement. To avoid aggregation of cells, cells were passed through Cell Strainer after trypsinizing, and then plated on low-attachment plate with the conditioned medium.

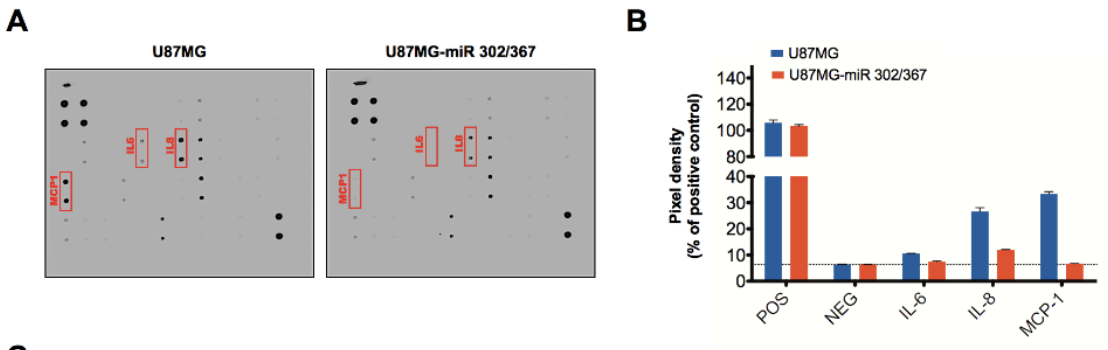
4.4.4. Effects of the miRNA 302/367 cluster on inflammatory cytokine expression and secretion in GBM cells

Signaling pathways which contribute to EMT of tumor cells through the up-regulation of mesenchymal marker genes also enhance their aggressive phenotypes and promote metastasis (Michael Zeisberg, 2009; Moustakas and Heldin, 2007). The signaling pathways can be triggered by extracellular signals, e.g. cytokines, soluble growth factors or hormones present in inflammatory microenvironments (Fernando et al., 2011; Mathias et al., 2010; Raychaudhuri and Vogelbaum, 2011; Sabbah et al., 2008; Yadav et al., 2011). Most of these extracellular signals are secreted from normal or from tumor cells present in the tumor tissue. They include cancer associated fibroblasts, immune cells or adjacent tumor cells (Joyce and Pollard, 2008; López-Novoa and Nieto, 2009; Wu and Zhou, 2009). Cancer cells secret cytokines, soluble growth factors or other soluble mediators which affect EMT through an autocrine loop (Fernando et al., 2011; Rokavec et al., 2012; Yadav et al., 2011) and miRNAs are able to regulate the expression and secretion of inflammatory

cytokines in cancer and immune cells (Bak and Mikkelsen, 2010; Chou et al., 2013; Rebane and Akdis, 2013). For these reasons, the effects of the miR302/367 cluster on the expression and secretion of inflammatory cytokines were evaluated. RT-PCR and cytokine array experiments were carried out with GBM cells.

4.4.4.1. Effects of the microRNA 302/367 cluster on inflammatory cytokine secretion by U87MG cells

To assess the effects of miR 302/367 cluster expression on inflammatory cytokine secretion, cytokine array analyses were carried out. U87MG cells and miR302/367 cluster expressing U87MG cells were incubated in serum free DMEM medium for 72 hours. Then the medium was collected and cytokine array analyses were carried out. Cell debris was removed by centrifugation of the medium and the supernatant was used for the cytokine analysis. The medium supernatant was incubated with a membrane onto which cytokine specific anti-bodies were bound and the signals were detected with fluorescing secondary antibodies. The fluorescence signal was analyzed by the Odyssey imaging system. The pixel density of the detected cytokines was standardized with the indicated positive and negative control pixel densities.



C

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    3' gaugaUUUUGUACCUUCGUGAAu 5' hsa-miR-302b
          :| |::|||||||||||
577:5' gugagGACAUGUGGAAGCACUUu 3' IL8
    3' ggugaCUUUGUACCUUCGUGAAu 5' hsa-miR-302c
          || |::|||||||||||
577:5' gugagGACAUGUGGAAGCACUUu 3' IL8
    3' ugugagUUUGUACCUUCGUGAAu 5' hsa-miR-302d
           | |::|||||||||||
577:5' gugaggACAUGUGGAAGCACUUu 3' IL8
    3' aguggUUUUGUACCUUCGUGAAu 5' hsa-miR-302a
          :| |::|||||||||||
577:5' gugagGACAUGUGGAAGCACUUu 3' IL8
```

Figure 38. The miR 302/367 cluster suppresses the secretion of pro-inflammatory cytokines by U87MG cells. Secretion of cytokines in U87MG cells by the miR 302/367 cluster was detected (A) and quantified (B) by cytokine array kits with Odyssey system. Data were standardized by pixel density of positive control and normalized to non-infected U87MG cells. N=2. Error bars represent mean ± S.D. (C) IL-8 is a predicted target of the miR 302/367 cluster. Target mRNA is predicted from microRNA target predict database (www.microrna.org; www.mirdb.org).

The results from the cytokine array analyses showed that the secretion of IL-6, IL-8 and MCP-1 by miR302/367 cluster expressing U87MG cells was strongly suppressed when compared to the parental control cells (Fig. 38A). The quantitation of the data confirms this conclusion (Fig. 38B). To gain insights into the mechanism by which the miR302/367 cluster regulates the cytokine expression, we searched for miR 302/367 cluster target sequences in the miRNA target predict database (www.microrna.org; www.mirdb.org; Fig. 38C). We found that the 3'UTR of the IL-8 mRNA contains the target sequence of the miR302/367 cluster (Fig. 38C). These findings indicate that IL-8 mRNA is a target for the miR302/367 cluster, and its expression results in the suppression of expression and secretion of IL-8. No miR302/367 cluster target sequences were found in the mRNAs encoding IL-6 and MCP-1.

4.4.4.2. Effects of the miRNA 302/367 cluster on the mRNA expression of inflammatory cytokines in GBM cells

I determined the mRNA levels of cytokines in GBM cell lines upon expression of the miR 302/367 cluster. RT-PCR was carried out with IL-6, IL-8 and MCP-1 specific primers. The cells were incubated in DMEM medium with 10% serum and 2 ug/mL doxycycline for 5 days, and then mRNAs were isolated. After cDNA synthesis from mRNAs the PCR reactions were performed.

