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# One step ahead: miRNA-34 in colon cancer-future diagnostic and therapeutic tool?

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ABSTRACT

The discovery that microRNAs (miRNAs) - short, non-coding RNA molecules which regulate gene expression - are implicated in many types of cancer has revolutionised cancer research, giving hope for a new perspective in diagnostics and treatment. Dysregulation of miRNAs occurs in various malignancies, including colorectal cancer (CRC). CRC is one of the leading causes of cancer-related death and in most countries its incidence is still rising. Among several miRNAs which have been linked to CRC, miR-34 has attracted particular attention. This miRNA is involved in the regulation of cell cycle and apoptosis through multiple signaling pathways such as p53, Ra and Wnt signaling. Understanding its role in CRC may facilitate its future use as a diagnostic tool and therapeutic target.

## 1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide. The last global statistics were provided for 2012, with approximately 1.4 million new cases and 0.7 million deaths (Ferlay et al., 2013). In 2017 in the United States alone approximately 135,430 new cases and 50,260 deaths were reported, while in 2015 in China around 350,000 new cases and 190,000 deaths were noted (Chen et al., 2016; Siegel et al., 2017). The majority of cases occur in more developed regions, which points to the importance of environmental risk factors in colorectal carcinogenesis, such as improper dietary habits, insufficient physical activity, excessive body weight, smoking and alcohol consumption (Ferlay et al., 2013). The non-modifiable risk factors comprise age, genetic predisposition and intestinal inflammation. Inflammatory bowel disease (IBD) significantly increases the risk of cancer development, as the cumulative risk for colitis-associated cancer (CAC) equals 18% in ulcerative colitis (UC) and 8.3% in Crohn's colitis after 30 years of disease (Eaden et al., 2001; Canavan et al., 2006; Rubin et al., 2012).

MicroRNAs (miRNAs) are short, non-coding RNA molecules which regulate gene expression through binding to the target mRNA – usually at the 3′UTR, although interactions with 5′UTR and coding region have also been reported (Arora et al., 2013). These miRNA-mRNA interactions result in either degradation of mRNA or translational repression (Bajan and Hutvagner, 2014). Since miRNAs regulate basic cellular functions such as proliferation, differentiation and apoptosis, they may have a role in carcinogenesis. In fact, upregulation or downregulation of many miRNAs has been reported in various cancers (Ma et al., 2012; Zhu et al., 2014; Berindan-Neagoe et al., 2014; Guo et al., 2014; Yang et al., 2014, 2015; van Schooneveld et al., 2015). Depending on the function of miRNAs and their altered expression in cancer, some of the miRNA-encoding genes could be considered as oncogenes or tumor suppressor genes (Yamakuchi et al., 2008; Hermeking, 2010; Chi and Zhou, 2016).

Since miRNAs dysregulation was first detected in cancer, it has been extensively studied with the hope of discovering biomarkers for early diagnosis and prognosis, as well as new treatment possibilities. Over the years, more than 160 miRNAs were reported to be dysregulated in CRC, either within the cancer cells or in the peripheral blood. The most commonly reported include miR-20a and miR-31 (upregulated), miR-143 and miR-145 (downregulated) in tissue samples, while miR-92a was upregulated both in tissue and plasma (Luo et al., 2011). Moreover, the role of many miRNAs in CRC development with regard to their involvement in different cellular pathways was analyzed (for review see Chi and Zhou 2016). Additional evidence pointing to the importance of

*Abbreviations:* AOM, azoxymethane; APC, adenomatous polyposis coli; BTG-4, B-cell translocation gene; CAC, colitis associated cancer; CSCs, cancer stem cells; CRC, colorectal cancer; DSS, dextran sodium sulfate; GSK-3, glycogen synthase kinase-3; EMT, epithelial-mesenchymal transition; FU, fluorouracil; HA, hyaluronic acid; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; KO, knockout; miRNAs, microRNAs; MM, multiple myeloma; MTA, methylthioadenosine; NO, nitric oxide; NOS2, nitric oxide synthase-2; SAMe, S-adenosylmethionine; siRNA, small interfering RNA; SIRT1, silent information regulator 1; SNALPs, stable nucleic acid lipid particles; TCF/LEF, T cell factor and lymphoid enhancer factor; UC, ulcerative colitis; WT, wildtype

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**Table 1**
The miR-34 expression in human CRC studies.

