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**Review**

# The p53/miR-34 axis in development and disease

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**The tumor suppressor p53 is one of the most frequently mutated genes in human cancers. MicroRNAs (miRNAs) are small non-protein coding RNAs that regulate gene expression on the post-transcriptional level. Recently, it was shown that p53 regulates the expression of several miRNAs, thereby representing an important mechanism of p53 signaling. Several independent studies identified the members of the miR-34 family as the most prevalent p53-induced miRNAs. miR-34s are frequently silenced in variety of tumor entities, suggesting that they are important tumor suppressors. Indeed, ectopic expression of miR-34s inhibits proliferation, epithelial to mesenchymal transition, migration, invasion, and metastasis of various cancer cell entities. Moreover, delivery or re-expression of miR-34 leads to notable repression of tumor growth and metastasis in cancer mouse models, and may therefore represent an efficient strategy for future cancer therapeutics. Besides their crucial functions in cancer, members of the miR-34 family also play important roles in spermatogenesis, stem cell differentiation, neuronal development, aging, and cardiovascular functions. Consequently, miR-34 has also been implicated in various non-cancerous diseases, such as brain disorders, osteoporosis, and cardiovascular complications.**

**Keywords:** p53, miR-34, miR-34a, miR-34b/c, cancer, metastasis, tumor suppression

## Introduction

The p53 tumor suppressor protein is a transcription factor that regulates the expression of stress response genes and mediates a variety of anti-proliferative processes (Vogelstein et al., 2000). p53 represents one of the most important proteins that protect against cancer. Accordingly, the *p53* gene is mutated or dysfunctional in the majority of human tumors (Levine et al., 2004). MicroRNAs (miRNAs) are a class of small non-coding regulatory RNAs that regulate gene expression by guiding target mRNA cleavage or translational inhibition (Bartel, 2004). Seven years ago, it was shown that p53 is an important regulator of miRNA expression (Hermeking, 2007). Moreover, many tumor suppressor functions of p53 are mediated by p53-regulated miRNAs. In 2007, several groups identified the members of the miR-34 family as the most prevalent p53-induced miRNAs (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007; Tazawa et al., 2007). Since then, a large number of studies have shown that the members of the miR-34 family are important mediators of tumor suppression. They have been implicated in the regulation of various cancer-related processes, such as proliferation, apoptosis, epithelial to mesenchymal transition (EMT), migration, invasion, and metastasis. In this review, we summarize the roles of miR-34 and its network of targets in cancer development. Moreover, we also describe the involvement of miR-34 in normal development and non-cancerous diseases. Since the p53/miR-34 axis was characterized as a central mediator of tumor suppression, miR-34 delivery or re-expression in tumors may represent an efficient strategy for future cancer therapeutics.

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## The p53 tumor suppressor protein

p53 is a transcription factor that is activated in response to DNA damage or other types of cellular stress and exerts multiple, anti-proliferative functions (Vogelstein et al., 2000). Activated p53 induces apoptosis and/or cell cycle arrest, which can be transient or permanent (senescence) (Oren, 2003). During cancer development, these properties of p53 suppress tumorigenesis by eliminating damaged cells that could otherwise progress to tumors (Goh et al., 2011). Therefore, it is not a surprise that over 80% of tumors display dysfunctional p53 signaling (Levine et al., 2004). *TP53*, the gene that encodes p53, is one of the most frequently mutated genes in human cancers. Up to 50% of all tumors contain mutations in both alleles of the *TP53* gene, with the mutation frequency ranging from ~10% in hematopoietic malignancies to 50%–70% in ovarian, colorectal, and head and neck cancers (Brosh and Rotter, 2009). *TP53* mutations can be somatic or germline, where patients that inherit a mutant *TP53* allele (Li–Fraumeni syndrome) develop a variety of early onset malignancies with 100% penetrance (Petitjean et al., 2007). Besides the induction of cell cycle arrest and apoptosis, p53 also suppresses tumor development by regulating EMT, differentiation, stemness, DNA repair, antioxidant defense, and metabolic pathways (Lane and Levine, 2010). p53 exerts these tumor suppressive functions by regulating the expression of its

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target genes, mainly by directly binding to specific DNA sequences called p53-responsive elements located in target promoters (el-Deiry et al., 1992). Prominent examples of p53 targets are Bax (Miyashita et al., 1994) and Puma (Nakano and Vousden, 2001), which are inducers of apoptosis, and p21 (el-Deiry et al., 1992) and 14-3-3σ (Hermeking et al., 1997), which induce G1 and G2 cell cycle arrest, respectively. Besides protein coding genes, miRNAs as important mediators of p53 functions have been added to the list of p53 targets recently (Hermeking, 2007, 2012).

**MicroRNAs**

miRNAs are short non-protein coding RNAs that are key post-transcriptional regulators of gene expression (Bartel, 2004). They contain a seed region between positions 2 and 8 from the 5′-end through which they bind to partially complimentary sequences in 3′-untranslated regions (3′-UTR) of their target messenger RNAs (mRNA) of protein coding genes. Once bound, miRNAs inhibit protein expression from their target mRNAs through two main mechanisms: suppression of translation and mRNA degradation (Pasquinelli, 2012). The generation of mature miRNAs is a multi-stage process (Krol et al., 2010). In Figure 1, this is illustrated for the miR-34 miRNAs. First, miRNA encoding genes are transcribed as primary transcripts (*pri-miRNA*) in the nucleus by RNA polymerase II or III. Next, *pri-miRNAs* are subjected to the first cleavage, which is carried out by the RNase III enzyme Drosha in complex with the dsRNA-binding protein DGCR8 (also known as Pasha). The result is a ~70 nucleotide stem-loop-structured miRNA precursor molecule (*pre-miRNA*). *Pre-miRNAs* are transported to the cytoplasm by Exportin 5, where they undergo the second and final cleavage, catalyzed by the RNase Dicer. The resulting 20–25 bp RNA duplex consists of the mature miRNA and its antisense strand (miRNA*), which is released and degraded. Mature miRNAs are incorporated into an RNA-induced silencing complex (RISC), which mediates miRNA-induced silencing of target mRNAs (Yates et al., 2013). The regulation by miRNAs has been described for almost all biological processes, including numerous studies that have revealed important roles of miRNAs in cancer. It is estimated that >60% of human protein coding genes are subject to regulation by miRNAs (Friedman et al., 2009). Therefore, the expression of miRNAs has to be tightly controlled. Recently, p53 has been described as an important regulator of miRNAs (Hermeking, 2007). In 2007, several groups including us identified several miRNAs as direct transcriptional targets of p53 (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007; Tazawa et al., 2007). Since then, many of these miRNAs have been implicated as important mediators of p53 functions (Hermeking, 2012). p53 not only regulates miRNAs at the transcriptional level, but also regulates the processing/maturation of certain miRNAs. It was demonstrated that p53 interacts with the DEAD-box RNA helicase p68 (also known as DDX5) and enhances its interaction with the DROSHA complex, thereby promoting the maturation miRNAs (Suzuki et al., 2009).

**MicroRNAs of the miR-34 family**

Among all miRNAs, members of the miR-34 family show the highest induction by p53 (Hermeking, 2007). The miR-34 family consists of three members: miR-34a, miR-34b, and miR-34c. MiR-34a is encoded in the second exon of a gene located on chromosome 1p36.22, whereas miR-34b and miR-34c share a common host gene located on chromosome 11q23.1 (Figure 1A). Both genes do not encode any other non-coding RNAs or proteins. Interestingly, the 1p36 locus where *miR-34a* is located is commonly deleted in neuroblastoma or other tumors (Welch et al., 2007). Moreover, expression of all members of the miR-34 family is frequently repressed in tumors compared with normal tissue of patients in a variety of tumor types (Hermeking, 2012; Wang et al., 2013b). Both *miR-34* genes contain several p53-responsive elements, since chromatin immunoprecipitation and luciferase assays showed that p53 can bind directly to these elements and activate transcription (Raver-Shapira et al., 2007; Tarasov et al., 2007). Other inducers of *miR-34* expression are ELK1 and FoxO3a, which bind to the promoter regions of *miR-34a* and *miR-34b/c*, respectively (Christoffersen et al., 2010; Kress et al., 2011).

In mice, miR-34a is ubiquitously expressed, with highest levels in the brain and testes, whereas miR-34b and miR-34c are expressed mainly in the brain, lungs, and testes (Bommer et al., 2007; Bouhallier et al., 2010). Thus, miR-34a is expressed at higher levels than miR-34b/c in most tissues, except in lungs, where miR-34b and miR-34c are predominant. Interestingly, miR-34a and miR-34c have identical seed sequences, whereas the miR-34b seed sequence is similar, but not identical, suggesting that miR-34a and miR-34c share similar mRNAs target, whereas miR-34b targets might be slightly different from these (Figure 1B).

**Functions of miR-34 in cancer**

The discovery of miR-34s as p53-regulated miRNAs prompted many researchers to investigate their role in cancer. Since miR-34 expression is induced by p53, it has been suggested that miR-34s may have tumor suppressing properties. Indeed, all members of miR-34 family were shown to suppress tumor growth and metastasis by inhibiting processes that promote cancer development, including cell cycle, EMT, metastasis, and stemness, and by promoting processes that inhibit carcinogenesis, such as apoptosis and senescence (Figure 2). MiR-34s regulate these processes through downregulation of their target mRNAs. As of today, more than 77 miR-34 targets have been validated (Table 1).