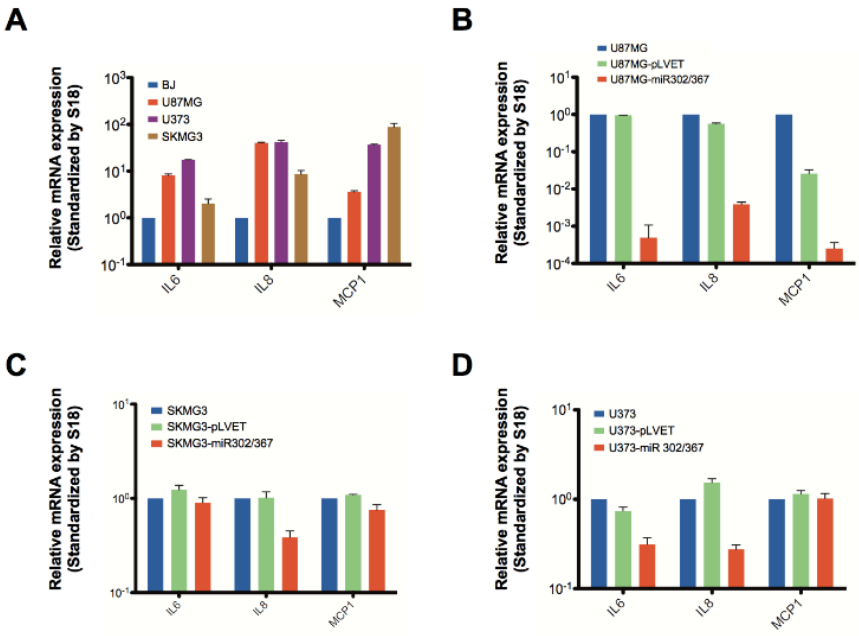

Figure 39. The miR 302/367 cluster suppresses the expression of cytokine encoding mRNA in GBM cells. (A) mRNA expression of cytokines in GBM cell lines. RT-PCR data shows mRNA expression of pro-inflammatory cytokines. Data were standardized by S18 and

normalized to BJ cells. N=3. Error bars represent mean ± S.D. (B)(C)(D) mRNA expression of cytokines in U87MG (B), SKMG3 (C), and U373 (D) upon over-expression of the miR 302/367 cluster. Data were standardized by S18 and normalized to their own non-infected cells. N=3. Error bars represent mean ± S.D.

Glioblastoma cell lines expressed high levels of cytokine mRNAs when compared to normal human foreskin BJ cells (Fig. 39A). We also measured the mRNA expression of cytokines in GBM cell lines upon expression of the miR 302/367 cluster. Fig. 39 shows that the miR 302/367 cluster strongly suppressed mRNA expression of IL-8 in U87MG (Fig. 39B), SKMG3 (Fig. 39C) and U373 cells (Fig. 39D). mRNA expression of MCP-1 was only inhibited in U87MG cells and not in SKMG3 or U373 cells. IL-6 mRNA was down-regulated in both U87MG and U373 cells, but not in SKMG3 cells. Our results show that the miRNA302/367 cluster inhibits mRNA expression of IL-8 and suppresses secretion of IL-8 in glioblastoma cell lines. In contrast, IL-6 and MCP-1 mRNA were not affected by the miR 302/367 cluster in SKMG-3 and U373 cells.

IL-8 plays a central role in the induction of tumor cell invasiveness through the activation of EMT (Fernando et al., 2011; Palena et al., 2012). Our results suggest that the inhibition of expression of IL-8 and other pro-inflammatory cytokines might be the mechanism by which the miR 302/367 cluster changes the phenotype of GBM cells and inhibits EMT.

4.4.4.3. Effects of a pro-inflammatory, paracrine cytokine loop on U87MG cell invasiveness upon expression of the miRNA 302/367 cluster

The secretion of inflammatory cytokines or soluble growth factors from cancer cells effect EMT by autocrine and paracrine loops (Palena et al., 2012). The cytokine array experiments show that the miR302/367 cluster influences the expression and secretion of inflammatory cytokines in U87MG cells (Fig. 38). Secreted inflammatory cytokines from neighboring cancer cells can induce EMT and tumor metastasis through a paracrine mode of action (López-Novoa and Nieto, 2009; Rokavec et al., 2012; Waerner et al., 2006). For these reasons, we evaluated the paracrine effects cell on invasiveness after changing the U87MG cell microenvironment through miR302/367 cluster expression.

To investigate the effects of the changes in the release of inflammatory molecules upon miR 302/367 cluster expression in U87MG cells on cell invasiveness, U87MG cells were co-cultured with U87MG cells, vector infected U87MG control cells, miR302/367 cluster expressing U87MG cells or no cells (Fig. 40B). The upper side of

a plate insert was coated with ECM, and U87MG cells were seeded on top of the coated insert. To assess possible paracrine effects of released molecules, different cells were seeded at the bottom of the plate. These cells were U87MG, U87-pLVET, U87MG expressing the miR302/367 cluster and no cells as controls. The effect of the cells present in the bottom compartment on the invasiveness of the cells present in the top insert were evaluated and compared (Figure 40).

Figure 40A shows the experimental arrangement. First, cells were plated on the bottom of the well. The next day, the medium was changed and the cells were incubated with fresh serum containing medium for 1 day at 37°C in a 5% CO2 incubator. The ECM coated plate insert was then placed in the top well, seeded with U87MG cells. The combinations were incubated for 18 hours at 37°C in a 5% CO2 incubator. After removing non-invasive cells from the inside of the insert, the bottom of the insert, with the invasive cells, was stained with crystal violet and stained cells were counted under the microscope (Fig. 40B).

The quantitation of invasive cells showed that more U87MG cells were found at the bottom of the top layer when they were cultured in the presence of regular U87MG cells or vector infected U87MG cells (Fig. 40B, C). The invasiveness of the U87MG cells in the upper compartment was reduced when miR302/367 cluster expressing U87MG cells were present in the lower compartment. The same was observed when the lower compartment was left without cells (Fig. 40B, C). These data suggest U87MG cells secrete soluble factors which are able to enhance the invasiveness of the cells in their vicinity. The miRNA302/367 cluster downregulates the secretion of such factors and possibly can affect EMT by mechanisms involving autocrine and paracrine loops.