| Reference | Number of patients (stage I/I/III/IV/adenoma tissue;number of samples) | Specimen | miR | | Method |
|---|---|---|---|---|---|
| | | | subtype | expression | |
| Roy et al. (2012) | Not applicable | formalin-fixed paraffin-embedded tissues from normal colonic mucosa and colon tumors | miR34a<br>miR34c | downregulated<br>downregulated | Real time RT-PCR |
| Arndt et al. (2009) | 45 (stage I, II, III, IV; 4:19:20:2) | Colon tumors | miR34a | upregulated | Real time Quantitative RT-PCR |
| Monzo et al. (2008) | 22 (stage I and II 6:16:0:0) | Colon tumors | miR34a | upregulated | Real time RT-PCR |
| Gao et al. (2015) | 10 (stage II and III) | Colon tumors | miR34a-5p | downregulated | Real time Quantitative RT-PCR |
| Tazawa et al. (2007) | 25 | Colon tumors | miR34a | downregulated | Real time RT-PCR |
| Ma et al. (2012) | 30 | Colon tumors | miR34a | downregulated | Real time RT-PCR |
| Bu et al. (2013) | 5 (stage I, II, III, IV; 2:1:1:1) | Colon tumors | miR34a | downregulated | Quantitative RT-PCR |
| Zhang et al. (2014) | 100 (stage I + II, stage III + IV; 44:56) | Colon tumors | miR34a | downregulated | Real time Quantitative RT-PCR |
| Akao et al. (2010) | 63 (stage I, II, III, IV; 12:19:24:8) | Colon tumors | miR34a | downregulated | Real time Quantitative RT-PCR |
| Wang et al. (2012) | 109 (stage III and IV) | Colon tumors | miR34a | upregulated | Taqman Real time PCR |

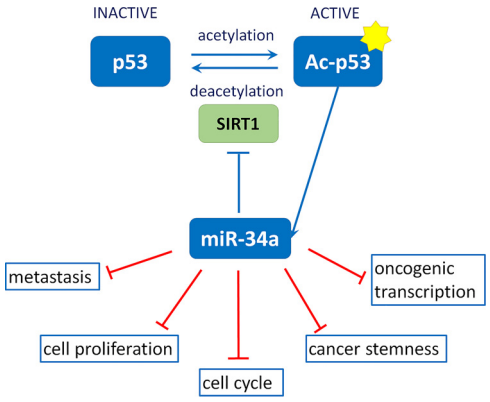


**Fig. 1.** The p53, SIRT-1 and miR-34a circuit. While acetylated p53 is the active form of this transcription factor, SIRT1 inhibits apoptosis through deacetylation of p53. Acetylated p53 induces miR-34a expression, which in turn inhibits SIRT1. Consequently, there is an increase in p53 acetylation, and increased p53 stability enhances miR-34a production. Therefore, miR-34a-mediated inhibition of SIRT1 can counteract the cancer process causing various effects on tumor cells. The positive feedback loop between p53, SIRT1 and miR-34a may thus become a new therapeutic target for the treatment of cancer.

miRNAs in CRC was provided by a study which reported that impaired DICER1 gene function and consequent downregulation of several miRNAs lead to cancer stem cell generation (Iliou et al., 2014). Among the studied miRNAs, miR-34 attracts particular attention (Table 1).

In this review we will analyze the role of miR-34 in the pathogenesis of CRC, its influence on the course of the disease and the pathways miR-34 is involved in.

## 2. The role of selected members of miR34 family in cancer: regulation of processes through different signaling pathways

The miR-34 family attracted attention when its members (miR-34a, miR-34b and miR-34c) were recognized as p53 targets. While miR-34a is encoded by its own gene and is expressed in all mouse tissues, with highest abundance in brain, miR-34b and miR-34c come from a single transcript and are mainly expressed in lungs. Except for the lung, where miR-34b/c are predominant, miR-34a is generally expressed at higher levels than the other members of the miR-34 family (Hermeking, 2010).

Overexpression of miR-34 leads to apoptosis and G1-phase cell cycle arrest, which suggests a link between miR-34 and p53. In fact, p53 regulates the expression of miR-34, as there are p53 binding sites within miR-34a and miR-34b/c promoter regions (He et al., 2007). Consequently, the antioncogenic action of miR-34 is regulated by p53.

Experiments based on ectopic introduction of miR-34 as well as bioinformatics approach allowed to identify numerous miR-34 targets, which primarily included mRNAs responsible for cell cycle control and response to DNA damage. Although miR-34a and miR-34b/c mostly target the same mRNAs, there is a difference in affinity for specific targets between miR-34 family members. Such system allows for an effective regulation of multiple processes by p53 (Hermeking, 2010). So far, over 77 of miR-34 targets have been validated, including factors controlling cell cycle (CDK4, CDK6, c-Myc, E2F3), apoptosis regulators (Bcl2, survivin, CREB), proteins engaged in invasion (c-Met, Axl receptor, RAS-oncogene homolog RRAS), factors related to epithelial-mesenchymal transition (EMT-inducing transcription factor SNAIL or zinc finger 281 protein), cancer stem cells formation (Notch1-4, WNT1, WNT3, β-catenin, CD44) and regulation of metabolism (hexokinase 1 and 2, glucose-6-phosphate isomerase, pyruvate dehydrogenase kinase 1, lactate dehydrogenase A) (Rokavec et al., 2014a).

Another miR-34 target, silent information regulator 1 (SIRT1), is involved in a feedback loop consisting of miR-34a, SIRT1 and p53, as demonstrated in an experiment conducted on the human colon cancer cell line, HCT116. SIRT1 is a NAD-dependent deacetylase involved in cellular response to oxidative stress and DNA damage. P53 is among SIRT1 target proteins, and deacetylation decreases p53 activity. Acetylated p53 induces miR-34a expression which inhibits SIRT1 and consequently activates p53-dependent apoptosis (Fig.1) (Yamakuchi and Lowenstein, 2009). It can be thus concluded that miR-34a regulates p53 activity through its direct target, SIRT1.