*Cell cycle*

The cell cycle represents a sequence of events that ultimately contribute to cell proliferation. It consists of four phases: G1-, S-, G2-, and M-phase. In normal cells, the cell cycle is tightly controlled; however in cancer this regulatory process fails, resulting in uncontrolled cell proliferation. Several important factors that regulate cell cycle machinery are targets of miR-34s. It has been shown that miR-34a inhibits the cell cycle and proliferation of lymphoma cells by repressing its target MAP2K1 (MEK1), which is a central component of MEK/ERK signaling (Ichimura et al., 2010). Furthermore, it was reported that ectopic expression of all members of the miR-34 family induces cell cycle arrest in a variety of cancer cell lines by repression of their targets Cyclins D1 and E2, and the cyclin-dependent kinases CDK4 and CDK6, which regulate the transition from G1- to S-phase (He et al., 2007; Sun et al., 2008; Toyota et al., 2008). Moreover, the transcription factors c-Myc, N-Myc, and E2F3, which are inducers of

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cell cycle progression and proliferation, are directly repressed by miR-34s (Wei et al., 2008; Christoffersen et al., 2010; Pulikkan et al., 2010). Interestingly, the expression of miR-34s can also be regulated by c-Myc via the c-Myc/MK5/FOXO3a/miR-34b/c and c-Myc/ARF/HDM2/p53/miR-34 axes, thereby forming two negative feedback loops: the MK5/FOXO3/miR-34b/c/Myc loop (Figure 3A) and the miR-34a/c-Myc/ARF/HDM2/p53 loop (Figure 3B) (Kress et al., 2011; Sotillo et al., 2011). Since HDM2 inhibits p53, a result of elevated miR-34a expression is a decrease in p53 protein levels. On contrary, a positive feedback loop of miR-34a and p53 that enforces their activities was suggested by Mandke et al. (2012), since they showed that MDM4 is a target of miR-34a. MDM4 binds to p53 and inhibits its transcriptional activity. Since miR-34a represses MDM4, this may lead to stabilization of p53 and sustainable expression of miR-34a (Figure 3B: miR-34a/MDM4/p53 loop). Furthermore, SIRT1 was also shown to connect miR-34a back to p53. SIRT1 is a $NAD^+$-dependent deacetylase, which represses p53 activity by post-transcriptional

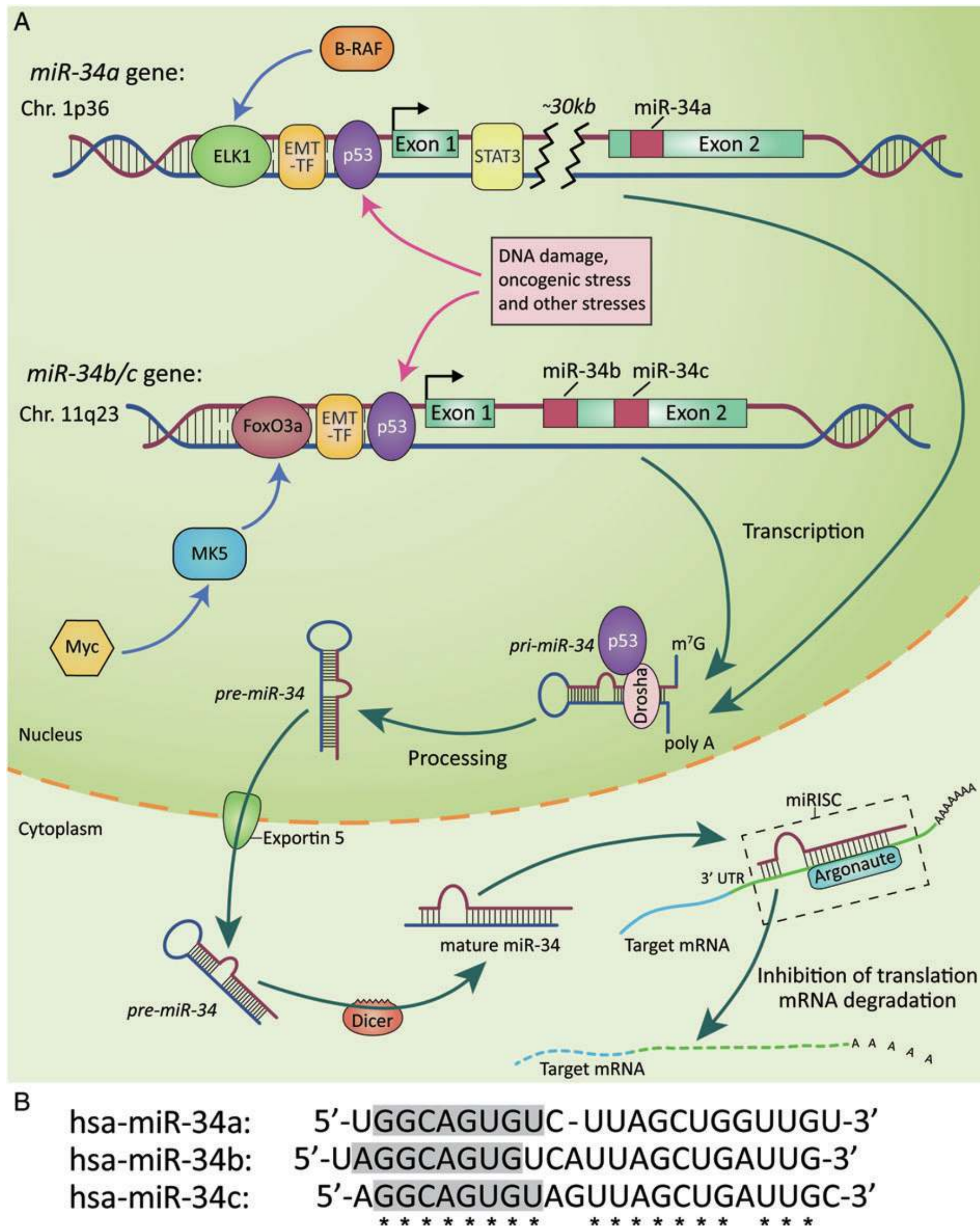


**Figure 1** Genomic structure and regulation of the miR-34 family members. (**A**) Structure of *miR-34a* and *miR-34b/c* genomic loci. Green and red boxes represent exons and miRNA hairpins, respectively. Horizontal arrows mark transcription start sites. The transcription factors p53, ELK1, and FoxO3a induce, whereas STAT3 and EMT-TF repress the transcription of *miR-34* genes. EMT-TFs represent EMT-inducing transcription factors, such as SNAIL and ZEB1/2. p53 also induces the expression of miR-34 at the post-transcriptional level by binding to the DROSHA complex. (**B**) Sequence alignment of the mature miR-34a, miR-34b, and miR-34c. The seed sequences are highlighted by gray shading. Asterisks indicate identical nucleotides in miR-34a, miR-34b, and miR-34c.

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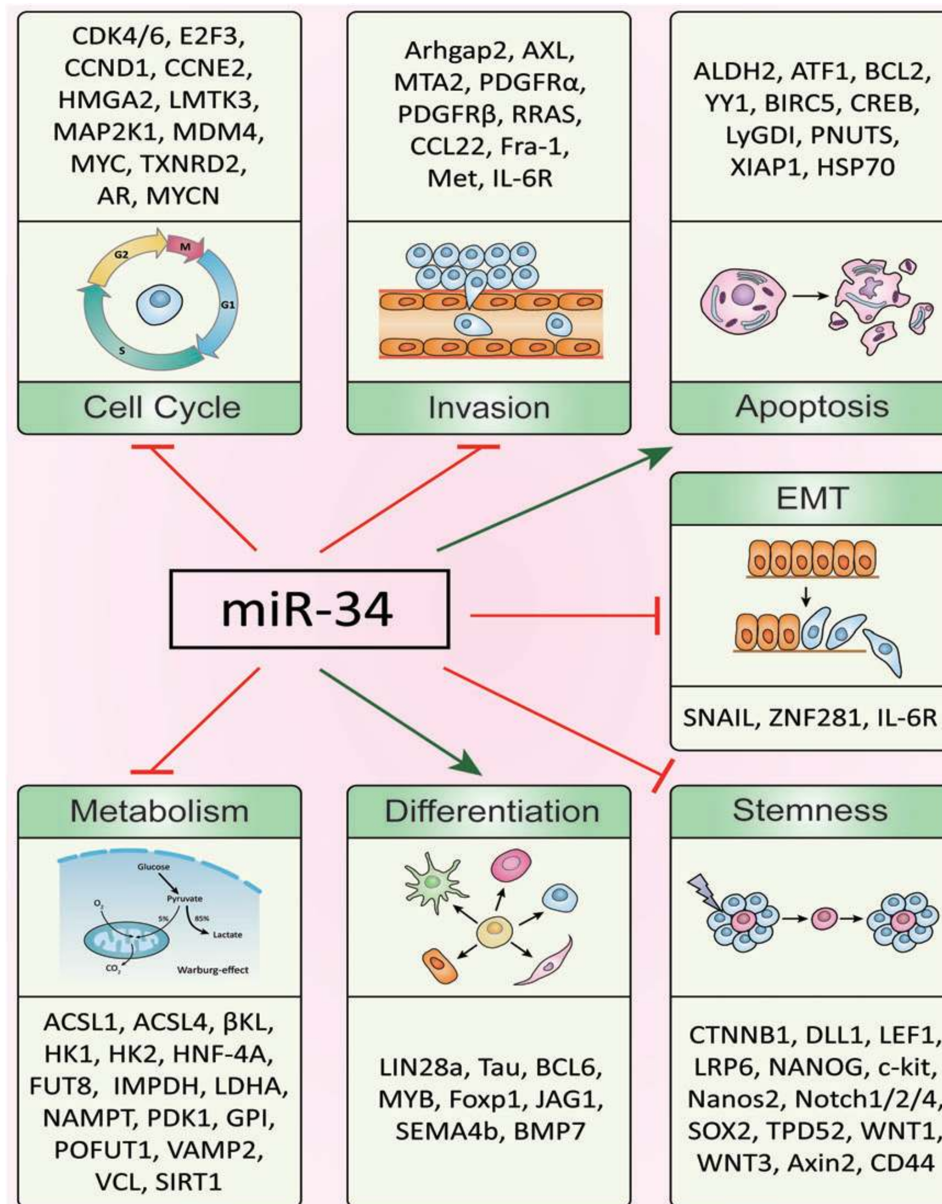