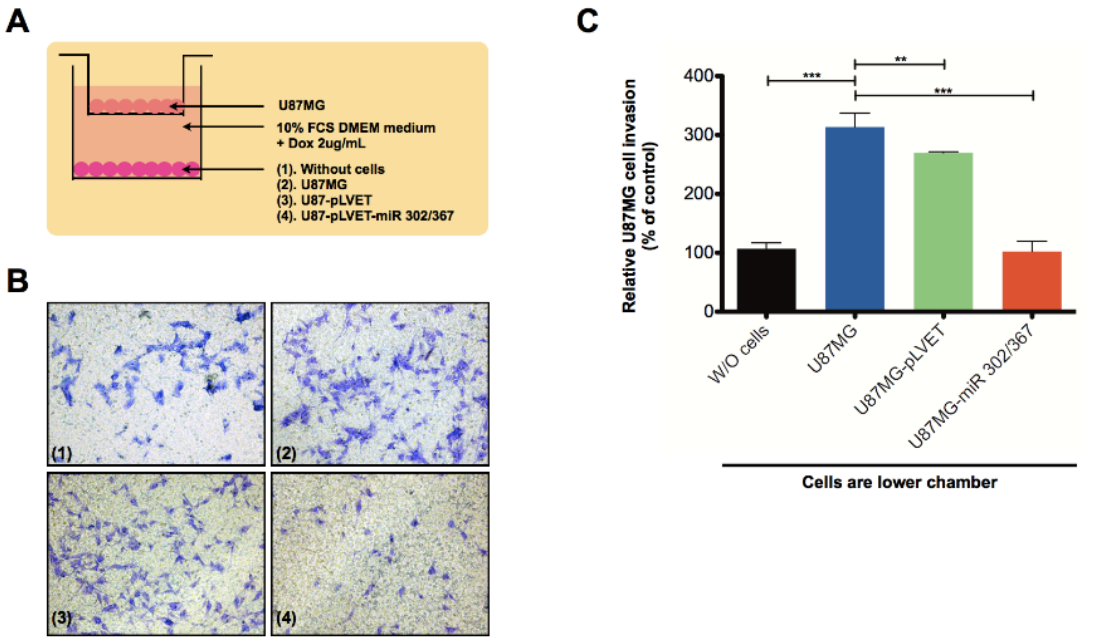

Figure 40. The miR 302/367 cluster blocks the secretion of cytokines that induce GBM cells invasiveness. (A) Scheme of the experiment. Same numbers of U87MG cells were plated on ECM gel coated upper chambers, and manipulated U87MG cells were plated on the bottom chambers with same numbers. Upper chamber U87MG cells were incubated for 16 hours, and then stained with crystal violet (B). (C) Numbers of invaded cells were quantified under the microscope with 5 different areas. N=3. Error bars represent mean ± S.D. $^{**}P<0.01$, $^{***}P<0.001$.

4.5. Effects of the miRNA302/367 cluster on U87MG tumor cell growth and metastasis formation

We showed above that miR302/367 cluster expression suppresses transformation related phenotypes of glioblastoma cells through the inhibition of PI3K/AKT and STAT3 signaling. It also enhances the expression of tumor suppressor genes and suppresses U87MG cell invasiveness through the downregulation of inflammatory cytokine secretion. We also evaluated the effects of the miR302/367 cluster expression on the properties of U87MG cell *in vivo*. For this purpose tumor growth and metastasis formation was monitored in NSG mice upon tumor cell transplantation.

The cells were introduced into NSG mice by subcutaneous injection. After injection, tumor volumes were measured in regular time intervals with a vernier caliper (Fig. 41B). The volume was calculated by the Ellipsoid equation method. Fig. 41A shows that the miR302/367 cluster strongly suppressed tumor growth *in vivo* when U87MG cells, vector infected U87MG cells and U87MG cells expressing the miR302/367 cluster were compared. The miR302/367 cluster expressing U87MG cells did not form tumors *in vivo* during the 30 day observation period (Fig. 41B).

30 days after tumor cell inoculation, the mice were sacrificed mice, dissected and metastasis formation in distant organs was investigated. We found liver metastases

in 3 out of 7 mice inoculated with U87MG cells. The same result was observed in mice injected with control cells infected with the control vector. No metastases were found in the group of mice injected with miR302/367 cluster expressing U87MG cells.

The tumor cells were introduced into the mice via the subcutaneous route. To confirm the loss of their metastatic potential, orthotopic application of the cells should be evaluated. However, the miR302/367 cluster inhibits granulocyte-macrophage colony-stimulating factors (GM-CSFs), IL-6, IL-8 and MCP1, which have been reported to regulate gliomagenesis and tumor progression in GBM patients (Mueller et al., 1999; Revoltella et al., 2012; Xie et al., 2008). The inhibition of these factors might indicate that miR302/367 cluster expression could become a tool to suppress metastasis formation.

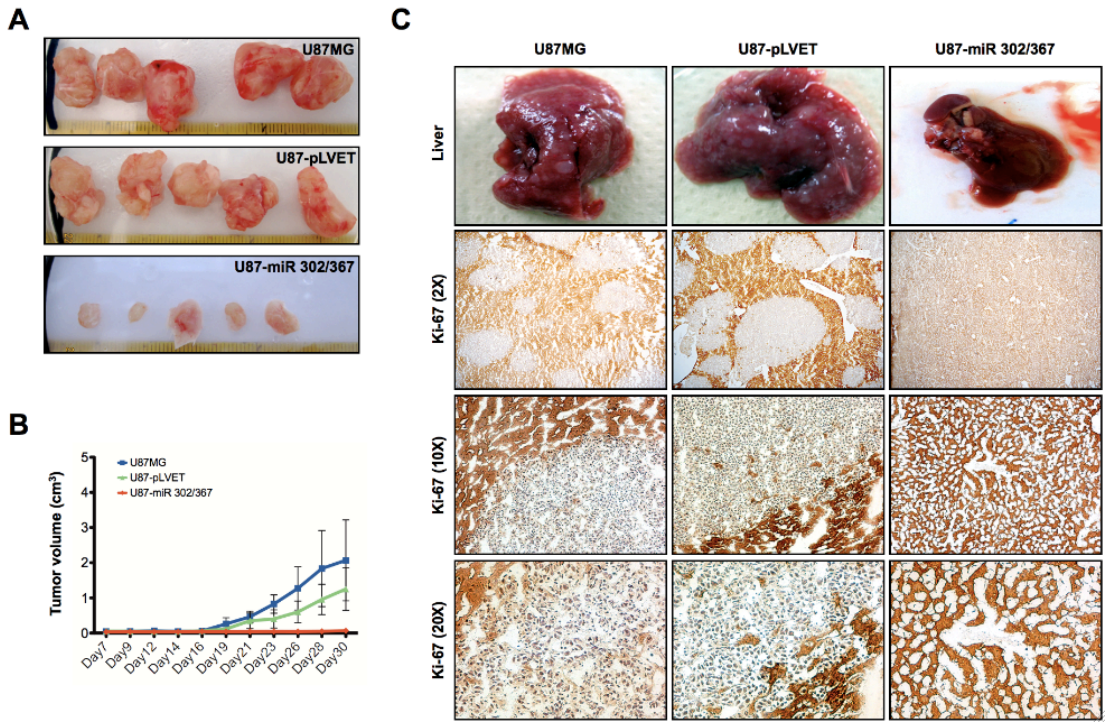

Figure 41. The miR 302/367 cluster suppresses U87MG tumor growth and liver metastasis formation *in vivo*. (A) Male 6- to 8- week-old NSG immnodeficient mice were injected *S.C.* in the flank with 5 X 106 viable cells in 0.1 mL of PBS with 20% matrigel. After 1 week later from the injection, tumor sizes were determined on the date indicated by measuring two dimensions (length (a) and width (b)). (B) Tumor volumes of each date estimated using the formula $V=aXb^2XPi/6$. N=7. Error bars represent mean ± S.D. (C) Liver metastasis was detected by immune-peroxidase staining with Ki-67 proliferation marker. Hematoxyline staining was used as a counter staining.