There are several arguments pointing to the role of miR-34 in cancer. Firstly, deletions in the regions where miR-34 genes are located (1p36 for miR-34a and 11q23 for miR-34b/c in humans) are detected in various types of cancer (1p36 deletion in neuroblastoma, glioma, melanoma, breast, colorectal, non-small cell and small cell lung cancers and 11q23 deletion in breast, lung, cervical and prostate cancers) (Agostini and Knight, 2014). Secondly, miR-34a downregulation is found in cancer cell lines (including breast, lung, colon, kidney, bladder and pancreatic carcinoma cell lines) and several types of primary cancer (such as colorectal, pancreatic, mammary, ovarian, urothelial, renal cell, nasopharyngeal and lung carcinomas and soft tissue sarcomas) (Toyota et al., 2008; Lodygin et al., 2008; Vogt et al., 2011; Siemens et al., 2013b; Wang et al., 2015a). The expression of miR-34a is also reduced in cancer stem cells (CSCs), in particular in glioblastoma, prostate, pancreatic and gastric CSCs, while its reintroduction suppresses EMT phenotype (Liu and Tang, 2011; Bao et al., 2012).

In particular, the regulation of Notch1 by miR-34a was shown to have a crucial role for colon CSCs (CCSCs) division. Notch1 signaling determines whether CCSCs will self-renew or differentiate at division. High miR-34a levels diminish Notch signaling and promote asymmetric division, where one daughter stem cell and one more differentiated non-CCSC is produced. Low miR-34a levels induce Notch signaling, favor symmetric division and as a result two daughter CCSCs are generated. Since CCSCs have higher proliferation rate, high miR-34a levels serve a tumor suppressive purpose through this mechanism (Bu et al., 2013).

In a meta-analysis, Wang et al. analyzed 9 studies regarding altered expression of miR-34 in colorectal carcinoma samples as compared to corresponding pericarcinous tissues and reported that they brought inconsistent results. While in two studies miR-34a was found to be upregulated, in 7 downregulation was described. In all the considered studies miR-34b and miR-34c were downregulated (Wang et al., 2015a). In another study it was reported that miR-34a/b/c were all increased in human colon cancers when compared to adjacent non-cancerous tissues. Moreover, raised miR-34b/c expression occurred in a more advanced stage, primarily in stroma as compared to epithelia, and was a marker of poor prognosis (Hiyoshi et al., 2015).

The contradictory results might be a consequence of the fact that miR-34a is involved in multiple cellular pathways and it is regulated in various mechanisms. miR-34a interacts with p53 and E2F, RAS, inducible nitric oxide synthase (iNOS) and Wnt pathway. It also influences EMT and undergoes epigenetic regulation (Li et al., 2017).

## 3. miR-34a-mediated modulation of selected signaling pathways

### 3.1. P53&E2F transcription factors

Tazawa et al. observed that upon exposure of HCT116 cells to a DNA-damaging agent, adriamycin, p53 induced miR-34a expression and it was increasing in time, suggesting that miR-34a was involved in a positive feedback loop through the p53 pathway (Tazawa et al., 2007). However, miR-34b/c overexpression did not influence p53 activity significantly. On the other hand, approximately a fourth of p53 network mRNAs (p53 transcriptional targets, human p53-interacting proteins, or transcription factors that regulate p53 expression), are regulated by miR-34a (Navarro and Lieberman, 2015). The same authors revealed that even p53 itself is a target of noncanonical regulation by miR-34a (with a binding site in 5′UTR), therefore p53 network undergoes both positive and negative regulation by miR-34a (Navarro and Lieberman, 2015). When HCT116 and RKO colon cancer cell lines were transfected with miR-34a, a strong growth inhibition with a decrease in expression of the E2F family of transcription factors together with senescence-like phenotype were observed. Moreover, miR-34a overexpression (through induction by adriamycin in HCT116 p53-knockout cell line or transfection in p53-mutated SW480 cell line) caused growth inhibition independently of p53, which may be explained by the repression of E2F proteins, involved in the regulation of cell cycle (Tazawa et al., 2007). E2F family consists of 8 members, E2F1-8, divided into three groups, E2F1-3 (activating group), E2F4-5 (repressor group), E2F6 (repressor with ability to interact with mammalian polycomb complex), while E2F7-8 have been discovered most recently and their functions are largely unknown (Tsantoulis and Gorgoulis, 2005). miR-34a represses the first group of E2F transcription factors, which are capable of triggering S-phase entry in quiescent cells. Downregulation of E2F may be related to the induction of p53 pathway, as observed previously in *E2f1-E2f3* knockout cells (Tazawa et al., 2007).