**Figure 2** miR-34 regulates cancer-relevant pathways and processes by targeting key factors. Proteins down-regulated due to direct targeting by miR-34 were grouped according to their main functions. The net effect of regulation by miR-34 on each pathway/process is indicated by a green arrow (activation) or a red inhibitory symbol.

deacetylation of p53 protein. Yamakuchi et al. (2008) showed that SIRT1 is a miR-34 target, thereby suggesting that miR-34 can induce the activity of p53 by repressing SIRT1 (Figure 3C: miR-34a/SIRT1/p53 loop). Moreover, miR-34 represses not only the expression of SIRT1 but also its activity by targeting NAMPT, the rate-limiting enzyme of the $NAD^+$ salvage pathway (Choi et al., 2013). It was recently shown that SIRT1 and Myc regulate each other via a positive feedback loop (Marshall et al., 2011; Menssen et al., 2012). Notably, c-MYC directly induces NAMPT expression to activate SIRT1 (Menssen et al., 2012). The mutual regulations of MYC and SIRT1 have been reviewed by Menssen and Hermeking (2012). Therefore, by repressing the c-Myc/SIRT1 axis, miR-34 may represent a central mediator of cell cycle suppression by p53. SIRT1 itself is an important regulator of cell cycle, and it has been suggested that by repression of SIRT1, miR-34 induces senescence (permanent cell cycle arrest) in endothelial cell (Ito et al., 2010; Zhao et al., 2010). Senescence-inducing functions of miR-34 have been confirmed in other cell types including colorectal cancer cells, where introduction of miR-34a caused complete suppression of cell proliferation and induced senescence by down-regulation of the E2F pathway (Tazawa et al., 2007). Interestingly, Christoffersen et al. (2010) showed that during B-RAF oncogene-induced senescence, miR-34a was regulated independently of p53. Instead, up-regulation of miR-34a was mediated by the ETS family transcription factor, ELK1.

*Apoptosis*

Apoptosis or programmed cell death is a physiological process whereby damaged or unwanted cells are eliminated and removed

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**Table 1 List of validated miR-34 target mRNAs.**

| Gene | Full name | miR-34 (a, miR-34a; c, miR-34c) | SMS | Validation method | Cellular process | Reference |
|---|---|---|---|---|---|---|
| *ACSL1* | Acyl-CoA synthetase long-chain family member 1 | All | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kaller et al. (2011) and Li et al. (2011a) |
| *ACSL4* | Acyl-CoA synthetase long-chain family member 4 | a | No | luc. reporter, qPCR, WB | Metabolism | Kaller et al. (2011) |
| *ALDH2* | Aldehyde dehydrogenase 2 | a | No | luc. reporter, qPCR, WB | Apoptosis | Fan et al. (2013) |
| *AR* | Androgen receptor | a, c | No | luc. reporter, qPCR, WB | Cell cycle | Östling et al. (2011) and Kashat et al. (2012) |
| *ARHGAP2* | Rho GTPase activating protein 1 | a | Yes | luc. reporter (mut), qPCR, WB | Invasion | Ahn et al. (2012) |
| *ATF1* | Activating transcription factor 1 | c | Yes | luc. reporter (mut), qPCR, WB | Apoptosis | Liang et al. (2012) |
| *AXIN2* | Axin 2 | All | Yes | luc. reporter (mut), qPCR, WB | Stemness, migration, invasion | Kim et al. (2013b) |
| *AXL* | AXL receptor tyrosine kinase | All | Yes | luc. reporter (mut), qPCR, WB | Migration, invasion, metastasis | Kaller et al. (2011), Mackiewicz et al. (2011), and Mudduluru et al. (2011) |
| *βKL* | β-Klotho | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Fu et al. (2012) |
| *BCL2* | B-cell leukemia/lymphoma 2 | All | Yes | luc. reporter (mut), qPCR, WB, IHC | Apoptosis | Bommer et al. (2007), He et al. (2007), and Cole et al. (2008) |
| *BCL6* | B-cell leukemia/lymphoma 6 | All | Yes | luc. reporter, qPCR, WB | Differentiation | Bernardo et al. (2012) |
| BIRC5 | Survivin | a | No | luc. reporter, qPCR, WB | Apoptosis | Kaller et al. (2011) and Shen et al. (2012) |
| *BMP7* | Bone morphogenetic protein 7 | a | No | qPCR, WB, IF | Differentiation | Wan et al. (2012) |
| *CCL22* | C–C motif chemokine 22 | a | Yes | luc. reporter (mut), qPCR, WB | Invasion | Yang et al. (2012) |
| *CCND1* | Cyclin D1 | a | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle, G1-arrest | Fujita et al. (2008) and Sun et al. (2008) |
| *CCNE2* | Cyclin E2 | All | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle, G1-arrest | He et al. (2007) and Toyota et al. (2008) |
| *CD44* | Heparan sulfate proteoglycan | a | Yes | luc. reporter (mut), qPCR, WB | Stemness | Liu et al. (2011) and Zhao et al. (2013b) |
| *CDK4* | Cyclin-dependent kinase 4 | All | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle, G1-arrest | He et al. (2007) and Toyota et al. (2008) |
| *CDK6* | Cyclin-dependent kinase 6 | a | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle, G1-arrest | Sun et al. (2008) and Toyota et al. (2008) |
| *c-kit* | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog | All | Yes | luc. reporter (mut), qPCR, WB | Stemness, migration, invasion | Siemens et al. (2013a) |
| *CREB* | Cyclic AMP-responsive element binding protein | All | Yes | luc. reporter (mut), qPCR, WB, IHC | Apoptosis, proliferation | Pigazzi et al. (2009) and Tivnan et al. (2011) |
| *CRFR1* | Corticotropin releasing factor receptor type 1 | a, c | Yes | luc. reporter (mut), qPCR, WB | Stress response | Haramati et al. (2011) |
| *CTNNB1* | Beta-catenin | All | Yes | luc. reporter (mut), qPCR, WB | Wnt signaling, metastasis, EMT, stemness | Kim et al. (2011a) |
| *DLL1* | Notch ligand delta-like 1 | a | Yes | luc. reporter (mut), qPCR, WB, IHC | Notch signaling, stemness | de Antonellis et al. (2011) and Pang et al. (2013) |
| *E2F3* | E2F transcription factor 3 | a | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle, G1-arrest | Tazawa et al. (2007) and Welch et al. (2007) |
| *Foxp1* | Forkhead box P1 | a | Yes | luc. reporter (mut), qPCR, WB | Differentiation | Rao et al. (2010) and Craig et al. (2011) |
| *Fra-1* | Fos-related antigen 1 | a, c | Yes | luc. reporter (mut), qPCR, WB | Migration and invasion | Wu et al. (2012) |
| *FUT8* | Fucosyltransferase 8 | a | No | luc. reporter, WB | Metabolism | Bernardi et al. (2013) |
| *GPI* | Glucose-6-phosphate isomerase | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kim et al. (2013a) |
| *HK1* | Hexokinase 1 | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kim et al. (2013a) |
| *HK2* | Hexokinase 2 | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kim et al. (2013a) |
| *HMGA2* | High mobility group AT-hook 2 | a | Yes | luc. reporter (mut) | Cell cycle | Tazawa et al. (2007) and Ji et al. (2008) |
| *HNF-4A* | Hepatocyte nuclear factor-4α | All | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Wang and Burke (2013) and Wirsing et al. (2011) |
| *HSP70* | Heat shock protein 70 | a | Yes | luc. reporter (mut), WB | Apoptosis, stemness, differentiation | Feng et al. (2014) |
| *IL-6R* | Interleukin 6 receptor | a, c | Yes | luc. reporter (mut), qPCR, WB | EMT, invasion | Rokavec et al. (2014) |