5. Discussion

The aim of this study was to evaluate the possibility to use reprogramming as a means to suppress the tumorigenic phenotypes of cancer cells. Since tumor cells are partially de-differentiated cells, placed somewhere in between tissue stem cells and functionally differentiated cells, a shift in their differentiation state by reprogramming agents might cause them to lose some or all of their tumorigenic properties. For this purpose, I attempted to reprogram GBM cells into a more normal phenotype through epigenetic modifications triggered by the expression of the miRNA 302/367 cluster. I also monitored the signaling events occurring in GBM cells upon induction of the microRNA 302/367 cluster. I evaluated the global protein expression pattern and the expression of particular proteins indicative of a stem cell state, a mesenchymal or an epithelial cell state. Finally, I monitored the transformation characteristics and quantitated the capabilities of the cells for soft-agar colony formation, cell invasion, cytokine secretion, tumor formation and metastasis in experimental animals and compared these parameters to the ones observed with parental GBM cells.

The expression of the miRNA 302/367 cluster can exert similar effects on somatic cells as the reprogramming factors defined by Yamanaka and convert somatic cells into pluripotent stem cells. An important difference between the two sets of reagents concerns tumorigenicity. It has been reported that reprogramming by the Yamanaka factors can yield tumorigenic cells, whereas the reprogramming by the miRNA 302/367 cluster does not (Anokye-Danso et al., 2011). The miRNA 302/367 cluster is not only capable to reprogram normal cells, but can also change skin cancer cells into pluripotent stem cells, when grown in stem cell conditioned medium (Lin et al., 2008). In addition the miRNA 302/367 cluster can affect breast cancer cells and increase their radio-sensitivity, most likely through the inhibition of AKT signaling (Liang et al., 2012). The mode of action of the microRNA 302/367 cluster involves the regulation of epigenetic modifications. It targets DNMTs and MECP2, enzymes which are involved in DNA methylation (Gruber and Zavolan, 2013). I studied the consequences of miRNA 302/367 cluster expression in GBM cells. GBM is one of the most aggressive cancers in humans. Aberrations of epigenetic modifications are highly prevalent in GBM and are correlated with tumor grade and invasiveness (Kreth et al., 2014).

5.1. Epigenetic modifications triggered by the miR 302/367 cluster expression as a strategy for differentiation therapy

De-differentiation is strongly associated with GBM grade and prognosis (Ben-Porath et al., 2008; Schoenhals et al., 2009a). In this study, we found that most of the glioblastoma and breast cancer cell lines expressed a subset of the four reprogramming factors. We also could show that mRNA of stem cell genetic program related genes are present. GBM assume stem like phenotypes possibly through the endogenous expression of reprogramming factors.

Changing the differentiation state of tumor cells through the inhibition of stem cell genetic programs is an attractive option. Promoting differentiation has been used as a therapeutic principle and retinoids (RAs) are the most prominent cell differentiation agents in cancer therapy. They are a family of signaling molecules that are related to vitamin A in their chemical structures. They are able of to induce differentiation and arrest proliferation of cancer cells. Acute promyelocytic leukemia can be successfully treated with pharmacological doses of retinoids which trigger differentiation of the leukemic cells into normal granulocytes (Fenaux et al., 2007). 13-cis-RA, an isomer of all-trans RA, can cause the differentiation of human neuroblastomas and reduce tumor growth (Reynolds, 2000). Numerous preclinical and clinical studies have employed retinoids, alone or in combination therapy, for the treatment of breast, ovarian, renal, head and neck, melanoma, and prostate cancers. The combined treatment of liposomal RA with the HDAC inhibitor trichostatin A caused a significant growth inhibition in a xenograft model of human kidney cancer (Touma et al., 2005). Moreover, the combination of interferon-α and 13-cis-RA, compared with interferone-α alone, resulted in significantly longer progression-free and overall survival for metastatic renal carcinoma patients (Escudier et al., 2009). These studies, based on the potential of RA as a differentiating signal, show that differentiation therapy is a promising therapeutic strategy to reduce tumor cell malignancy and advance cancer treatment.

Despite these encouraging results, the possibility to change entire genetic programs of tumor cells and suppress cancer phenotypes has not been widely exploited yet. It could assume a complementary role to conventional chemotherapeutic strategies aimed at cancer cell death which is often only temporarily effective. The most widely used drug in chemotherapy of GBM is temozolomide. Temozolomide is an alkylating agent and used for the treatment of grad IV glioma. Temozolomide alkylates DNA and triggers apoptosis of tumor cells. However, the presence of MGMT, which repairs

DNA damage, diminishes the therapeutic efficacy of temozolomide (Hegi et al., 2004; 2005). For this reason, a combination therapy with inhibitors affecting epigenetic gene regulation is required to increase the therapeutic efficacy of temozolomide in GBM patients (Hegi et al., 2009).