### 3.2. RAS –(RB)- > HBP1

Additionally, miR-34a evoked the induction of HBP1 gene expression (Tazawa et al., 2007). HBP1 is a transcription factor, which was reported to participate in premature senescence induction. It acts as a downstream effector of RAS and p38 MAPK and is regulated by the tumor suppressor retinoblastoma (RB) (Zhang et al., 2006; Li et al., 2010). HBP1 also acts as a tumor suppressor and its knockdown promotes tumorigenesis. Mutations and deletions in the region of HBP1 gene have been reported in breast and colon cancers. Decreased expression of HBP1 has been linked to poor prognosis in breast cancer and non-small cell lung cancer (NSCLC) (Li et al., 2010; Tseng et al., 2014).

### 3.3. NO → p53 → miR-34

High nitric oxide (NO) concentrations which occur during chronic inflammation due to iNOS induction may contribute to carcinogenesis through p53 protein inactivation or a mutation within p53 gene (Lala and Chakraborty, 2001). Li et al. showed that NO stimulates miR-34a and miR-34c expression of RKO colon cancer cells and therefore induces their apoptosis. This miRNA upregulation is presumably mediated through p53. *in vivo*, CAC is studied in the well-established murine azoxymethane/dextran sodium sulfate (AOM/DSS) model (Neufert et al., 2007; Rokavec et al., 2014b; Li et al., 2015). In the AOM/DSS model, in the course of cancer development, iNOS expression was remarkably increased. In contrast, miR-34c levels varied depending on the stage: at an early stage of adenoma or hyperplasia miR-34 was significantly upregulated, while later in the development, when adenocarcinoma was observed, miR-34c levels were lower, when compared with control samples. Moreover, the expression of p21, a downstream target gene of p53, was decreased in adenocarcinoma in comparison to the earlier stage, adenoma, which showed that the drop in miR-34c expression was a consequence of p53 inactivation during the transformation from adenoma to adenocarcinoma (Li et al., 2015). In order to identify downstream effectors of miR-34c involved in apoptosis, the expression of two antiapoptotic proteins – c-Met (tyrosine-protein kinase Met) and survivin - was assessed in RKO cells transfected with miR-34c mimic (Li et al., 2015). Survivin, which belongs to the inhibitor of apoptosis protein family, is overexpressed in most types of cancers (Peery et al., 2017). Induction of miR-34c downregulates c-Met and survivin on the protein level, but does not decrease the mRNA levels. The modulation of apoptosis through p53 and miRNA may have a role in the development of CRC associated with inflammation (Li et al., 2015). Consistent results were obtained by Mathe et al. who showed that inflammatory microenvironment regulates miRNA expression *in vivo* in cooperation with p53 (miR-34b/c) and NO (Mathé et al., 2012). In line, an overexpression of miR-34a/b/c was observed in the spleen following *C.parvum*-induced inflammation in C57BL mice, and was associated with either cancer development or a strong immune response. Additionally, in nitric oxide synthase-2 (NOS2)- knockout mice the expression of miR34a/b/c was also increased, which confirmed a negative feedback regulation between p53 and NOS2 during inflammatory process and thus suggests other than NOS2-dependent regulation of miR-34a/b/c (Mathé et al., 2012).

### 3.4. Wnt signaling

P53 was also reported to act through miR-34 on Wnt signaling pathway. Activation of the canonical Wnt signaling pathway results in β-catenin accumulation, which allows for its translocation to the nucleus where β-catenin coactivates transcription together with T cell factor and lymphoid enhancer factor (TCF/LEF) family of transcription factors (Fig. 2).

When Wnt pathway is upregulated in cancer cells, this series of events induces EMT and leads to cancer progression (Kim et al., 2011). miR-34 interacts with Wnt pathway genes (WNT1/3, LRP6, β-catenin, AXIN2 and LEF1) and thereby inhibits the transcriptional activity of β-catenin-TCF/LEF complex (Kim et al., 2011; Peng et al., 2017). On the other hand, mutations of Wnt pathway components, such as Adenomatous polyposis coli (APC) or β-catenin can be counterbalanced by miR-34 (Kim et al., 2011). Worth mentioning, Jiang et al. generated