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**Table 1** *Continued*

| Gene | Full name | miR-34 (a, miR-34a; c, miR-34c) | SMS | Validation method | Cellular process | Reference |
|---|---|---|---|---|---|---|
| *IMPDH* | IMP (inosine 5 -monophosphate) dehydrogenase | All | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kim et al. (2012) |
| *JAG1* | Jagged 1 | a, c | No | luc. reporter (mut), WB | Notch signaling, differentiation | Hashimi et al. (2009) and Bae et al. (2012) |
| *LDHA* | Lactate dehydrogenase A | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kaller et al. (2011) |
| *LEF1* | Lymphoid enhancer binding factor 1 | All | Yes | luc. reporter (mut), qPCR, WB | Wnt signaling, metastasis, EMT, stemness | Kaller et al. (2011) and Kim et al. (2011a) |
| *LIN28a* | LIN28a | a | Yes | luc. reporter (mut), WB | Differentiation | Jain et al. (2012) |
| *LMTK3* | Lemur tyrosine kinase 3 | a | Yes | luc. reporter, WB | Cell cycle | Zhao et al. (2013a) |
| *LRP6* | Low-density lipoprotein receptor-related protein 6 | All | Yes | luc. reporter (mut), qPCR, WB | Wnt signaling, metastasis, EMT, stemness | Kim et al. (2011a) |
| *LyGDI* | Rho GDP dissociation inhibitor (GDI) beta | a | Yes | qPCR, WB, | Apoptosis | Duan et al. (2013) |
| *MAP2K1* | Mitogen-activated protein kinase kinase 1 (MEK1) | a | Yes | luc. reporter (mut), qPCR, WB | Cell cycle | Ichimura et al. (2010) |
| *MAP3K9* | Mitogen-activated protein kinase kinase kinase 9 | a | Yes | luc. reporter (mut), qPCR, WB | Cell cycle | Tivnan et al. (2011) |
| *MDM4* | MDM4 | a | Yes | luc. reporter (mut), qPCR, WB | Cell cycle | Mandke et al. (2012) |
| *Met* | Met proto-oncogene | All | Yes | luc. reporter (mut), qPCR, WB, IHC | Migration and invasion | He et al. (2007) and Li et al. (2009a) |
| *MTA2* | Metastasis associated 1 family member 2 | a | Yes | luc. reporter (mut), qPCR, WB | Migration and invasion | Kaller et al. (2011) |
| *MYB* | v-myb myeloblastosis viral oncogene homolog | a | Yes | luc. reporter (mut), qPCR, WB | Differentiation | Navarro et al. (2009) |
| *MYC* | v-myc myelocytomatosis viral oncogene homolog | All | Yes | luc. reporter (mut), qPCR, WB | Cell cycle | Christoffersen et al. (2010) and Sotillo et al. (2011) |
| *MYCN* | v-myc myelocytomatosis viral related oncogene N | a | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle | Cole et al. (2008) and Wei et al. (2008) |
| *NAMPT* | Nicotinamide phosphoribosyltransferase | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Choi et al. (2013) |
| *NANOG* | NANOG homeobox transcription factor | All | Yes | luc. reporter (mut), qPCR, WB | Stemness | Choi et al. (2011) |
| *Nanos2* | Pyruvate dehydrogenase kinase 1 | c | Yes | luc. reporter (mut), qPCR | Stemness | Yu et al. (2014) |
| *Notch1* | Notch homolog 1 | a, c | Yes | luc. reporter (mut), qPCR, WB, IHC | Notch signaling, stemness | Bae et al. (2012) and Du et al. (2012) |
| *Notch2* | Notch homolog 2 | a, c | No | luc. reporter (mut), qPCR, WB, IHC | Notch signaling, stemness | Li et al. (2009c) and Bae et al. (2012) |
| *Notch4* | Notch homolog 4 | a, c | No | luc. reporter (mut), WB | Notch signaling, stemness | Yu et al. (2012) |
| *PDGFR*$\alpha$ | Platelet-derived growth factor receptor, alpha | a, c | Yes | luc. reporter (mut), qPCR, WB | Invasion | Silber et al. (2012) and Garofalo et al. (2013) |
| *PDGFR*$\beta$ | Platelet-derived growth factor receptor, beta | a, c | Yes | luc. reporter (mut), qPCR, WB | Invasion | Garofalo et al. (2013) |
| *PDK1* | Pyruvate dehydrogenase kinase 1 | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Kim et al. (2013a) |
| *PNUTS* | Phosphatase nuclear targeting subunit | a | Yes | luc. reporter (mut), qPCR, WB | Apoptosis | Boon et al. (2013) |
| *POFUT1* | Protein O-fucosyltranferase 1 | All | No | luc. reporter, qPCR, WB | Metabolism | Bernardo et al. (2012) |
| *RRAS* | Related RAS viral oncogene homolog | A | No | luc. reporter, qPCR, WB | Invasion | Kaller et al. (2011) |
| *SATB2* | SATB homeobox 2 | All | Yes | luc. reporter (mut), qPCR, WB | Differentiation | Wei et al. (2012) |
| *SEMA4b* | Semaphorin 4B | All | No | luc. reporter, qPCR, WB | Differentiation | Bernardo et al. (2012) |
| *SIRT1* | Sirtuin 1, silent information regulator 1 | a | Yes | luc. reporter (mut), qPCR, WB, IHC | Cell cycle, metabolism | Yamakuchi et al. (2008) and Ito et al. (2010) |
| *SIRT6* | Sirtuin 6 | a | Yes | luc. reporter (mut), qPCR, WB | Differentiation | Lefort et al. (2013) |
| *SNAIL* | Snail family zinc finger 1 | All | Yes | luc. reporter (mut), qPCR, WB | EMT | Kim et al. (2011b) and Siemens et al. (2011) |
| *SOX2* | SRY (sex determining region Y)-box 2 transcription factor | All | Yes | luc. reporter (mut), qPCR, WB | Stemness | Choi et al. (2011) |
| *Tau* | Microtubule-associated protein tau | All | Yes | luc. reporter (mut), qPCR, WB | Differentiation | Dickson et al. (2013) and Wu et al. (2013) |

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**Table 1** *Continued*

| Gene | Full name | miR-34 (a, miR-34a; c, miR-34c) | SMS | Validation method | Cellular process | Reference |
|---|---|---|---|---|---|---|
| *TPD52* | Tumor protein D52 | a | Yes | luc. reporter (mut), qPCR, WB | Wnt signaling, stemness | Kaller et al. (2011) |
| *TXNRD2* | Thioredoxin reductase 2 | a | Yes | luc. reporter (mut), qPCR, WB | Cell cycle | Bai et al. (2011) |
| *VAMP2* | Vesicle-associated membrane protein 2 | a | Yes | luc. reporter (mut), qPCR, WB | Metabolism | Lovis et al. (2008) |
| *VCL* | Vinculin | All | Yes | luc. reporter, qPCR, WB | Metabolism | Bernardo et al. (2012) |
| *WNT1* | Wingless-related MMTV integration site member 1 | All | Yes | luc. reporter (mut), WB | Wnt signaling, stemness | Hashimi et al. (2009) and Kim et al. (2011a) |
| *WNT3* | Wingless-type MMTV integration site family member 3 | All | Yes | luc. reporter (mut), qPCR, WB | Wnt signaling, stemness | Kim et al. (2011a) |
| *XIAP1* | X-linked inhibitor of apoptosis protein | a | Yes | luc. reporter, qPCR | Apoptosis | Niederer et al. (2012) |
| *YY1* | Ying yang 1 transcription factor | a | Yes | luc. reporter (mut), qPCR | Apoptosis | Chen et al. (2011) and Kaller et al. (2011) |
| *ZNF281* | Zinc finger protein 281 | a | Yes | luc. reporter (mut), qPCR, WB | EMT | Hahn et al. (2013) |

For each target mRNA, the encoding gene, method of validation, and its biological function are provided. Furthermore, it is indicated whether the publication identified a seed-matching sequence (SMS) in the 3′-UTR. The regulation of the target gene has been validated for the indicated member of the miR-34 family (a, miR-34a; c, miR-34c). However, other miR-34 family members that have not been analyzed might also regulate the respective target mRNA. It is also indicated whether the reporter assay validated the direct regulation by using mutation (mut) of the SMS. IHC, immunohistochemistry; IF, immunofluorescence; luc. reporter, luciferase reporter assay; mut, mutagenesis of the SMS in the 3′-UTR-reporter construct; qPCR, quantitative real-time PCR; WB, western blotting analysis.

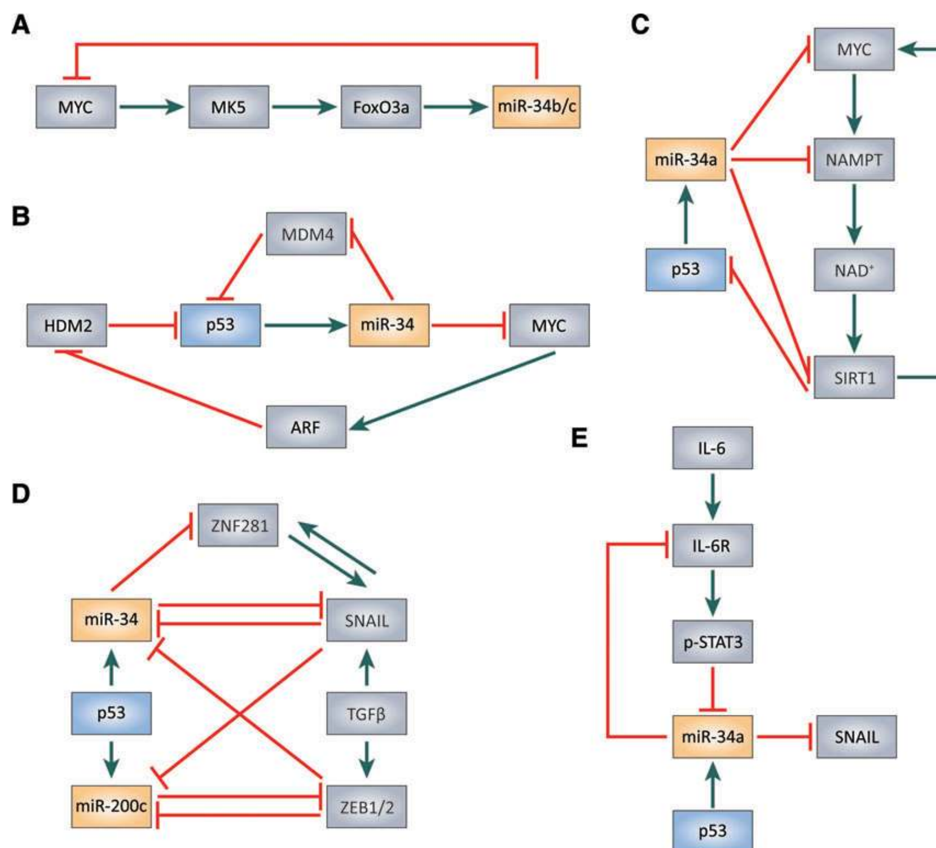


**Figure 3** Feedback loops involving miR-34. (**A**) MK5 phosphorylates and activates FoxO3a, and thereby induces expression of miR-34b/c. Both miRNAs inhibit translation of c-Myc. c-Myc in turn induces expression of MK5, thereby creating a negative feedback loop. (**B**) miR-34 represses its target MDM4 and induces HDM2 through inhibition of c-Myc/ARF. Since MDM4 and HDM2 both bind to p53 and inhibit its transcriptional activity, miR-34 can either activate or repress p53, depending on which mechanism prevails. (**C**) SIRT1 represses p53 activity by post-transcriptional deacetylation of p53 protein. SIRT1 is a miR-34 target. Therefore, miR-34 induces the activity of p53 by repressing SIRT1. MiR-34 also represses the activity of SIRT1 by targeting NAMPT, the rate-limiting enzyme for $NAD^+$ biosynthesis. (**D**) p53 induces the expression of miRNAs of the miR-34 and miR-200 family, forming two double-negative feedback loops with their targets SNAIL, ZEB1, and ZEB2. (**E**) Representation of the IL-6R/STAT3/miR-34a feedback loop identified and characterized by Rokavec et al. (2014). The loops in **D** and **E** function as bimodal switches, which stabilize either epithelial or mesenchymal states of cells. Activations are indicated by green arrows and inhibitions by red lines.