In this study, we found that the miR 302/367 cluster alters the gene expression pattern in U87MG cells. Protein profiling analysis indicated multiple changes which resulted in modified U87MG characteristics and a loss of transformation phenotypes. Both protein expression analyses and RT-PCR measurements suggest that stem cell genetic program related genes are affected by the miR 302/367 cluster. This shifted U87MG cells toward more differentiated cell phenotypes. Interestingly, these changes also increased the sensitivities for JAK and STAT3 inhibitors. Moreover, the loss of stem cell genetic programs in GBM by the miR 302/367 cluster suppressed tumor colony formation and invasiveness *in vitro* and *in vivo*, which are relevant cancer stem cell phenotypes. These experiments show that the microRNA 302/367 cluster can be used as a differentiation inducing agent, and can possibly supplement the conventional chemotherapy.

5.2. Different effects of miR 302/367 cluster expression in normal cells and GBM cells

The miRNA 302/367 cluster is an embryonic stem cell specific miRNA cluster (Barroso-delJesus et al., 2008). The main function of the miR 302/367 cluster in normal cells is the enhancement of stem cell gene expression and the establishment of pluripotency through epigenetic modifications. This also requires a special conditioned medium (Lipchina et al., 2012). It has been reported that the miRNA 302/367 cluster enhances the expression of pluripotency and embryonic stem cell genes through the inhibition of cell cycle, EMT and epigenetic regulators.

I evaluated the expression of stem cell genes in normal and glioblastoma cells upon expression of the miR 302/367 cluster. Western blotting analyses were carried out and the expression of the four reprogramming factors was monitored. I found that the miR 302/367 cluster enhanced the expression of the reprogramming factors in normal MEF cells, but inhibited the endogenous expression of the reprogramming factors in GBM cells. Protein profiling analyses and Western blotting showed that expression of the miR 302/367 cluster also inhibited PI3K/AKT signaling molecules in glioblastoma cells.

It has previously been reported that the miR 302/367 cluster inhibits AKT signaling in breast and cervical cancer cells (Cai et al., 2012; Liang et al., 2012), but the effects of AKT inhibition on the expression of the reprogramming factors in cancer cells have not yet been investigated. To study the effects of PI3K/AKT signaling on the expression of the reprogramming factors in cancer cells, U87MG and MCF7 cells were treated with the PI3K inhibitor LY294002. LY294002 caused a downregulation of the expression of the reprogramming factors in these cells. I conclude that PI3K/AKT signaling regulates the endogenous expression of the reprogramming factors in cancer cells and thus increases their stem cell like phenotypes.

When the miR 302/367 cluster was ectopically expressed in normal cells, we also found an inhibition of PI3 kinase. This inhibition, however, did not coincide with downregulation of the expression of the reprogramming factors, but on the contrary, caused an increase in their expression. We also observed that phosphorylated Stat3 was increased in BJ cells whereas Stat3 phosphorylation was suppressed in glioblastoma cell lines upon expression of the miR 302/367 cluster. Furthermore, inhibition of Stat3 by small molecule, Stattic, suppressed the expression of the reprogramming factors in breast cancer cells, and the treatment of U87MG cells and MCF7 with LY294002 also reduced phosphorylation of Stat3. These data indicate that PI3K/AKT/Stat3 signaling regulates the expression of the reprogramming factors. Inhibition of the PI3K/AKT/STAT3 signaling suppresses the de-differentiation phenotypes of GBM cells through the inhibition of reprogramming factor expression and stem cell specific gene sets.

The expression and activation of AKT in GBM cells is strongly down-regulated by the miR 302/367 cluster; however, AKT expression was not changed in normal BJ cells. Moreover, we found that phospho-Stat3 levels were increased upon expression of the miR 302/367 cluster in normal BJ cells. There is a published report that shows that the regulation of Stat3 activation via the PTEN/AKT/LIFRβ/Stat3 axis (la Iglesia et al., 2008). However, the activation of Stat3 by PTEN activation in GBM is still controversial, because most of the published studies indicate that PTEN over-expression inhibits the activation of Stat3. It will be necessary to gain additional insights into the molecular mechanism linking Stat3 activation to AKT signaling and its connection to the somatic cell reprogramming process by the miR 302/367 cluster.

GSK-3 is well known signaling molecule downstream of PI3K/AKT. Recently, it has been reported that somatic cells also can be induced into pluripotent stem cells by small molecular weight compounds, including epigenetic modulators and GSK-3 inhibitors (Hou et al., 2013). Moreover, inhibition of GSK-3 during reprogramming of somatic cells increases the efficacy of the reprogramming process (Li and Rana, 2012). This complements our observation that the inhibition of PI3K/AKT and Stat3 signaling in GBM cells, triggered by the expression of the miR 302/367 cluster, suppresses de-differentiated phenotypes of GBM through the inhibition of the reprogramming factors.

It will be useful to understand the exact mechanisms by which the miR 302/367 cluster affects the cellular signaling in normal and in cancer, and possibly develop these mechanisms into a strategy for cancer treatment. Moreover, our findings suggest that the combined inhibition of PI3K/AKT and Stat3 signaling could become a promising therapeutic approach.

5.3. Modification of inflammatory cytokine expression in the GBM microenvironment

Inflammatory cytokines regulate tumor invasion and metastasis through the regulation of epithelial to mesenchymal transition (EMT) (Grivennikov et al., 2010; Wu and Zhou, 2009). Pro-inflammatory cytokines such as TNF-, IL-1, IL-6 and IL-8, produced in the tumor microenvironment, enhance cell proliferation, survival, migration, and tumor angiogenesis (Lu et al., 2006; Mantovani et al., 2008; Wu and Zhou, 2009). Stat3 signaling increases EMT in cancer cells through the stimulation of type I/II cytokine receptors by e.g. IL-6 (Xie et al., 2012; Yadav et al., 2011). IL-8, also known as an inflammatory cytokine, regulates EMT in cancer cells through the activation of PI3K/AKT signaling (Waugh and Wilson, 2008). IL-8 effects tumor metastasis by autocrine and paracrine activities (Fernando et al., 2011; Palena et al., 2012). Both, autocrine and paracrine factors play an important role in the GBM microenvironment. Autocrine factors sustain glioma cell proliferation and invasion, and paracrine factors increase the invasiveness of glioma cells (Hoelzinger et al., 2007). Recent studies on auto- and paracrine signals in breast cancer suggest that inflammatory cytokines can effect tumor initiation, progression, stem cell phenotypes, and EMT (Scheel et al., 2011).