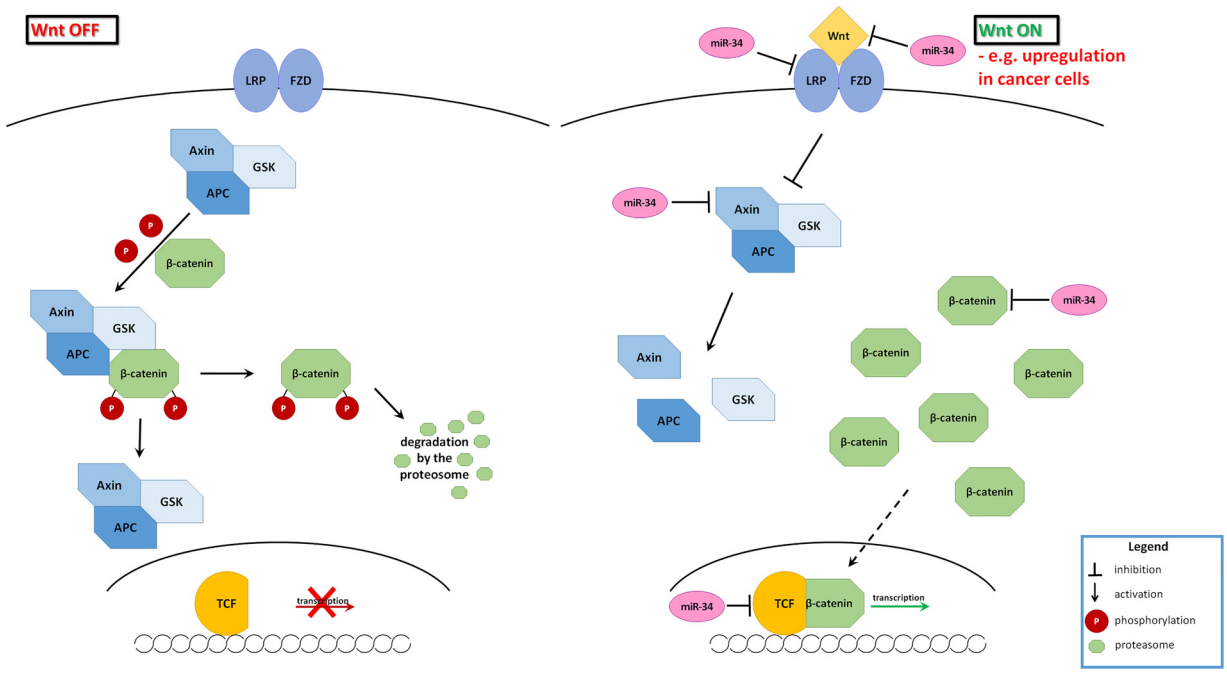


**Fig. 2.** Left – In the absence of Wnt ligand Axin, Adenomatous polyposis coli (APC) and Glycogen synthase kinase (GSK) form the „destruction complex", which phosphorylates β-catenin and marks it for degradation.
Right – When Wnt ligand binds to Frizzled (FZD) and its co-receptor LDL receptor-related protein (LRP), the destruction complex dissociates and β-catenin is released. β-catenin which shuttles between cytoplasm and nucleus interacts with T cell factor (TCF) and the complex induces the expression of Wnt-responsive genes, including CyclinD, c-Myc, CD44, IGF-2. miR-34 may inhibit Wnt signaling by targeting multiple components of the pathway (Schmitz et al., 2013; Peng et al., 2017).

*APC* $^{Min/+}$ mice with deletions of the miR34a and/or miR-34b/c genes separately or in combination (Jiang and Hermeking, 2017). Deletions of miR-34a/b/c increased the number of intestinal stem cells, Paneth and goblet cells which enlarged intestinal crypts and led to increased tumor burden. Moreover, mice with miR-34a/b/c-deficient genes had significantly decreased survival in comparison to control animals. Taken together, miR-34a/b/c act as global regulators *via* multiple cellular pathways, which are necessary to maintain proper homeostasis of intestinal epithelia (Jiang and Hermeking, 2017).

The two components of Wnt signaling, which have major importance for intracellular regulation, comprise glycogen synthase kinase-3 (GSK-3) and Axin, which, together with APC form the so-called destruction complex. Their basic function is β-catenin inactivation through phosphorylation, however, they also regulate the signaling through nucleo-cytoplasmic shuttling (Schmitz et al., 2013). miR-34 controls Wnt signaling by targeting multiple components of the pathway, therefore the expression of Wnt-regulated genes is balanced (Kim et al., 2013).

Moreover, Kim et al. reported that miR-34 targets the untranslated regions (UTRs) of Axin2 and decreases its expression in CRC cells. As a consequence, nuclear GSK-3 levels are increased and Snail expression is enhanced (Kim et al., 2013). Overexpression of Snail was reported in CRC surgical specimens and it was related to EMT induction and cancer stem cell (CSC)-like phenotype as well as chemoresistance (Fan et al., 2012).

### 3.5. EMT

In addition to the influence through Axin2, miR-34 was also reported to target the 3′-UTR of Snail mRNA directly. Through this process, miR-34 induced a change of phenotype in SW480 cells, inhibiting their motility and invasiveness, and downregulated transcription factors like SLUG and ZEB1 as well as stemness factors (*e.g.* c-Myc, CD44, BMI1). Ectopic expression of miR-34 prevented TGF-β-induced EMT. On the other hand, Snail was found to repress not only miR-34 but also miR-200 by binding to motifs called E-boxes, which are located within miR-34 promoter, therefore a double negative feedback loop is formed. A presumable additional effect of miR-34 is obtained through indirect regulation of ZEB1 and SLUG: downregulation of Snail allows for miR-200 expression, which in turn targets ZEB1 and SLUG (Siemens and Jackstadt, 2011).