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from the organism. However, repression of apoptosis may result in uncontrolled cell growth and tumor formation. Before miR-34a was identified as a p53 target, it was already reported that ectopic expression of miR-34a induces apoptosis in neuroblastoma cell lines (Welch et al., 2007). Subsequent studies confirmed the pro-apoptotic functions of miR-34a in various cancer entities and several anti-apoptotic genes were identified as miR-34 targets (Chang et al., 2007; Raver-Shapira et al., 2007; Cao et al., 2013). Inhibition of miR-34a protects cells to some extent from the DNA damage-induced apoptosis in wild-type p53-expressing cells, suggesting that miR-34a is at least in part required for p53-induced apoptosis (Raver-Shapira et al., 2007). Bcl-2, the prominent regulator of apoptosis, was identified as a direct target of miR-34 and pro-apoptotic functions of miR-34 are presumably mediated mainly via repression of Bcl-2 (Bommer et al., 2007). Furthermore, BIRC5 (Survivin), CREB, and YY1, which are additional proteins with strong anti-apoptotic properties, have been identified as miR-34 targets, which could be mediators of the pro-apoptotic activity of miR-34 (Pigazzi et al., 2009; Chen et al., 2011; Kaller et al., 2011; Shen et al., 2012). MiR-34a is presumably also involved in the regulation of apoptosis of non-transformed cells, since embryonic stem cells (ESC) lacking the *miR-34a* gene displayed reduced levels of baseline apoptosis after induction of differentiation (Bommer et al., 2007).

*EMT*

EMT is a crucial process during organ development, which converts epithelial cell types into cells with mesenchymal properties and allows their migration. EMT is also activated during cancer progression, where it is believed to represent the initial step of metastasis by transforming primary epithelial non-invasive tumor cells to mesenchymal cancer cells that can invade the surrounding tissues and spread to distant organs (De Craene and Berx, 2013). It was shown that repression of miR-34a is required for hypoxia- and TGF-β-induced EMT (Siemens et al., 2011; Du et al., 2012), suggesting that miR-34a is a suppressor of EMT. We and others showed that miR-34a suppresses EMT by directly targeting and repressing the EMT-inducing transcription factor (EMT-TF) SNAIL (Kim et al., 2011b; Siemens et al., 2011). Moreover, we demonstrated that SNAIL also represses miR-34a and miR-34b/c by directly binding to their promoters, thereby forming a double-negative feedback loop (Figure 1A) (Siemens et al., 2011). It was shown that besides miR-34, p53 also induces members of the miR-200 family (Kim et al., 2011c), which also represent EMT-regulating miRNAs that suppress EMT by a similar double-negative feedback loop involving the EMT-TFs ZEB1 and ZEB2 (Burk et al., 2008; Gregory et al., 2008). Thus, p53 might be a key regulator of cellular plasticity by controlling EMT and its counterpart MET through targeting the miRNAs of the miR-34 and miR-200 family, which together with SNAIL, ZEB1, and ZEB2 form two double-negative feedback loops that function as a bimodal switch to stabilize either the epithelial or the mesenchymal state (Figure 3D: miR-34a/miR-200c/SNAIL loop). Moreover, ZEB1 was also shown to repress miR-34a by binding to the same E-boxes in *miR-34* promoters as SNAIL, thereby adding more complexity and further connecting the miR-34/SNAIL and miR-200/ZEB loops (Siemens et al., 2011; Ahn et al., 2012). We recently showed that the zinc finger 281 protein (ZNF281) is an important miR-34 target with respect to EMT (Hahn et al., 2013). We demonstrated that the expression of ZNF281 is controlled by miR-34 and SNAIL in a coherent feed-forward-loop, where SNAIL induces ZNF281 directly, and indirectly by repressing miR-34, thereby derepressing ZNF281 (Figure 3D). In addition, ectopic ZNF281 induces EMT by directly activating SNAIL and thereby mediates increased migration and invasion.

*Cancer stem cells*

Cancer stem cells (CSCs) or tumor initiating cells are cancer cells with stem cell properties. They have the ability to form new tumors and are thought to be responsible for therapy resistance, cancer relapse, and metastasis. Liu et al. (2011) showed that the expression of miR-34a is lower in $CD44^+$ prostate CSCs when compared with $CD44^-$ non-stem cells purified from xenograft or primary tumors from cancer patients. miR-34a re-expression in $CD44^+$ prostate cancer cells blocked tumor regeneration and metastasis, whereas anti-miR-34a oligonucleotides (antagomirs) in $CD44^-$ cells promoted these processes. Moreover, in mice with orthotopically transplanted human prostate cancer cells, systemic delivery of miR-34a through tail veins inhibited metastasis and extended survival. Importantly, CD44 itself was identified and validated as a direct and functionally relevant target of miR-34a mediating these effects (Liu et al., 2011). Furthermore, miR-34a was identified as a cell-fate determinant in colon CSCs (Bu et al., 2013). Loss or gain of miR-34a resulted in a sharp bimodal threshold regulation of stem cell-associated signaling via the miR-34a target Notch1, which altered the balance between CSC self-renewal versus differentiation (Bu et al., 2013; Winton, 2013). Consistently, miR-34a and miR-34c levels are reduced in the stem cell population of pancreatic and breast cancer cell lines, respectively (Ji et al., 2009; Yu et al., 2012). Ectopic expression of miR-34a or miR-34c in these cells reduces self-renewal and inhibited EMT via silencing its target genes Bcl2 and Notch1−4. Interestingly, the repression of miR-34c in breast CSCs is mediated by CpG-methylation in the promoter region of *miR-34c* gene that decreases DNA binding activities of Sp1 (Yu et al., 2012). Besides the direct repression of Notch receptors, miR-34a was shown to regulate Notch signaling also via direct repression of the Notch ligands Delta-like 1 (Dll1) (de Antonellis et al., 2011) and JAG1 (Hashimi et al., 2009). Through this mechanism miR-34a suppressed proliferation, migration, invasion, and xenograft tumor growth of choriocarcinoma cells (Pang et al., 2013) and negatively affected $CD133^+/CD15^+$ medulloblastoma tumor-propagating cells (de Antonellis et al., 2011). We recently showed that the receptor tyrosine kinase c-kit, which is critically involved in the regulation of cancer cell stemness, is also a direct target of all members of the miR-34 family (Siemens et al., 2013a). Consistently, SCF, the ligand of c-kit, induced colonosphere formation, which was prevented by ectopic miR-34a. Moreover, ectopic miR-34a expression resulted in repression of stem cell markers CD44, CD133, and BMI1 in colorectal cancer cells (Siemens et al., 2011, 2013a). Furthermore, members of the miR-34 family may play a role in the generation of induced pluripotent stem cells (iPSC), which are a type of pluripotent stem cells artificially derived from somatic cells. Choi et al. (2011) showed that like *p53*-deficiency, loss of *miR-34* also enhances somatic

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reprogramming of mouse embryonic fibroblasts (MEFs). However, unlike the lack of p53, which enhances reprogramming at the expense of iPSC pluripotency, genetic ablation of miR-34a promoted iPSC generation without compromising self-renewal or differentiation of MEFs (Choi et al., 2011). The suppression of reprogramming by miR-34a was, at least in part, due to direct repression of pluripotency genes, including Nanog, Sox2, and N-Myc. Consistently, miR-34a mimics reduced and miR-34a inhibitors increased iPSC formation by MEFs (Lee et al., 2012). Since stemness has been linked to EMT (Mani et al., 2008), many of the effects of miR-34 on the regulation of EMT may also affect stemness. Among the miR-34 targets mediating these effects, components of the WNT/β-catenin signaling pathway, such as β-catenin, LEF1, LRP6, Axin 2, WNT1, and WNT3, may be especially relevant, besides those already mentioned above (Kaller et al., 2011; Kim et al., 2011a, 2013b).

*Cancer invasion and metastasis*

Several studies showed that low expression of miR-34 in primary tumors correlates with the presence of metastases in cancer patients, suggesting that miR-34 is a suppressor of metastasis (Dang et al., 2013; Siemens et al., 2013b; Yang et al., 2013b). Accordingly, in xenograft mouse models, ectopic expression of miR-34 or miR-34 mimics inhibited the formation of metastases (Lujambio et al., 2008; Guo et al., 2011; Ahn et al., 2012; Yan et al., 2012; Yang et al., 2013b). Head and neck, breast, lung, and osteosarcoma cancer cells stably expressing ectopic miR-34a formed markedly less pulmonary metastases than control cells when injected into the tail vein, subcutaneously, or into the proximal tibia of mice (Lujambio et al., 2008; Ahn et al., 2012; Yan et al., 2012; Yang et al., 2013b). Moreover, liver cancer cells transfected with miR-34a mimics generated significantly less and smaller lymph node metastases, when subcutaneously injected into mice (Guo et al., 2011). Conversely, inhibition of miR-34a with miR-34a-specific antagomirs promoted the formation of lung metastases of orthotopically implanted prostate cancer tumors (Liu et al., 2011). We and others showed that ectopic expression of miR-34 suppresses invasion and migration, which are processes required for metastasis, of colorectal, breast, liver, osteosarcoma, and lung cancer cells (Kim et al., 2011b; Mudduluru et al., 2011; Siemens et al., 2011; Dang et al., 2013; Yang et al., 2013b; Zhao et al., 2013b). The direct targets that mediate the suppression of cancer cell migration and invasion by miR-34 include the EMT-TF SNAIL (Kim et al., 2011b; Siemens et al., 2011), the RAS-oncogene homolog RRAS (Kaller et al., 2011), c-kit (Siemens et al., 2013a), Axl (Mackiewicz et al., 2011; Mudduluru et al., 2011), Arhgap1 (Ahn et al., 2012), PDGFR-α/β (Garofalo et al., 2013), Fra-1 (Wu et al., 2012; Yang et al., 2013b), and c-Met (Li et al., 2009a; Yan et al., 2012; Dang et al., 2013; Siemens et al., 2013b). Interestingly, c-Met is also directly repressed by p53 via the inhibition of SP1 binding to its promoter (Hwang et al., 2011). Yang et al. (2012) showed that CCL22 is an important miR-34a target with respect to cancer progression. The chemokine CCL22 is a chemo-attractant that predominantly attracts regulatory T cells. They proposed a mechanism whereby repression of *miR-34a* leads to enhanced levels of CCL22 and consequent recruitment and tumor infiltration of T cells, which affect tumor progression. Accordingly, ectopic expression of miR-34a in mouse liver and breast cancer cells resulted in less metastases when these cells were inoculated in the spleen or the mammary fat pads of syngeneic, immunocompetent mice. Complementary expression of non-targetable CCL22 abolished the effect of miR-34a and resulted in infiltration of T cells into the tumor and enhanced metastasis formation. Consistently, this effect was not observed in athymic nude mice that lack T cells, thereby supporting the important role of T cells during cancer progression mediated by loss of miR-34a.