Micro RNAs are small non-coding RNAs that regulate the expression of a large number of genes. miRNAs also can regulate inflammation through their effects on cytokine and cytokine receptor expression (Hu and O'Connell, 2013; Olivieri et al., 2013; Rebane and Akdis, 2013). From the miRNA target prediction database, we found that IL-8 mRNA is a possible target of the miR 302 family. To evaluate the effects of miR 302/367 cluster expression in GBM on IL-8 secretion, cytokine array analyses and RT-PCR were carried out. The results showed that the miR 302/367 cluster strongly downregulated IL-8 mRNA expression levels in GBM and suppressed the secretion of IL-8. We also found that the miR 302/367 cluster suppressed expression and secretion of IL-6 and MCP-1 in U87MG cells. These pro-inflammatory cytokines are known as EMT regulators. We designed an experiment to demonstrate the effects of the pro-inflammatory cytokines in the microenvironment of U87MG cell on their invasiveness mediated by a paracrine loop. U87MG cells were co-cultured with parental U87MG cells, vector infected U87MG cells, or miR 302/367 cluster expressing U87MG cells. The data showed that the miR 302/367 cluster expressing U87MG cells modified cytokine microenvironment in a way which resulted in a decrease of the U87MG cell invasiveness; whereas the parental U87MG cells and the control cells stimulated invasiveness. The cytokine array analyses and the U87MG cell invasion assays suggest that U87MG generate a microenvironment which promotes invasiveness through the expression of pro-inflammatory cytokines and the activation of auto- and paracrine effects. miR 302/367 cluster expression suppresses expression and secretion of e.g. IL-8 and thus inhibits GBM invasiveness.

Expression of the miR 302/367 cluster also suppressed GBM colony formation and invasion *in vitro*; effects which were confirmed by *in vivo* observations. The subcutaneous injection of miR 302/367 cluster expressing U87MG cells did not support tumor growth and metastasis formation, whereas non-infected or vector infected U87MG cells generated tumor and liver metastasis in NSG mice.

These findings show that the expression of the miR 302/367 cluster blocks the expression and secretion of pro-inflammatory cytokine signals and their auto- and paracrine effects, inhibits PI3K/AKT and Stat3 signaling and results in the loss of transformation phenotypes.

5.4. Development of miR 302/367 cluster delivery systems and the brain blood barrier

The experiments described above provide the perspective that the miR 302/367 cluster or drugs based on its mechanism of action could become beneficial for GBM therapy. Therapy, however, requires efficient delivery and bioavailability. The blood brain barrier (BBB) is a protective system that separates the circulating blood from the brain's extracellular fluid in the central nervous system (CNS). BBB occurs along all capillaries and consists of tight junctions around the capillaries that do not exist in the normal circulation. BBB restricts the passage of substances from the bloodstream to brain tissues. This protective system makes it difficult to deliver drugs into specific regions of the brain. Overcoming the difficulty of delivering therapeutic agents to specific regions of the brain presents a major challenge to treatment of most brain disorders.

Chemotherapy for GBM is restricted, because although numerous effective reagents were developed for GBM, delivery is still a problem. The development of a delivery system that can pass through the BBB is needed and various approaches have been taken: direct injection and implantation, chemical modifications, permeability enhancers, intranasal delivery, and nanoparticle-enabled delivery platforms via the intravenous route.

When injection and implantation are used, the delivery of drugs into the brain can be accomplished through the drilling of a hole into the head, by direct intrathecal injection into the cerebrospinal fluid (CSF), or by interstitial delivery through intracerevral implantation. These methods allow the circumvention of the BBB and the delivery of drugs into specific regions of the brain. However, the rapid clearance of CSF restricts the diffusion of drugs into the CNS tissues (Pathan et al., 2009).

Lipophilicity is an propensity of a chemical compound to dissolve in fats, oils, lipids, and non-polar solvents such as hexane or toluene. Interestingly, lipophilic molecules can pass the BBB by passive diffusion (Mensch et al., 2009). Several chemical modifications such as lipidation, cationization and a prodrug approach have been used to modify drugs to improve the penetration into brain. Another drug delivery system to penetrate the BBB are permeability enhancers. The permeability enhancers generally target GAP junction proteins, which are modulating the barrier function of brain endothelial cells (Deli, 2009). It has been shown that vasoactive compounds

such as histamine, bradykinin, or leukotrienes increase BBB permeability, especially in blood vessels in brain tumor tissue (Black, 1995).

Intranasal delivery is also a possible route to pass the BBB into brain tumors. Intranasal delivery provides a rapid onset of action, direct nose-to-brain delivery through the olfactory and trigeminal nerves, and minimization of systemic exposure and metabolism. However, drugs still need to pass a mucus layer and an epithelial membrane to efficiently reach the brain tissues.
Nano-technology could possibly provide alternative solutions for the delivery problem. Nano-enabled delivery platforms mimick lipid-based carriers and are of a size between 1 and 100 nm. Once the drug-nanocarrier conjugates reach the diseased tissues, the therapeutic agents are released. A controlled release of drugs from nanocarriers can be achived through changes in the physiological environment such as temperature, pH, osmolality, or via an enzymatic activity (Hwang and Kim, 2014).

In this study, we found that the miR 302/367 cluster has anti-cancer potential small. We believe that the miR 302/367 cluster could become a GBM anti-cancer compound if a controlled delivery system can be developed which penetrates the BBB and transports the miR 302/367 cluster into its targets cells.

5.5. Development of small molecules mimicking the effects of the miR 302/367 cluster

An alternative to the admittedly difficult miR 302/367 cluster delivery problem might be the derivation of small molecules which have the same effects as the miR 302/367 cluster in GBM cancer cells. We found that the miR 302/367 cluster has the potential as an anti-tumor compound in GBM and published data shows that the miR 302 family can induce skin cancer cells into pluripotent stem cells. It can also suppress tumor formation of iPS cells obtained through reprogramming of somatic cells (Lin et al., 2008; Lin and Ying, 2013). In this study, we showed that the miR 302/367 cluster strongly suppress stem cell phenotypes of GBM through the inhibition of PI3K/AKT and Stat3 signaling and the modification of pro-inflammatory cytokine microenvironments. For these reasons, we believe that a combination therapy, including small molecules which inhibit PI3K/AKT and Stat3 signaling might suppress proinflammatory cytokines and could become successful in GBM treatment.

5.6. Understanding the signaling events in the miR 302/367 cluster mediated reprogramming process

Micro RNAs regulate large numbers of genes through post-transcriptional regulation and genomic mutations can effect on the micro RNA mediated regulation of gene expression. We still have no information about the exact function of the miR 302/367 cluster in normal cells. It is possible that GBM cancer cell lines have genomic mutations that could result in unexpected gene regulation events by the miR 302/367 cluster. Computational analysis from online databases and our data strongly indicate that the miR 302/367 cluster regulates expression of the PI3K, AKT and IL-8 mRNAs. These results suggest that inhibition of AKT signaling reduce the potential transformation of iPS cells during somatic cell reprogramming.