Snail is also linked to miR-34 through ZNF281, a transcription factor whose expression is up-regulated in human CRC tissue samples. ZNF281 induces EMT, migration and invasion and together with Snail forms a positive feedback loop by enhancing each other's expression, while miR-34a directly represses ZNF281. ZNF281 may likely be required to maintain mesenchymal state of CRC cell lines and proved necessary for metastatic activity in a xenograft model (Hahn et al., 2013).

The inhibition of stemness markers by miR-34 may also be mediated by c-Kit. c-Kit is a receptor tyrosine kinase, whose ligand is stem cell factor. Stimulation of c-Kit activates multiple intracellular pathways, which may result in increased metastatic potential (Siemens et al., 2013a). Involvement of miR-34 in EMT is important with regard to metastasis. Silencing miR-34a by the methylation of its promoter leads to upregulation of factors involved in metastasis, such as c-Met, Snail and β-catenin. Indeed, this dysregulation was strongly associated with liver and lymph node metastases of colon cancer (Siemens et al., 2013b).

Since chronic inflammation significantly increases the risk of carcinogenesis, the possible involvement of miR-34 in this process and its interaction with IL-6, a proinflammatory cytokine, has been studied (Rokavec et al., 2014b). IL-6 promotes EMT, invasiveness and metastatic properties of CRC cell lines and primary colorectal tumors through the action of its effector, STAT3, on miR-34. While STAT3 directly represses miR-34, miR-34 targets IL-6 receptor, therefore a feedback loop is formed. The induction of miR-34 by p53 disrupts the feedback loop, which is important for its tumor suppressive action (Rokavec et al., 2014b). Rokavec et al. compared CAC development in miR-34a wildtype (WT) and miR-34a knockout (KO) mice in the AOM/

DSS model of CAC. miR-34a KO mice showed increased incidence and size of tumors with increased cell proliferation and decreased apoptosis. Moreover, miR-34 KO tumors were more invasive and spread easily through the *muscularis mucosa*, exhibited increased phosphorylation of STAT3 and higher expression of IL-6 receptor, SNAIL and ZEB1. Consequently, miR-34a inhibits IL-6R/STAT3 pathway responsible for EMT and invasion, which can promote metastasis (Rokavec et al., 2014b). Tomasi et al. examined the influence of S-adenosylmethionine (SAMe) and methylthioadenosine (MTA) on miR-34a/b expression and cancer metastasis, since SAMe and MTA were earlier reported to inhibit IL-6/STAT3 pathway (Li et al., 2012; Tomasi et al., 2017). In the AOM/DSS model SAMe and MTA significantly reduced tumor load (Li et al., 2012). SAMe and MTA also increased miR-34a/b expression in SW620 (colon cancer cell line derived from lymph node metastasis) and RKO cells. While the expression of methionine adenosyltransferase 2 A (MAT2 A) is upregulated in CRC, where it enhances cancer cell growth and survival, treatment with SAMe, MTA, miR-34a or miR-34b decreased MAT2 A expression. miR-34a or miR-34b overexpression inhibited cancer cell migration and invasion. These results demonstrate that SAMe and MTA act through miR34a/b and miR-34a/b inhibit cancer metastasis by targeting MAT2 A (Tomasi et al., 2017).

### 3.6. E2F3 → SIRT1(p53dep), PI3K/Akt

Yamakuchi et al. proved that miR-34a decreases SIRT1 expression through binding the response element in its 3′UTR in HCT116 cells (Yamakuchi et al., 2008). Transfection of the cells with miR-34a increased apoptosis only if they were wildtype with regard to p53, suggesting the effect is p53-dependent, consistent with the positive feedback loop described above. Additionally, it was noticed that downregulation of SIRT1 is associated with a rise in apoptosis after 5-fluorouracil (FU) treatment, indicating that SIRT1 counteracts 5-FU induced apoptosis (Yamakuchi et al., 2008). Akao et al. further studied the role of miR-34a in the response of CRC cells to 5-FU treatment. Expression of miR-34a was downregulated, while E2F3 and SIRT1 upregulated in 5-FU resistant cells in comparison to parental DLD-1. Additionally, exposure to 5-FU increased miR-34a expression in parental DLD-1 cells, but not in their 5-FU resistant counterparts. miR-34a overexpression in 5-FU resistant cell line increased the sensitivity to the chemotherapeutic agent and decreased the level of E2F3 and SIRT1. At the same time, PI3K/Akt signaling activation was observed in the course of 5-FU treatment (Akao et al., 2011). It was concluded that miR34a/E2F3/SIRT1 cascade, which was earlier reported to contribute to apoptosis induction (Kumazaki et al., 2013), and the inhibition of miR-34a expression through PI3K/Akt signaling might participate in the development of 5-FU resistance (Akao et al., 2011).