Recently, we could show that STAT3 directly represses *miR-34a* after exposure of colorectal cancer cells to the pro-inflammatory cytokine IL-6 (Figure 1A) (Rokavec et al., 2014). Furthermore, we identified the IL-6R as a miR-34 target and characterized an IL-6R/STAT3/miR-34a feedback loop (Figure 3E). Activation of this circuitry was associated with EMT, invasion, and metastasis in colorectal cancer cell lines and primary colorectal tumors. Moreover, in contrast to wild-type mice, colitis-associated intestinal tumors of *miR-34a*-deficient mice progressed to invasive colon carcinomas and displayed elevated levels of phosphorylated STAT3, IL-6R, and SNAIL expression (Rokavec et al., 2014). Notably, these results represent the first genetic evidence of a tumor suppressor function of *miR-34a*.

*Metabolic regulation*

The metabolic switch from oxidative phosphorylation to anaerobic glycolysis (also known as the Warburg effect) is a hallmark of cancer cells (Hanahan and Weinberg, 2011). It was recently demonstrated that the suppression of glycolysis and glucose uptake is a pivotal function of p53 with respect to tumor suppression (Li et al., 2012). Several genes involved in the regulation of glycolysis and metabolism were identified as miR-34 targets. Kim et al. (2013a) reported that p53 regulates glycolysis and glucose metabolism via miR-34a-mediated repression of several key enzymes: hexokinase 1 (HK1), hexokinase 2 (HK2), glucose-6-phosphate isomerase (GPI), and pyruvate dehydrogenase kinase 1 (PDK1). Correspondingly, miR-34a-mediated inhibition of these enzymes suppressed glycolysis and enhanced mitochondrial respiration. Another important target of miR-34a is the transcription factor HNF4a, which regulates the expression of genes involved in glucose metabolism and has been associated with liver cancer (Wirsing et al., 2011; Wang and Burke, 2013). We showed that lactate dehydrogenase A (LDHA), an enzyme that converts pyruvate to lactate and is required for aerobic glycolysis, is a miR-34a target (Kaller et al., 2011). Interestingly, pharmacological or siRNA-mediated inhibition of LDHA resulted in suppression of cancer cell proliferation (Fantin et al., 2006). Furthermore, miR-34a directly represses inosine 5′-monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme of *de novo* GTP biosynthesis (Kim et al., 2012). Consequently, suppression of IMPDH by miR-34a resulted in decreased activation of the GTP-dependent Ras signaling pathway. The acyl-CoA synthetase long-chain family members ACSL1 and ACSL4 have been identified as miR-34a targets as well (Kaller et al., 2011; Li et al., 2011a). ACSL plays a central role in the metabolism of lipids and fatty acids, which are critically involved in cancer development (Li et al., 2011a). Finally, miR-34a targets Fucosyltransferase 8 (FUT8), which is an enzyme critically involved in fucosylation (Bernardi et al., 2013). Since

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fucosylation is essential for signaling of several growth factors such as EGF and TGF-β, miR-34 might also exhibit anti-cancer functions by inhibition of fucosylation.

**Functions of miR-34 in normal development and non-cancerous diseases**

Besides their functions in tumor suppression, members of miR-34 family also play important roles in tissue development and non-cancerous diseases. There is strong evidence that miR-34s are involved in spermatogenesis, which is a cyclic process, whereby male primordial germ cells differentiate into spermatozoa. The sharp increase of miR-34c expression from newborn to sexually mature mouse testes suggests that miR-34c is involved in germ cell differentiation and sperm production (Bouhallier et al., 2010; Liang et al., 2012; Zhang et al., 2012). Yu et al. (2014) showed that miR-34c enhances the differentiation of mouse spermatogonial stem cells by targeting Nanos2, suggesting that miR-34c may promote meiosis. Using miR-34c mimics and inhibitors, miR-34c was shown to promote apoptosis of primary spermatocytes and male germline stem cells, a process that is important during normal spermatogenesis in mammals and plays a pivotal role in sperm output (Liang et al., 2012). Interestingly, members of the miR-449 family, which share many targets with the miR-34 family, were also preferentially expressed in the mouse testis, and their levels were drastically up-regulated upon meiotic initiation during testicular development and in adult spermatogenesis (Bao et al., 2012). However, *miR-449*-null male mice exhibited normal spermatogenesis and fertility. Levels of miR-34b/c were significantly up-regulated in miR-449-null testes, which suggests that the miR-449 cluster and miR-34b/c function redundantly in the regulation of male germ cell development in murine testes. MiR-34c was also shown to regulate the differentiation of mouse ESC into male germ cells, a process that is a prerequisite for normal spermatogenesis (Zhang et al., 2012). In mammals, the sperm delivers various mRNAs and miRNAs into the oocytes during fertilization. Mouse sperm contains miR-34c that is required for the first cell division, since injection of a miR-34c inhibitor into zygotes inhibited DNA synthesis and significantly suppressed the first cleavage division (Liu et al., 2012b). The levels of miR-34b and miR-34c in seminal plasma were also associated with male infertility. In one study, miR-34c was significantly decreased in men with azoospermia (no measurable level of sperm in semen), but interestingly increased in men with astenozoospermia (reduced sperm motility) when compared with fertile control individuals (Wang et al., 2011a). In another study, miR-34b and miR-34c levels were decreased in patients with oligoasthenozoospermia (a combination of reduced sperm number in semen with decreased motility) or astenozoospermia (Abu-Halima et al., 2013).

The involvement of miR-34 in CSC functions suggests that it might also play a role in normal stem cell differentiation. Indeed, miR-34a was induced during the differentiation of human ESC, where it together with another p53-induced miRNA—miR-145 repressed the stem cell factors OCT4, KLF4, LIN28A, and SOX2, thereby preventing reversal to pluripotency (Jain et al., 2012). Furthermore, miR-34 has been implicated in cell differentiation during organ development. All members of the miR-34 family are induced during osteoblast differentiation in bone development (Bae et al., 2012; Wei et al., 2012). *In vivo* osteoblast-specific gain- and loss-of-function analyses revealed that miR-34b and miR-34c affect skeletogenesis during embryonic development, as well as bone mass accrual after birth, through two complementary mechanisms. MiR-34b/c inhibits osteoblast proliferation by suppressing Cyclin D1, CDK4, and CDK6 and suppresses terminal differentiation of osteoblasts through the inhibition of SATB2, a nuclear matrix protein that is a critical determinant of osteoblast differentiation (Wei et al., 2012). Interestingly, transgenic mice with osteoblast-specific, ectopic miR-34c expression developed age-dependent osteoporosis due to defective mineralization and proliferation of osteoblasts and increased osteoclastogenesis (Bae et al., 2012). It was suggested that this function of miR-34c is mediated by targeting multiple components of the Notch signaling pathway. Notch and TGF-β signaling is also affected by miR-34a during tooth development, since miR-34a regulates dental papilla cell differentiation by targeting NOTCH1 and BMP7 (Wan et al., 2012). In a zebrafish model, Wang et al. (2013a) showed that the expression of miR-34b is enriched in multiciliated cells (cells that regulate fluid movement in the intercellular space) of both the kidney and the olfactory system, and that miR-34b is required for normal kidney morphogenesis and olfactory organ development. MiR-34a expression was induced during differentiation of monocytes to dendritic cells and its inhibition suppressed this process and led to reduced endocytic capacity, a key function of dendritic cells (Hashimi et al., 2009). JAG1 was identified as the miR-34a target responsible for this function (Hashimi et al., 2009). On the other hand, constitutive expression of miR-34a led to a block in B cell development at the pro-B cell to pre-B cell transition, resulting in a reduction in mature B cells (Craig et al., 2011). This block appeared to be mediated primarily by miR-34a-mediated inhibition of the Forkhead transcription factor, Foxp1. Foxp1 was also identified as the main miR-34a target that mediates the tumor suppressive effects of miR-34a in B cell lymphomas (Craig et al., 2011). Finally, miR-34a expression increases during keratinocyte differentiation, while it is suppressed in skin and oral squamous cancer cells. SIRT6 was proposed as the mediator of this effect, since SIRT6 is a direct target of miR-34a, and suppression of SIRT6 was sufficient to reproduce the pro-differentiation effects of miR-34a (Lefort et al., 2013).