In cancer research, PI3K/AKT signaling is well known for its oncogenic potential that regulates transformation, invasion and survival. Moreover, in the transcription factor transduction protocol for somatic cell reprogramming, activation of AKT signaling has been recognized to be sufficient to maintain the pluripotency of cells in the absence of leukemia inhibitor factor (LIF) (Nakamura et al., 2008; Watanabe et al., 2006).

Although activation of AKT signaling is necessary for stem cell pluripotency upon transduction of the transcription factors, the somatic cell nuclear transfer (SCNT) methods has shown that AKT signaling can also have the opposite effect. In the SCNT mediated reprogramming procedure, transduction of an active allele of the AKT gene in recipient cells disrupts oocytes development at the morula/blastocyst stage. The active AKT mutant arrests oocytes transition from the two to eight-cell stage (Nakamura et al., 2008). In addition, somatic cell reprogramming by using small molecule compounds showed that inhibition of GSK-3, which is down-stream signaling molecule of the PI3K/AKT signaling pathway, enhances reprogramming efficacy (Hou et al., 2013; Li and Rana, 2012). Those data indicate that the miR 302/367 cluster mediated reprogramming method may employ different mechanisms than the transcription factor based reprogramming method; moreover, other extrinsic factors also may effect on reprogramming process.

To induce somatic cells into pluripotent stem cells, special conditioned media is necessary. FGF and LIF are the most important cytokines in the stem cell conditioned media. LIF maintains pluripotency of stem cells through activation of the JAK/Stat3 signaling pathway (Kawazoe et al., 2009; Pera and Tam, 2010). As

previous shown, signaling events downstream of FGF and LIF stimulate type I/II cytokine receptor that in turn activate Stat3 signaling. Although the miR 302/367 cluster inhibits PI3K/AKT signaling regulating stem cell pluripotency, extrinsic factors in the stem cell conditioned media, including LIF and FGF, supplement the stem cell pluripotency through activation of Stat3 signaling pathway. For these reasons, we need to understand both the roles of the PI3K/AKT and the Stat3 signaling pathways in stem cell pluripotency and the reprogramming process by the miR 302/367 cluster and the generation of non-tumorigenic iPS cells.

5.7. A model for the cellular reprogramming of glioblastoma tumor cells by the miR 302/367 cluster

In this study, I showed that the expression of the miR 302/367 cluster in GBM cells resulted in the inhibition of tumor growth and invasiveness. These effects are mediated through the suppression of stem cell genetic programs and PI3K/AKT/Stat3 signaling. Although this was not systematically pursued, we did not detect the transition of GBM cells into pluripotent stem cells. It is possible that the cell culture conditions and the loss of PTEN in GBM cells could explain this observation.

Extrinsic factors, including LIF and FGF, affect the regulation of stem cell pluripotency through activation of the Stat3 signaling pathway. We tried to avoid effects on GBM cells by extrinsic factors and cultured the cells in serum containing, general GBM culture media without feeder cells. Such cells are used in somatic cell reprogramming protocols to supply extrinsic factors. The experiments in this study were carried out under regular cell culture conditions.

Fig. 42 shows a model of cellular reprogramming of GBM cells by the miR 302/367 cluster. It has been reported that the reprogramming factors induce expression of the miR 302/367 cluster through binding to the promoter of the miR 302/367 cluster (Anokye-Danso et al., 2012; Barroso-delJesus et al., 2008; Card et al., 2008). In this study, we found that GBM cells exhibit has de-differentiated phenotypes and the expression of stem cell genetic programs, including the reprogramming factors. However, the miR 302/367 cluster expression was not detected in GBM cells. These data suggest that epigenetic alterations increase the expression of stem cell genetic programs, but not of the miR 302/367 cluster in GBM cells; the epigenetic

modifications induced by the expression of the reprogramming factors probably causes de-differentiation phenotypes of GBM cells.

The activation of Stat3 signaling promotes somatic cell reprogramming (Tang et al., 2012). In this study, the miR 302/367 cluster expressing GBM cells were not grown in stem cell conditioned media, including LIF and FGF. These factors are known extrinsic simulators of Stat3 signaling. Moreover, we also found that the miR 302/367 cluster suppressed activation of Stat3 through the inhibition of PI3K/AKT signaling in GBM cells. The regulatory mechanisms of Stat3 activation by PI3K/AKT signaling have not been clearly detailed yet; however, the experiments in this study, involving the inhibition of PI3 kinase with LY294002, clearly showed that PI3K/AKT signaling regulates the activation of Stat3. Stat3 inhibition subsequently results in the suppression of the expression of the reprogramming factors.

Reprogramming of human somatic cells takes 3~4 weeks when standard reprogramming methods are used and stem cell differentiation takes around 2 weeks in specialized conditioned media. In general, in all reprogramming methods, the presence of extrinsic factors, including LIF and FGF, is important to sustain stem cell properties. The absence of the extrinsic factors during the reprogramming process prevents the maintenance of stem cell properties and initiates the cellular differentiation of iPS cells.

From the available information in the literature and our data, we hypothesized that the miR 302/367 cluster regulates epigenetic modifications in GBM cells which then results in the loss of transformation and stem cell properties. The epigenetic modifications suppress de-differentiated phenotypes of GBM cells and initiate more differentiated, normal cell phenotypes through suppression of PI3K/AKT/Stat3 signaling in the absence of extrinsic factors.

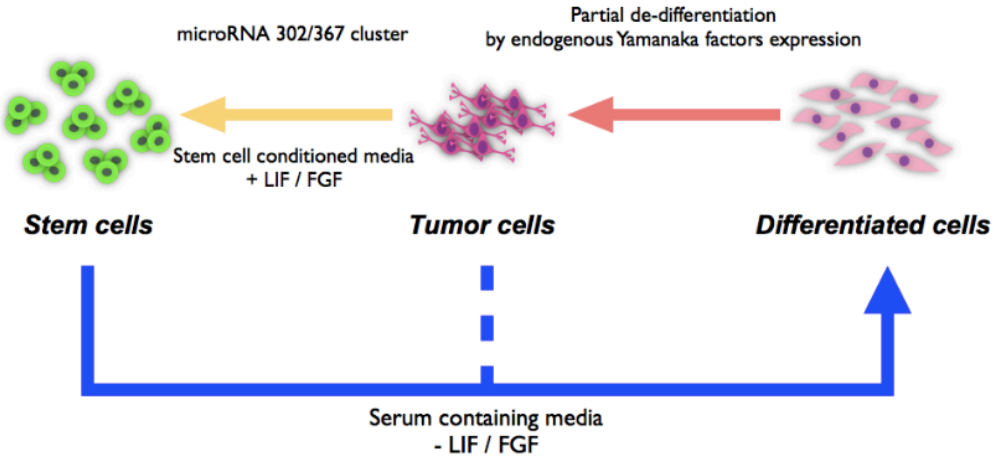

Figure 42. A model of cellular reprogramming by the miR 302/367 cluster. Transformation phenotypes of U87MG glioblastoma cells can be suppressed by the microRNA 320/367 cluster under cell culture condition with absence of LIF and FGF.