### 3.7. Epigenetic regulation

Epigenetic regulation of miR-34 expression may be central for its down-regulation in cancer. Aberrant CpG methylation of miR-34a promoter leading to transcriptional silencing was observed in melanoma as well as prostate, pancreatic and ovarian cancers (Toyota et al., 2008; Lodygin et al., 2008; Vogt et al., 2011; Siemens et al., 2013b). In CRC not only miR-34a, but also mi-34b/c were downregulated by this mechanism. Toyota et al. reported that miR-34b and miR-34c, which are silenced in CRC, are epigenetically regulated through methylation of CpG islands (Toyota et al., 2008). The same site regulates expression of B-cell translocation gene (BTG4). *in vitro*, BTG4 inhibited colony formation in CRC cell lines. The results suggested that miR-34 and BTG4 act as tumor suppressors and may undergo epigenetic silencing through the same CpG island in CRC (Toyota et al., 2008). In another study, treatment of CRC cells with a methyltransferase inhibitor increased the expression of miR-34a and miR-34c, therefore proving that the promoter hypermethylation is responsible for its downregulation. Re-expression of miR-34 led to reduction in the expression of Notch1, one of miR-34 targets (Roy et al., 2012).

Since miR-34 is commonly suppressed by CpG methylation in tumors and further transcriptional silencing, it can represent a tumor suppressor gene itself (Siemens et al., 2013b). The aberrant hypermethylation process that occurs in the colorectal tissues drives initiation and the progression of the cancerous cells towards more invasive and advanced stage of tumors. The study by Siemens et al. addressed the involvement of epigenetically silenced miR-34 s/b genes in CRC cancer development and showed the upregulation of Snail, c-Met and β-catenin in primary tumors (Siemens et al., 2013b). This led to the conclusion that epigenetic silencing of miR-34a in primary tumors may become a prognostic marker (especially with simultaneous detection of Snail, c-Met, and β-catenin) for distant metastases in CRC.

## 4. Future prospects of miR-34 in cancer prevention and treatment

Altered expression of miR-34 in CRC and its involvement in numerous cellular pathways implicated in carcinogenesis indicate the diagnostic and therapeutic potential of this microRNA. Decreased level of circulating miR-34a in CRC patients makes it a possible non-invasive biomarker (Nugent et al., 2012). Moreover, restoration of miR-34 expression decreased the proliferative potential of CRC cells, therefore miR-34 could be used as a therapeutic agent (Toyota et al., 2008).

To date, most studies have analysed tissue and cell-based miR-34 expression; there are relatively little data examining its expression in human body fluids. However, recently significantly higher DNA methylation of miR-34b and miR-34c has been detected in the mucosal wash fluids from patients undergoing colonoscopy (Kamimae et al., 2011). Furthermore, higher frequency of methylation was reported in patients with invasive when compared to those with noninvasive tumors, which may suggest that DNA methylation of miR-34b/c in colorectal washing fluid from CRC patients could be applied to determine the invasiveness of colorectal lesions (Kamimae et al., 2011). The presence of miRNAs has also been observed in whole blood specimens, in which the expression of circulating miR-34a was significantly lower in CRC patients *vs*. control individuals. It has been suggested that the ability to detect miRNAs in circulation was possible due to cancer cells death, or their release into the cancer microenvironment and subsequent translocation to a newly formed blood vessels of the systemic circulation. Of note, independently of time necessary to analyse blood samples, the material remained stable when stored in the unprocessed form at 4 °C (whole blood sample in the EDTA vacuette tubes) or semi-processed form *i.e.* extracted RNA stored at −80 °C (Nugent et al., 2012). This information opens up an important practical implication for future applications of blood-based miRNA assays but more detailed work needs to be done, especially when it comes to the sensitivity and specificity of the method in a larger group of CRC patients. Besides changes in the level of miR-34 in fluids of CRC patients, it is also possible to detect alterations in fecal specimens. In line, Kalimutho et al. performed a DNA analysis of feces from CRC patients in search of changes in the miR-34b/c methylation (Kalimutho et al., 2011). Although the method confirmed the presence of colon lesions, the percentage of detection of miR-34 in feces was lower when compared to its evaluation in tumor samples. The evaluation of the frequency of miR-34 methylation in colon cancer cannot be used for prognostic purposes, however, this method may represent a novel non-invasive way for early detection of colon cancer or can serve as an additional test in clinical settings *e.g.* in combination with endoscopic examination. In light of the widespread changes in miR-34 expression in human colon cancer, modulation of deregulated miRNAs in tumor cells by the restitution of their downregulated level has emerged as a promising therapeutic strategy. Although up to now the systemic delivery of miR-34 has been performed only in mouse models of cancer, the approach proved to be successful in attenuating cancer progression (Pramanik et al., 2011; Siemens et al., 2013a). Additionally, miR-34 may be particularly advantageous in the case of chemoresistance, as it may improve the

efficacy of chemotherapy by increasing sensitivity to 5-FU (Akao et al., 2011).