Several reports showed that miR-34a plays important roles in neuronal development. In zebrafish, repression of miR-34 led to developmental defects in the neuronal system with an enlargement of the hindbrain during early embryonic development (Soni et al., 2013). Furthermore, miR-34a contributes to cellular motility and oxidative phosphorylation in neural precursors derived from human umbilical cord mesenchymal stem cells, which may be crucial for stem cells to home to the target sites (Chang et al., 2011). MiR-34a also regulates neurite outgrowth and spinal morphology, and is associated with both morphological and electrophysiological changes in mouse ESC and in mouse models (Agostini et al., 2011). Interestingly, this process was initiated by p73, which drives the expression of miR-34a, but not miR-34b or miR-34c, suggesting a specific role of miR-34a in neuronal development. Ectopic expression of miR-34a modulates neural differentiation by increasing the percentage of post-mitotic neurons and

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neurite elongation of mouse neural stem cells, whereas miR-34a-specific antagomirs had the opposite effect, suggesting that miR-34a is required for proper neuronal differentiation. These effects were mediated in part by the repression of SIRT1 and modulation of p53 activity (Aranha et al., 2011). On the contrary, Fineberg et al. (2012) showed that ectopic expression of miR-34a in neural progenitor cells significantly reduces the neuron yield upon *in vitro* induction of differentiation. These contradicting results suggest that the role of miR-34a in neuronal development is complex and that miR-34a may act on distinct targets in a sequential fashion to regulate and fine-tune neuronal differentiation kinetics.

Members of the miR-34 family have also been implicated in brain disorders and aging. In *Drosophila melanogaster,* mouse, and rat brains, expression of miR-34a increases with age (Li et al., 2011b; Liu et al., 2012a; Yang et al., 2013a). In *Drosophila*, loss of miR-34 decreases survival and accelerates brain aging and degeneration, whereas miR-34 up-regulation extends survival and diminishes neurodegeneration induced by human pathogenic polyglutamine disease protein (Liu et al., 2012a). On the other hand, in *Caenorhabditis elegans*, a miR-34 loss-of-function mutation prolonged lifespan, delayed the age-related physiological decline, and increased resistance to heat and oxidative stress (Yang et al., 2013a). Similar miR-34 functions have also been observed in age-associated brain disorders, where miR-34 can be either protective or causative. Using parallel sequencing and integrative genetics, Zovoilis et al. (2011) showed that miR-34c levels are elevated in the hippocampus of Alzheimer's disease patients and that miR-34c is a negative constraint of memory consolidation in mouse models. Consistently, miR-34 seed inhibitors rescued learning ability in these mouse models. Similarly, expression of miR-34a was elevated in the APPswe/PSΔE9 mouse model of Alzheimer's disease compared with age-matched controls (Wang et al., 2009). Interestingly, increased levels of miR-34a circulating in blood reflected age-dependent changes in brain (Li et al., 2011b) and miR-34b was shown to be significantly elevated in plasma from Huntington's disease gene carriers prior to symptom onset (Gaughwin et al., 2011). On the other hand, compared with healthy individuals, levels of miR-34b and miR-34c were decreased in brains of patients with Parkinson's disease (PD) and coupled to a decrease in the expression of DJ1 and Parkin, two proteins associated with familial forms of PD that also have a role in idiopathic cases (Miñones-Moyano et al., 2011). The members of the miR-34 family have also been implicated in brain trauma and epilepsy. A decrease of miR-34a coupled with an increase of NOTCH1 was observed after brain trauma induced with a weight drop device on exposed rat skulls (Wang et al., 2012). However, after kainic acid or lithium–pilocarpine induced epileptical seizures, an up-regulation of miR-34a in mouse and rat hippocampal subfields was observed (Hu et al., 2012; Sano et al., 2012). In the kainic acid model of epilepsy, the induction of miR-34a was only temporary, and intracerebroventricular injection of antagomirs targeting miR-34a had only a minor modulatory effect on apoptosis-associated signaling and did not prevent hippocampal neuronal death (Sano et al., 2012), whereas in the pilocarpine model, miR-34a induction lasted for several weeks, and miR-34a antagomir treatment blocked caspase-3 signaling and also protected against seizure-induced neuronal death (Hu et al., 2012). An induction of miR-34c in central amygdala of rat brains was observed after acute restraint stress induced by introducing the rats into a perforated 50 ml conical tube (Haramati et al., 2011). Moreover, rats with a lentiviral-mediated expression of miR-34c within the central amygdala showed increased anxiety behavior after dark/light transfer tests. A possible target that mediates the effects of miR-34c on anxiety is the stress-related corticotropin releasing factor receptor type 1 (CRFR1) (Haramati et al., 2011). Finally, increased levels of miR-34a were significantly associated with severity of age-related cataracts, which are the most common cause of blindness (Chien et al., 2013).

MiR-34a has been also associated with metabolic diseases, such as obesity and diabetes (Li et al., 2009b; Kong et al., 2011). An up-regulation of miR-34 with corresponding down-regulation of ACSL1 was observed in liver fibrosis, a process that can lead to liver cirrosis and hepatic cancer (Li et al., 2011a). Elevated levels of miR-34a were reported in serum or liver tissue of patients with nonalcoholic steatohepatitis (fatty liver disease), which is characterized by inflammation with concurrent accumulation of fat in the liver (Cheung et al., 2008; Cermelli et al., 2011; Yamada et al., 2013). Several miRNA screens identified miR-34a as the most significantly elevated miRNA in liver and pancreatic islets of dietary and genetic (db/db) obese mouse models (Lovis et al., 2008; Li et al., 2009b; Lee et al., 2010). Moreover, compared with healthy individuals, miR-34a levels were elevated in serum of patients with type 2 diabetes (Kong et al., 2011). A plausible mechanism of miR-34a pro-diabetic functions has been recently provided by Fu et al. (2012). They showed that miR-34a directly represses the co-receptor β-Klotho, which together with FGF19 receptor 4 and FGF19 hormone constitutes a unique endocrine metabolic regulatory axis that is normally activated after a meal and regulates glucose metabolism. Elevated levels of miR-34a in patients with metabolic disorders might therefore result in reduced FGF19 signaling and impaired glucose metabolism. Other studies suggest that the pro-diabetic and pro-obesity functions of miR-34a depend on the repression of its targets vesicle-associated membrane protein 2 (VAMP2) and SIRT1 (Lovis et al., 2008; Castro et al., 2013; Choi et al., 2013).

Recently, several studies reported an important role of miR-34 in cardiovascular functions and myocardial infarction. MiR-34a is induced in human and mouse ageing hearts (Ito et al., 2010; Boon et al., 2013) and *in vivo* silencing or genetic deletion of miR-34a in mouse reduced age-associated cardiomyocyte cell death (Boon et al., 2013). Moreover, all members of miR-34 family are also strongly induced after acute myocardial infarction in mice and rats (Bernardo et al., 2012; Boon et al., 2013; Fan et al., 2013). Consistently, inhibition of miR-34 reduced cell death and fibrosis following acute myocardial infarction and improved recovery of myocardial function (Bernardo et al., 2012; Boon et al., 2013). The improved outcome of anti-miR-34-treated mice was accompanied by up-regulation of several miR-34 targets, including vascular endothelial growth factors, vinculin (VCL), protein O-fucosyltranferase 1 (POFUT1), Notch1, semaphorin 4B, PNUTS, BCL6, and ALDH2 (Bernardo et al., 2012; Boon et al., 2013; Fan et al., 2013). Furthermore, elevated levels of

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miR-34a were detected in serum (Fan et al., 2013) and bone marrow–mononuclear cells (BMC) (Xu et al., 2012) from patients with myocardial infarction compared with healthy subjects. Moreover, injection of *ex vivo* anti-miR-34a pre-treated BMC directly after induction of acute infarction in nude mice improved the therapeutic benefit of BMC cell therapy (Xu et al., 2012). Additionally, increased levels of miR-34a and decreased expression of its target SIRT1 were observed in endothelial progenitor cells from patients with coronary artery disease (CAD) (Tabuchi et al., 2012). In a randomized clinical study, it was shown that atorvastatin treatment, which has anti-atherosclerotic effects, resulted in markedly decreased levels of miR-34a and increased expression of SIRT1, suggesting that the beneficial effects of statins on CAD are at least in part mediated by the repression of miR-34a (Tabuchi et al., 2012). Recently, Feng et al. (2014) showed that repression of miR-34a improves stem cell therapy for ischemic heart diseases. Interestingly, the repression of miR-34a was induced by exposing the Sca-1$^{+}$ stem cells to a heat shock, which induces the heat shock factor 1 (HSF1). HSF1 binds to the *miR-34a* promoter, which leads to reduction of enrichment of methylated histone H3K4me3 and increase of enrichment of H3K27me3, collectively resulting in remodeling of the chromatin structure of *miR-34a* promoter and repression of miR-34a expression. The repression of miR-34a resulted in an increase of its target heat shock protein 70 (HSP70), which was shown to represent the key chaperone mediating the cytoprotective effect of heat shock in Sca-1$^{+}$ stem cells. Altogether, these findings suggest miR-34s as potential therapeutic target and diagnostic marker of myocardial infarction and other cardiovascular diseases.

**Therapeutic and diagnostic potential of miR-34**

The tumor suppressor functions of miR-34 detected in cell culture experiments suggest that re-expression of miR-34 in tumors may represent an efficient approach for cancer treatment. The most common approach for miRNA delivery relies on lipid-based nanoparticles, coupled with vectors expressing miRNAs, or ~19–23-nt double-stranded miRNA mimics that correspond to mature miRNA. These can be administered systemically by intravenous injection or locally into tumors. Several studies showed that systemic miR-34a delivery suppressed tumor growth *in vivo*. Using xenograft or genetically engineered mouse models of melanoma, lymphoma, multiple myeloma, breast, prostate, pancreatic, and non-small cell lung cancer, a tumor growth repression between 20% and 83% was observed (Bader, 2012). No severe toxicity of systemic miR-34a delivery in mouse model has been shown, based on phenotypic and pathologic observations (Pramanik et al., 2011; Craig et al., 2012). Likewise, no unwanted immune response has been detected, based on serum cytokine levels in immune-competent mice (Wiggins et al., 2010). The Texas-based company Mirna Therapeutics has initiated a preclinical development program of nanoparticle-based delivery of miR-34a, and they anticipate to initiate clinical trials in 2013 (Bader, 2012). Therefore, miR-34a may be one of the first miRNA mimics to reach the clinic.