6. Literature

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7. Figures

8. Tables

9. Abbreviations

Abbreviation	
Akt	Serine/threonine-specific protein kinase
Dax1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DNMT	DNA methyl transferase
E2F1	Transcription factor E2F1; Retinoblastoma-assocated protein 1
E2F4	Transcription factor E2F4
EMT	Epithelial to mesenchymal transition
FGF	Fibroblast growth factor
FN1	Fibronectin 1
FOXO3	Forkhead in rhabdomyosarcoma-like 1
GBM	Glioblastoma multiform
HAT	Histone acetyltransferase
HDAC	Histone de-acetyltransferase
IL-6	Interleukin-6
IL-8	Interleukin-8
iPS cells	Induced pluripotent stem cells
JAK	Janus Kinases
Jarid2	Protein Jumonji
Klf4	Krueppel-like factor 4
LIF	Leukemia inhibitory factor
Max	Myc-associated factor X
MCP-1	Monocyte chemotactic protein-1
MET	Mesenchymal to epithelial transition
Mtf2	Metal-response element-binding transcription factor 2
Myc	Myc proto-oncogene protein
NANOG	Homeobox transcription factor Nanog
NEDD4	Neural precursor cell expressed developmentally down-regulated protein 4

NSG mice	NOD scid IL-2 receptor gamma chain knockout mice
Oct4	POU domain, class 5, transcription factor 1
PI3K	Phosphatidylinositol 3-kinase 3
PrC	Polycomb repressive complex
PTEN	Phosphatidylinositol 3,4,5-triphosphate 3-phosphatease and dual-specificity protein phosphatase PTEN
Rnf2	E3 ubiquitin-protein ligase RING2
Sall4	Sal-like protein 4
SILAC	Stable isotope labeling by amino acids in cell culture
Sox2	Transcription factor SOX-2
Stat3	Signal transducers of activator of transcription 3
Suz12	Polycomb protein SUZ12
TCF3	Transcription factor 3
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Trrap	Transformation/transcription domain-associated protein

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11. Curriculum vitae

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Personal data

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Education

Since 06/2010	PhD student, Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy and Goethe University of Frankfurt, Frankfurt am Main, Germany **PhD thesis:** Reprogramming of tumor cells: Signaling events and phenotypes Supervisor: Prof. Dr. Bernd Groner
2008-2010	Student at Kyonggi University Department of Life Sciences, Suwon, South Korea Master of Science degree **Master's thesis:** Development of a STAT3 pathway inhibitor: extracts from *Alpinia officinarum* arrest MCF-7 human breast cancer cells in the S-phase of the cell cycle and induce apoptosis Supervisor: Prof. Dr. Shung Ho Ghil
2000-2007	Undergraduate student at Kyonggi University Department of Life Sciences, Suwon, South Korea Bachelor of Science degree

Research skills

During the course of my graduate studies, I have been working in the field of tumor biology and have applied a wide array of molecular and cellular biological methods and techniques. These include:

Extensive experience:

- ✓ DNA, RNA, microRNA and protein isolation and purification.
- ✓ RT-qPCR
- ✓ Stem-loop RT-PCR
- ✓ Western blot analysis
- ✓ Cell proliferation assays
- ✓ Cell culture techniques
- ✓ Soft agar colony formation assay
- ✓ Tumor sphere formation assays in low adherent culture dishes
- ✓ Cell invasion assays
- ✓ Cytokine assay analysis
- ✓ Reporter gene-luciferase assays
- ✓ Apoptosis assays
- ✓ Cloning procedures
- ✓ Immunocytochemistry
- ✓ Gene transfer through the generation of lenti-viral particles and infection of mammalian cells
- ✓ Preparation of histology samples by using microtome
- ✓ H&E staining

Intermediate experience:

- ✓ Protein expression in *E-coli* including purification
- ✓ Experimental work with laboratory animals
- ✓ Protein profiling analysis (SILAC labeling)
- ✓ FACs analysis

Basic experience:

- ✓ Silica Column fractionation assays
- ✓ TLC (Thin Layer Chromatography)
- ✓ GC (Gas Chromatography), GC/MS
- ✓ LC/MS (Liquid Chromatography / Mass)
- ✓ HPLC (High Performance Liquid Chromatography)
- ✓ Yeast two hybrid analysis
- ✓ Handling of microorganisms

Computer skills:

Dnastar, DNA Lasergene, Amplify 3, Adobe Photoshop, Image J, GraphPad Prism, Sigma plot, Cell Quest Pro, Papers, MS Office.

Languages

- ✓ English: Good
- ✓ German: Basic
- ✓ Korean: mother tongue

Conferences

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Oral presentation / Poster presentation
Reprogramming of tumor cells: Signaling events and phenotypes

03/2013
17th International AEK Cancer Congress
Heidelberg, Germany
Poster presentation
Reprogramming of tumor cells: Signaling events and phenotypes

10/2011; 09/2012
UCT Science Day
Klinikum der Goethe Universität Frankfurt a. M., Germany
Poster presentation
Reprogramming of tumor cells: Singaling events and phenotypes

12. Publications

Chul Min Yang, Tomohiro Chiba, and Bernd Groner. “Expression of Reprogramming Factors in Breast Cancer Cell Lines and the Regulation by Activated Stat3.” *Hormone Molecular Biology and Clinical Investigation* 10, no. 2 (2012). doi:10.1515/hmbci-2012-0003.

13. E R K L Ä R U NG

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung unterzogen habe.

Frankfurt am Main, den_____ _____

Chul Min Yang

14. Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorgelegte Dissertation über

Reprogramming of tumor cells: Signaling events and phenotypes

selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.
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