Unfortunately, the use of miRNA in treatment is limited by technological barriers. miRNA molecules are unstable, rapidly eliminated from bloodstream and absorbed by the cells only to a little extent, therefore numerous techniques have been designed for miRNAs delivery (Di Martino et al., 2014). Several lipid-based delivery systems have been used to introduce miR-34 into cancerous tissue. The use of nanocarriers, *e.g.* liposomes, is a promising solution to the problem. Liposomal formulation allows for delivery of the nucleic acid to the tumor site. Unfortunately, there are some impediments to clinical application of liposomes, such as low specificity and toxicity. Nevertheless, when a miR-34a mimic was delivered within an amphoteric liposomal formulation, a considerable tumor growth inhibition was noted in two mouse models of liver cancer (Daige et al., 2014). Liposomes can be modified to improve their properties, for example, in attempt to deliver miR-34a to murine model of B16F10 lung metastasis, liposome-polycation-hyaluronic acid nanoparticles were modified with a tumor-targeting monoclonal antibody (Chen et al., 2010). Suppression of gastric cancer cells proliferation and motility *in vitro* and reduced tumor volume *in vivo* (in mouse model) was achieved with the use of PEGylated lipid vesicles containing poly(L-lysine-graft-imidazole)/miR-34a complexes. In this system, endosomal release of miR-34a was facilitated thanks to the buffering capacity of imidazole residues in the acidic, endosomal environment (Jang et al., 2016). Another strategy, which overcomes some of the limitations of liposomal delivery, involves stable nucleic acid lipid particles (SNALPs) (Misso et al., 2014). SNALPs have previously been validated for small interfering RNA (siRNA) delivery. Di Martino et al. proved the effectiveness of SNALPs for miR-34a mimics delivery to multiple myeloma (MM) cells *in vitro* and in mouse xenografts, and observed tumor growth inhibition and increased survival rates in treated mice (Di Martino et al., 2014). Modifications of the basic system may improve the effectiveness of treatment: when miR-34a was 2′-O-methylated to enhance encapsulation efficiency and SNALPs were conjugated with transferrin (Tf) to target MM cells which overexpress transferrin receptors, the resultant increase in survival was the highest (Scognamiglio et al., 2014). Another lipid-based delivery system was used to deliver miR-34 to mouse model of non-small cell lung cancer. In this study, synthetic miR-34a mimics in complex with a neutral lipid emulsion caused miR-34a accumulation in tumor tissue and a 60% reduction in tumor area when compared to control (Trang et al., 2011). Although many attempts of miRNA delivery have been proposed and verified in animal models, there has been only one clinical trial so far evaluating the safety, pharmacokinetics and pharmacodynamics of a liposomal nanoparticle formulation of a naturally occurring tumor suppressor miR34a, MRX34 (ClinicalTrials.gov Identifier: NCT01829971). In line, Phase I, open-label, multicenter, dose-escalation study investigated MRX34 in 155 participant with refractory advanced solid tumors. The trial has been terminated due to serious adverse immunological events, with other frequently non-laboratory adverse effects such as fever, fatigue chills and back pain. Additionally, a large group with hepatocellular carcinoma patients had higher frequency of laboratory liver-related abnormalities, in comparison to the group of patients with other cancers (*e.g.* pancreatic cancer or cholangiocarcinoma). Other MRX-34-related adverse effects tended to occur later post-infusion *e.g.* diarrhea/enteritis, altered mental status or dyspnea (Beg and Brener, 2017).

In addition to lipid-based nanocarriers, other systems have also been examined. To study the effect of miR-34a on PANC-1 pancreatic cancer cells, β-cyclodextrin-polyethylenimine (PC) nanoparticles conjugated with a tumor-targeting bifunctional peptide CC9 were used. The treatment with such nanocomplexes induced cancer cell apoptosis both *in vitro* and *in vivo*, where it also markedly inhibited tumor growth (Hu et al., 2013). Cancer cells often overexpress receptors which bind hyaluronic acid (HA), such as CD44, therefore incorporating HA into nanocarriers helps target tumor cells. miR-34a delivered to MDA-MB-231 breast cancer cells in hyaluronic acid/protamine sulfate interpolyelectrolyte complexes (HP-IPECs) showed anticancer effects both *in vitro* and *in vivo* (Wang et al., 2015b). Moreover, when miR-34a was co-encapsulated with doxorubicin into HA-chitosan nanoparticles, the treatment of the same breast cancer cell line brought a synergistic antitumor effect (Deng et al., 2014). In another study, a combination of miR-34a and paclitaxel were used to treat B16F10-CD44+ melanoma stem-like cancer cell line. It proved effective both *in vitro*, where the two components showed synergistic action, and in a murine model of lung metastases *in vivo,* where the combination inhibited tumor growth more efficiently than either of the agents alone (Shi et al., 2014).

Nanotechnology-based formulations provide an effective manner for the administration of miRNA to different experimental tumor models; however, whether they prove to be an adequate tool for the treatment of CRC is unknown. Further studies investigating the potential of miR-34 in CRC may bring about new therapeutic alternatives, especially for tumors showing chemoresistance.

## Conflicts of interest

The authors declare no conflicts of interest.

## Author contributions

Julia Krajewska, Paula Mosińska, and Jakub Fichna provided the overall concept and framework of the manuscript; Julia Krajewska and Paula Mosińska researched and identified appropriate articles, Julia Krajewska wrote the manuscript; Julia Krajewska, Paula Mosińska, and Jakub Fichna revised the manuscript. All authors approved the final version of the manuscript.

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