Conventional anti-cancer therapies, such as chemotherapy and radiation, induce miR-34 expression in human cancer cells with wild-type p53 (Tazawa et al., 2007). However, since the majority of human tumors lack normal p53 function, addition of ectopic miR-34 may enhance the efficacy of standard cancer therapies. Indeed, in prostate, colorectal, and bladder cancer cells, ectopic expression of miR-34a using miR-34a precursors attenuated chemoresistance to camptothecin, paclitaxel, 5-fluorouracil, and cisplatin (Fujita et al., 2008; Kojima et al., 2010; Vinall et al., 2012). The repression of the miR-34a targets SIRT1, E2F3, BCL2, and CDK6 was the main mechanism of this beneficial function of miR-34a. Furthermore, lentiviral transduction of miR-34a sensitized gastric and pancreatic cancer cells to radiation and chemotherapeutic drugs docetaxel, gemcitabine, cisplatin, and doxorubicin (Ji et al., 2008, 2009). Moreover, we recently showed that c-Kit is a miR-34a target, the downregulation of which mediates the beneficial effects of miR-34a with respect to chemosensitivity (Siemens et al., 2013a). Thus, co-treatment with miR-34 mimics may enhance the beneficial effects of conventional cancer therapies.

Many natural compounds from dietary sources have anti-cancer properties. Recently, several compounds that are used in traditional medicines were shown to modulate the expression of miR-34. Resveratrol, a phytoalexin present in a wide variety of plant species including grapes, berries, and peanuts, induces expression of miR-34a and down-regulation of its targets SIRT1 and E2F3 in breast and colorectal cancer cells (Hagiwara et al., 2012; Kumazaki et al., 2013). In non-small cell lung cancer cell lines, Rhamnetin that is present in cloves and Cirsiliol present in white wormwood enhanced radiosensitization and inhibited EMT. The effects of Rhamnetin and Cirsiliol were mediated by up-regulation of miR-34a and consequent repression of its target Notch-1 (Kang et al., 2013). Furthermore, in pancreatic and non-small cell lung cancer cells, miR-34a-mediated inhibition of cell growth and induction of apoptosis was observed after treatment with Genistein, a compound found in a number of plants including soybean and coffee (Xia et al., 2012), and Delta-Tocotrienol, a member of the vitamin E family (Ji et al., 2012). Hui et al. (2012) found that oral administration of the flavonoid compound 3, 6-dihydroxyflavone leads to enhanced expression of miR-34a associated with reduced MNU-induced breast carcinogenesis in rats. Up-regulation of miR-34a and miR-34c was also observed after treatment of colorectal cancer cells with difluorinated curcumin (CDF), a synthetic analog of curcumin, found in the root of turmeric plant and a component of popular Indian spices (Roy et al., 2012). Finally, treatment with diindolylmethane (DIM), which is found in cruciferous vegetables such as broccoli and cabbage, resulted in increased expression of miR-34a in prostate cancer cells (Kong et al., 2012). Interestingly, the CDF- and DIM-induced up-regulation of miR-34a was mediated by a decrease of miR-34 promoter methylation. Collectively, these findings demonstrate that many natural compounds could potentially lead to re-expression of miR-34a in tumors, and thus should be a potentially fruitful focus of future anti-cancer research studies.

Besides intra-cellular miRNAs, circulating miRNAs have also been found in blood serum. These circulating miRNAs are extremely stable both in blood and after isolation, where even introduction of RNases or storage of serum samples at room temperature did not markedly alter their concentrations. In cancer patients, these miRNAs mainly originate from microvesicles or exosomes released from tumor cells (Healy et al., 2012). Therefore, circulating miRNAs

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may represent valuable biomarkers for prognosis or prediction of therapy outcome. In a study of 63 colorectal cancer patients and 45 controls, levels of circulating miR-34a in blood were significantly lower in patients (Nugent et al., 2012). On contrary, in another study with 80 breast cancer patients and 40 controls, circulating miR-34a levels were elevated in serum of patients (Eichelser et al., 2013). Similarly, in a study from Roth et al. (2010), elevated miR-34a was significantly associated with metastasis in a collection of 89 breast cancer patients. Furthermore, in a rat model of liver cancer, an up-regulation of circulating miR-34a in serum has been observed during cancer progression (Sukata et al., 2011). Thus, current studies on miR-34 provide conflicting results, with more studies associating higher levels of circulating miR-34 with increased lymph node invasion, metastasis, and worse outcome. At first sight, these data seem contradictory, since cell culture studies showed that miR-34s are suppressors of EMT, invasion, and metastasis. Yet, recent studies showed that when metastatic cells colonize host tissues and form metastases, they undergo MET and re-express EMT suppressing genes and miRNAs (Tsai et al., 2012). Therefore, elevated levels of EMT suppressing circulating miRNAs might originate from metastases and indicate metastatic dissemination. Indeed, Toiyama et al. (2013) recently reported that elevated levels of circulating EMT suppressing miRNA miR-200c are associated with enhanced distant metastasis and worse prognosis in colorectal cancer patients. Besides cancer, circulating miR-34 has also been associated with other diseases. Compared with healthy individuals, miR-34a levels were elevated in serum of patients with type 2 diabetes (Kong et al., 2011), fatty liver disease (Cermelli et al., 2011), and acute myocardial infarction (Matsumoto et al., 2013), whereas levels of mR-34c were reduced in patients with chronic obstructive pulmonary disease (Akbas et al., 2012).

We and others demonstrated that CpG islands in the *miR-34a* and *miR-34b/c* promoters are frequently methylated in primary tumors and cell lines from various cancer entities (Lodygin et al., 2008; Toyota et al., 2008; Suzuki et al., 2010; Vogt et al., 2011). Methylation has a significant impact on miR-34 expression, since treatment with the de-methylating agent 5-aza-2′-deoxycytidine leads to miR-34 re-expression (Toyota et al., 2008; Suzuki et al., 2010). Moreover, a strong inverse correlation between *miR-34a* methylation and expression has been observed in colon and non-small cell lung tumors (Gallardo et al., 2009; Siemens et al., 2013b). These findings suggest that methylation plays a crucial role in *miR-34* silencing in tumors. Therefore, miRNA cancer treatment strategies may rely not only on delivery of ectopic miRNAs into tumors, but also on re-expression of miRNAs using anti-methylation agents. Indeed, treatment with BioResponse 3,3′-Diindolylmethane (BR-DIM), an experimental anti-androgen prostate cancer drug, resulted in de-methylation and re-expression of *miR-34a* in prostate cancer cells (Kong et al., 2012). In a phase II clinical trial, treatment of prostate cancer patients with BR-DIM prior to radical prostatectomy led to the re-expression of *miR-34a*, which resulted in repression and nuclear exclusion of its target, the androgen receptor (Östling et al., 2011; Kong et al., 2012). Several reports showed that *miR-34* methylation may also have prognostic values. In our study, *miR-34a* methylation was significantly associated with increased lymph node and liver metastasis in colon cancer patients (Siemens et al., 2013b). Finally, in prostate and non-small cell lung cancer patients, aberrant DNA methylation of *miR-34b/c* significantly correlated with shorter disease-free and overall survival (Wang et al., 2011b; Majid et al., 2013).

## Future outlook

The members of the miR-34 family regulate numerous targets and thus influence various cellular processes, such as proliferation, apoptosis, and differentiation. Therefore, miR-34 has to be tightly regulated, but still responsive to extrinsic or intrinsic stimuli, in order to coordinate the balance between different cellular functions. In future studies, it will be important to investigate how miR-34 expression is regulated by various stimuli and how it cooperates with other miRNAs to control cell homeostasis. Given the importance of miR-34 in cancer, spermatogenesis, neuronal development, the cardiovascular system, and obesity, it is surprisingly that *miR-34* knockout mice, either single or triple *miR-34a/b/c* knockout mice, did not display an elevated frequency of spontaneous tumors nor any other obvious disease phenotype or viability issues (Concepcion et al., 2012; our unpublished observations). Since these mice are germline knockout, possibly there is a compensation of miR-34 functions by up-regulation of other miRNAs that were shown to regulate similar targets, like the miR-449 family. Therefore, more research is needed by generating tissue-specific and/or inducible miR-34 knockout mice that would provide tools for studying miR-34 functions *in vivo*. The *p53* gene is mutated in the majority of human tumors. Different mutations can lead to loss of function of the p53 protein that results in cancer progression (Rivlin et al., 2011). The p53 protein harboring a loss-of-function mutation cannot induce the expression of miR-34s. However, recent studies show that gain-of-function p53 mutations may lead to abnormal activation of miRNAs, such as miR-27a and miR-130b (Dong et al., 2013; Wang et al., 2013c). Therefore, it would be interesting to elucidate the impact of gain-of-function p53 mutations on the expression of miR-34 in future studies. The cancer treatment approach with miR-34a mimics shows great promise for therapeutics of cancers with loss of function of p53 or methylation of *miR-34* genes. However, the main obstacle here is the low efficiency of miRNA delivery into tumor cells with current methods. Therefore, further research is needed to develop more efficient strategies for *in vivo* miRNA delivery or re-expression of miR-34 that is frequently silenced by methylation. *miR-34* methylation may also be an important aspect of future cancer diagnostics, because it is strongly associated with cancer, and methylated DNA released by tumor cells can be detected in body fluids with high sensitivity, as recently shown for *miR-34b/c* in colorectal cancer (Kalimutho et al., 2011). However, utilizing circulating miRNAs as easily accessible markers for cancer prognosis and therapy outcome prediction needs further research, since current association studies provided conflicting results regarding miR-34 and other miRNAs. These inconsistencies may be due to discrepancies between profiles of circulating miRNAs in blood and tumors, which can be attributed to tumor heterogeneity or presence of metastases that exhibit different miRNA expression levels from primary tumors. Furthermore, circulating miRNAs are not only secreted by tumor cells, but also by other cells or released by cells undergoing apoptosis.

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